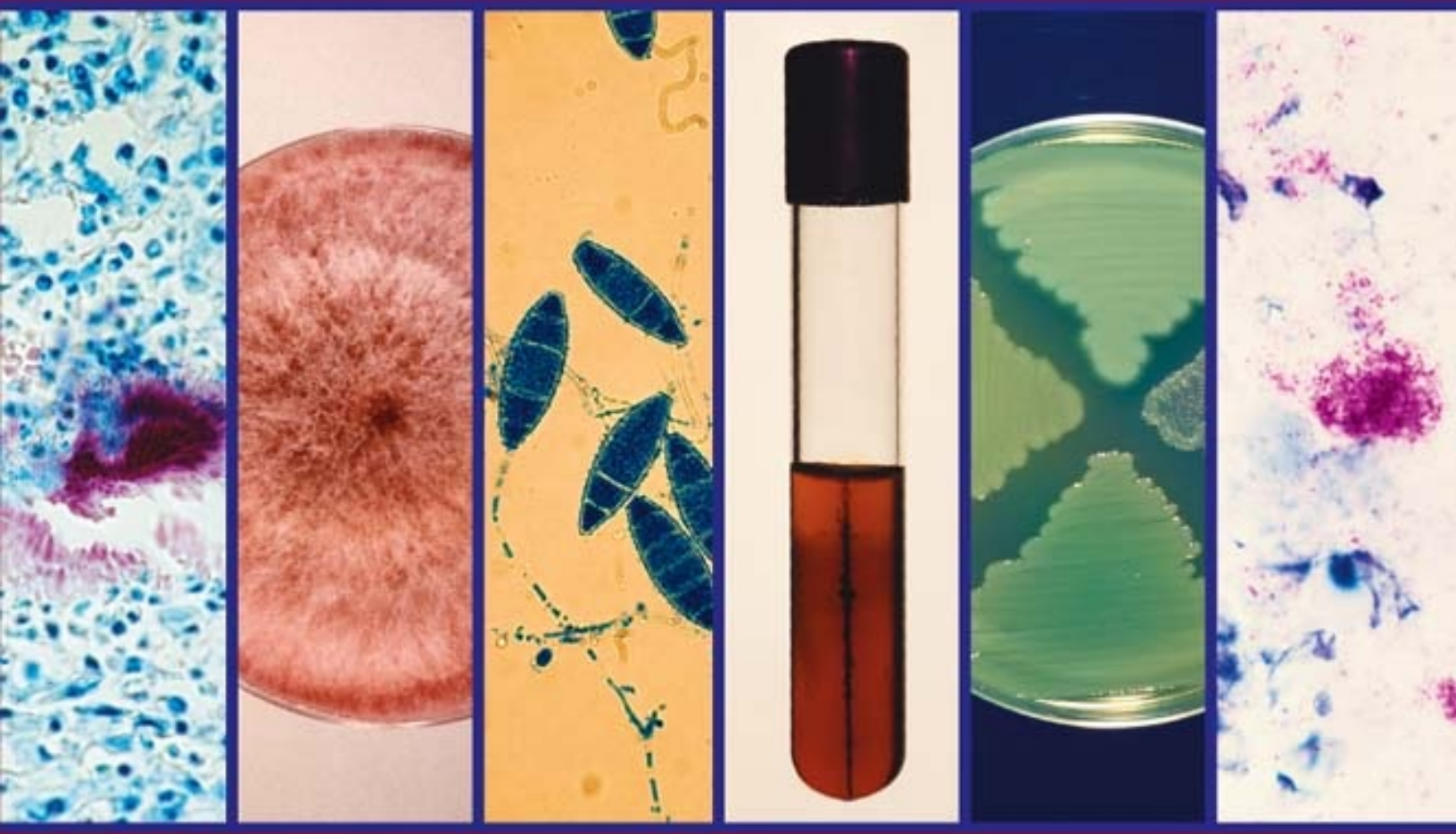
Veterinary Microbiology and Microbial Disease

P J Quinn
B K Markey
F C Leonard
E S FitzPatrick
S Fanning
P J Hartigan

Second Edition



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Contents

Section II Introductory Bacteriology

Section III Pathogenic Bacteria

Section IV Mycology

Section VII Microbial Agents and Disease Production

Index

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This book is dedicated to the memory of Margery E. Carter and W.J.C. (Bill) Donnelly, co-authors of the first edition

Preface

The pace of change in microbiology has accelerated in recent years as molecular techniques, applied to microbial pathogens, elucidate the pathogenesis of many infectious diseases and improve the reliability of diagnostic test procedures. Today, microbiology occupies a central position in the veterinary curriculum and has developed into a subject of vast complexity. Since the publication of *Veterinary Microbiology and Microbial Disease* in 2002, many changes have occurred in veterinary microbiology, some on the recommendations of international committees and others as a consequence of relevant research.

The second edition of our book incorporates changes in individual chapters which have been updated and expanded. In addition, new chapters on immunodeficiency diseases, vaccines and vaccination, molecular diagnostic methods, antibacterial resistance, antifungal chemotherapy, antiviral chemotherapy and microbial diseases of the urinary tract, cardiovascular system, musculoskeletal system and the integumentary system have been added.

This edition is divided into seven sections. The first section provides an introduction to microbiology, infection, immunity and molecular diagnostic methods. Section II contains chapters on introductory bacteriology. Pathogenic bacteria are dealt with in Section III. The twelve chapters in Section IV are concerned with mycology. Introductory virology is presented in Section V. Viruses and prions are covered in Section VI. The final section, Section VII, includes chapters on the interactions of microbial pathogens with body systems. A separate chapter in this section deals with bovine mastitis and the final chapter provides a comprehensive review of disinfection, biosecurity and other aspects of disease control.

To facilitate readers requiring additional information on topics included in the book, a list of websites is provided at the end of Section VII.

The use of colour in this edition enhances the quality of the illustrations and facilitates the interpretation of complex diagrams.

The authors would be pleased to receive notification of errors or inaccuracies in this edition of our book.

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We wish to acknowledge the constructive comments of the following colleagues who offered scientific, technical and editorial advice on individual chapters or who assisted in other ways: Hester McAllister, Marijka Beltman, Aidan Kelly, Paul Stanley, Carolyn Cummins, Eva Maischberger, Jane Irwin, Robert Shiel, Clodagh Kearney, Gráinne McCarthy, Hanne Jahns, Joe Cassidy, Yvonne Abbott, Dores Maguire, Frances LeMatti, Ruth Henry, Pauline Coyle, Sean Hogan, Jarlath Nally, Steve Gordon, Brian Sheahan, Mark Rogers, Shane Cooney, Orla Condell, Marta Martins, Matthew McCusker, Stephen O'Brien, Katie Solomon, Karen Power, Paul Whyte, Patrick Wall and Theo De Waal, School of Veterinary Medicine, University College Dublin; Cliona O'Farrelly, Tim Foster and Patrick Prendergast, Trinity College Dublin; Pat Lenihan, Maire McElroy, Kevin Kenny, Peter O'Neill and Pat Raleigh, Central Veterinary Research Laboratory, Backweston; Patrick Rogan, Department of Agriculture, Fisheries and Food; Hywel Ball, Agri - Food and Biosciences Institute, Stormont; Patrick McDonough, College of Veterinary Medicine, Cornell University; Helen O'Shea, Department of Biological Sciences, Cork Institute of Technology; Alan Reilly and Wayne Anderson, Food Safety Authority of Ireland; Brendan Crowley, Department of Medical Microbiology, St. James's Hospital, Dublin; Donal Walsh, School of Veterinary Medicine, University of California, Davis; Ross Fitzgerald, Universitry of Edinburgh; Davida Smyth, NewYork University; Clive Lee, Royal College of Surgeons in Ireland; James Buckley, Veterinary Department, Cork County Council.

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Justinia Wood, Nick Morgan, Lucy Nash and their colleagues at Wiley-Blackwell provided advice and assistance throughout this long project. The careful editing of the manuscript by Mary Sayers, copy editor, improved the accuracy of the text, illustrations and references. As Project Manager, Ruth Swan coordinated corrections and advised the authors on technical aspects of changes to the manuscript.

Dublin, July 2011

Author biographies

P.J. Quinn, MVB, PhD, MRCVS, was Professor of Veterinary Microbiology and Parasitology and Head of the Department in the Faculty of Veterinary Medicine, University College Dublin, from 1985 to 2002. After graduating from University College Dublin in 1965, he spent some time in veterinary practice before enrolling as a postgraduate student in Ontario Veterinary College, University of Guelph, Canada. In 1970, he was awarded a PhD for research in veterinary immunology and he remained on the staff of Ontario Veterinary College until his return to the Faculty of Veterinary Medicine, University College Dublin, in 1973.

His research interests have included allergic skin reactions in the horse to biting insects, the epidemiology of toxoplasmosis in sheep, immune mechanisms in the respiratory tract of calves, leptospirosis in dairy cattle, immunomodulation, mechanisms of immunity in the respiratory tract of specific pathogen-free and conventional cats, botulism in gulls around the Irish coastline, factors influencing the tuberculin test in cattle, airborne dispersal of bacteria during slurry spreading, and evaluation of the efficacy of chemical disinfectants against *Brucella abortus and Mycobacterium bovis*.

In addition to many refereed publications in journals and chapters in books, he edited *Cell-mediated Immunity* (1984), is senior co-author of *Animal Diseases Exotic to Ireland* (1992), *Clinical Veterinary Microbiology* (1994), *Microbial and Parasitic Diseases of the Dog and Cat* (1997), *Veterinary Microbiology and Microbial Disease* (2002) and *Concise Review of Veterinary Microbiology* (2003) and is co-author of *Veterinary Embryology* (2006).

He was awarded the title Professor Emeritus by University College Dublin in 2002. In 2006, he was recipient of the Association of Veterinary Teachers and Research Workers outstanding teaching award. For his contribution to teaching and faculty development in the Faculty of Veterinary Medicine in Tirana, he was awarded an honorary doctorate by the Agricultural University of Tirana, Albania, in May 2010.

Bryan K. Markey, MVB, PhD, MRCVS, Dip Stat, graduated from the Faculty of Veterinary Medicine, University College Dublin, in 1985. Following a short period in general practice he was appointed house surgeon in the Faculty of

Veterinary Medicine, University College Dublin. In 1986, he joined the academic staff as an assistant lecturer in the Department of Veterinary Microbiology and Parasitology. He spent one year on study leave at the Veterinary Sciences Division, Belfast, and enrolled for a PhD degree at Queen's University. He was awarded a PhD from Queen's University, Belfast in 1991 and was promoted to senior lecturer in veterinary microbiology in 1997. From 2002 to 2004 he served as Head of Department. In 2005 he was visiting professor at the College of Life Sciences, Queensland University of Technology, Brisbane.

His research interests include chlamydial infections of domestic animals and methicillin-resistant *Staphylococcus aureus* infection in veterinary species. He has contributed chapters to books on veterinary disinfection and is co-author of *Animal Diseases Exotic to Ireland* (1992), *Clinical Veterinary Microbiology* (1994), *Microbial and Parasitic Diseases of the Dog and Cat* (1997), *Veterinary Microbiology and Microbial Disease* (2002) and *Concise Review of Veterinary Microbiology* (2003).

Finola C. Leonard, MVB, PhD, MRCVS, graduated from the Faculty of Veterinary Medicine, University College Dublin, in 1983. She was house surgeon in the Department of Large Animal Medicine, Royal (Dick) School of Veterinary Studies, Edinburgh, for one year and engaged in veterinary practice for three years. She commenced postgraduate studies in the Faculty of Veterinary Medicine, University College Dublin, on leptospirosis in dairy cattle while based at Teagasc, Moorepark, Co. Cork, and was awarded a PhD for research on this topic in 1991. She remained in Moorepark as a postdoctoral research worker until 1997. Her research was concerned with foot lameness in dairy cattle and the influence of housing on the behaviour and welfare of cattle and pigs.

She was appointed college lecturer in the Department of Veterinary Microbiology and Parasitology in the Faculty of Veterinary Medicine, University College Dublin in 1997 and was promoted to senior lecturer in veterinary microbiology in 2002. Her research interests include *Salmonella* infection in pigs, other zoonotic infections, and antimicrobial resistance, including methicillin-resistant *Staphylococcus aureus* infection in farm and companion animals.

Eamonn S. FitzPatrick, FIBMS, was awarded Fellowship of the Institute of Biomedical Science in 1978 and was appointed to the post of Principal Technician in the Department of Veterinary Anatomy, University College

Dublin. He was appointed to the Histopathology Advisory Committee of the Irish Academy of Medical Laboratory Sciences in 1979. From 1987 to 1989 he was External Examiner for the Diploma in Medical Laboratory Science—Histopathology Option, at the Dublin Institute of Technology, where he also lectured for many years on electron microscopy in the Medical Laboratory Sciences Degree course. He was appointed Chief Technical Officer in the Veterinary Science Unit of the School of Veterinary Medicine, University College Dublin, in 2006. He has been teaching veterinary anatomy and histology for over 25 years.

Recent published work includes papers on hormone receptors in the bovine reproductive tract and the effect of diet supplements on the alimentary tracts of weanling pigs. His current research interests are centred mainly on mucins, mucus gels and the interaction of microbial pathogens with epithelial surfaces, especially of the bovine and equine reproductive tracts. He is co-author of *Veterinary Embryology* (2006).

Séamus Fanning, BSc, PhD, graduated in Biochemistry and Microbiology from University College Cork. He was awarded a Fulbright Fellowship in 1995 and worked at Baylor College of Medicine, Houston. In 2002 he was appointed as the Professor of Food Safety and Zoonoses at University College Dublin and set up the UCD Centre for Food Safety. Currently, his research interests include the application of molecular methods to food safety to aid in the control of zoonotic bacteria. A significant part of his research is related to the characterization of the genetic mechanisms contributing to the emergence of multiple drug resistance in food - borne pathogens. In particular, this work is related to strain virulence and its influence on survival in the food chain. His research group is involved in characterizing the emerging pathogen, *Cronobacter* species (formerly known as *Enterobacter sakazakii*), linked to powdered infant milk formula. The UCD Centre for Food Safety was designated as the World Health Organization (WHO) Collaborating Centre for Research, Reference and Training on *Cronobacter*.

Patrick J. Hartigan, BSc, MVM, MA, PhD, MRCVS, graduated from the Veterinary College of Ireland in 1955. After a decade in large animal practice in Co. Kerry, he registered as a graduate student at the School of Veterinary Medicine, Trinity College, Dublin. His studies on uterine pathology in repeat breeder cows were rewarded with a PhD in 1970. After 10 years as a pathologist in the School of Veterinary Medicine, he moved to a post as Senior Lecturer in

Reproductive Physiology at the Department of Physiology in the Faculty of Health Sciences at Trinity College, where he remained until retirement. At present, he is a Research Associate in the Department of Physiology.

Section I
Introduction to Microbiology, Infection, Immunity and Molecular Diagnostic Methods

Chapter 1

Microbiology, microbial pathogens and infectious disease

The earliest forms of life on this planet are presumed to have had characteristics resembling those of bacteria, most likely anaerobic bacteria. It is postulated that prokaryotes evolved from primitive forms of life and that the subsequent availability of oxygen resulting from photosynthesis contributed to microbial diversity. The chronological sequence of evolutionary events relating to the emergence of microbial life and, subsequently, eukaryotic cells is outlined in Fig. 1.1. This proposed scheme is based on limited factual information, some deriving from information gleaned from fossilized remains of prokaryotic cells approximately 3.5 billion years old and also from studies of ribosomal RNA among microorganisms.

Before the causes of infectious diseases could be discussed and evaluated in a rational manner, events associated with the emergence of life forms required explanation. Traditional views on the origin of life were strongly influenced by the writings of classical Greek and Roman scholars, many of whom espoused the view of spontaneous generation of small living entities. Disease was often attributed to evil forces associated with disturbances in the upper atmosphere, poisonous vapours called miasmas, supernatural events and other influences unrelated to biology. Awareness of the possible existence of forms of life not visible to the naked eye emerged slowly. As early as 1546, in his treatise *De Contagione*, Girolamo Fracastoro suggested that animate agents were responsible for disease. Concepts of infectious diseases were closely related to the demonstration of organisms too small to be observed without magnification and to the isolation and characterization of these small organisms, termed microorganisms. Major developments in microbiology, the study of these microorganisms, began with theories relating to the causes of infectious diseases and continued with the development of microscopy, which confirmed the existence of microorganisms visible only by substantial magnification. Towards the middle of the nineteenth century, the pioneering work of Louis Pasteur and

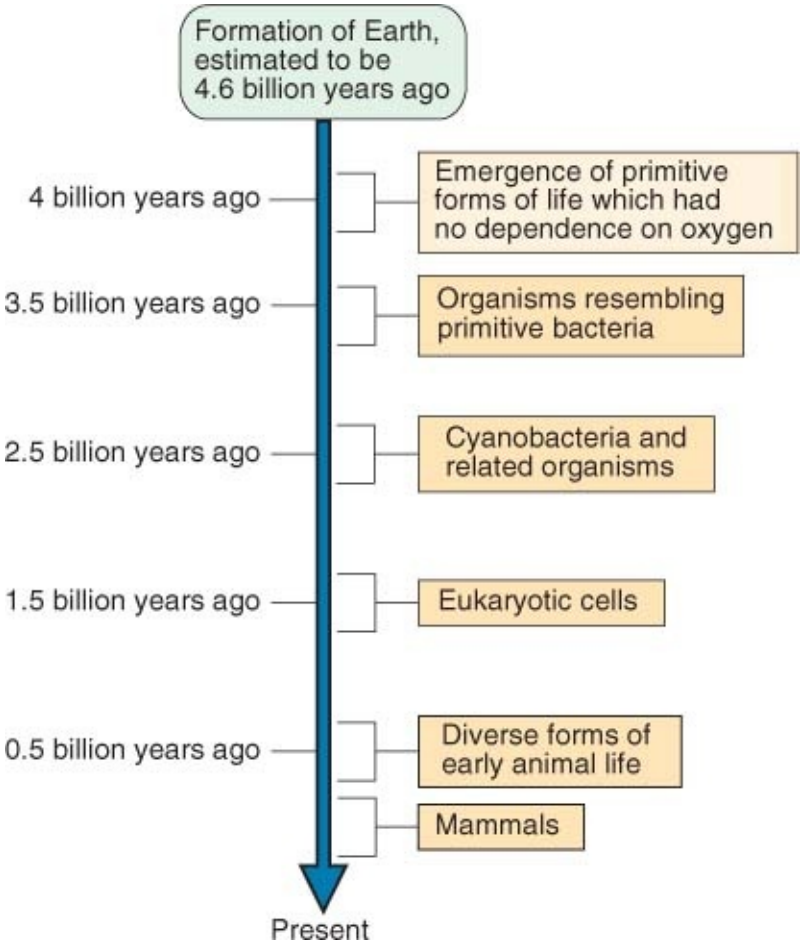
Robert Koch confirmed the microbial aetiology of infectious diseases. Progressive developments contributed to the rapid expansion of knowledge and the establishment of microbiology as a subject of major importance not only in human and animal health but also in food processing and preservation.

Spontaneous generation as an explanation for the emergence of life from decaying organic matter was a commonly held view for many centuries. It was postulated that life began as a consequence of putrefaction or some other associated change in organic matter. A number of practical experiments aimed at testing this concept were carried out, often with equivocal results. Improved scientific methodology and the availability of suitable instrumentation gradually challenged the acceptance of spontaneous generation. The development of the microscope around 1600 offered a means of exploring minute living entities, and the amateur Dutch scientist, Antonie van Leeuwenhoek, took a keen interest in the examination of water, fluids and organic material. In fluids he observed large numbers of motile structures, not visible to the naked eye, which he called ‘animalcules’. In 1675, van Leeuwenhoek recorded the structures he observed, which were probably bacteria, yeasts and protozoa. However, van Leeuwenhoek’s discovery of microorganisms did not resolve the issue of spontaneous generation.

The occurrence of maggots on putrefying meat was taken as evidence of spontaneous generation. The Italian physician and naturalist Francesco Reddi (1626–1697) carried out relevant experiments on this topic and demonstrated that maggots developed in meat only when flies laid their eggs on it. In the mid-eighteenth century, the English naturalist John Needham investigated the effect of boiling broth on the survival of microorganisms. He claimed to have detected microorganisms in boiled broth several days later. Needham’s experimental procedures were shown subsequently to have been unreliable. In 1769, Lazzaro Spallanzani repeated Needham’s experiments and demonstrated that no organisms survived in broth boiled for 1 hour. Needham argued that air was essential for all life and that Spallanzani had excluded air from the flasks containing broth. As a defined branch of science, microbiology could not advance until the concept of spontaneous generation was disproved. When the French chemist Louis Pasteur (1822–1895) became involved in investigations relating to microbiology, his careful planning and intuitive understanding of biology brought a new energy and appropriate methodology which conclusively refuted the prevailing theories of spontaneous generation. Pasteur’s interest in spontaneous generation was prompted by experiments which he had conducted

on spoilage during the fermentation of beet alcohol. He showed that a contaminating yeast that produced lactic acid during fermentation and which differed morphologically from brewers' yeast was responsible for the spoilage. He deduced that both alcoholic and lactic fermentation resulted from the metabolism and replication of the living yeast cells. The solution to the spoilage problem during fermentation of wine and beer products lay in heating the raw materials to about 120° F (49°C) in order to kill contaminating microorganisms prior to the addition of the appropriate yeast cells. This process, now known as pasteurization, is widely used to reduce microbial contamination in order to prolong the shelf-life of milk and some other foods.

Figure 1.1 Chronological sequence of biological events from the formation of Earth, relating to the evolution of different forms of microbial life and, later, eukaryotic cells. Although supporting scientific evidence documenting the earliest forms of microbial life is not currently available, data from microfossils confirm the existence of organisms resembling cyanobacteria approximately 3.5 billion years ago.



Pasteur effectively ended the controversy about spontaneous generation through definitive confirmation of Spallanzani's experiments. Furthermore, he demonstrated that contamination of nutrient broth when exposed to air resulted from microorganisms in dust particles settling on the fluid.

An important technical advance, which stemmed from Pasteur's fermentation studies, was the development of a fluid medium suitable for culturing yeast cells. He then developed other liquid media containing specific ingredients that favoured the growth of particular pathogenic bacteria. It was this development which eventually allowed him to formulate the germ theory of disease. The germ theory formed the**Figure 1.1**

Together with Pasteur, the German physician Robert Koch is considered to be a co-founder of modern microbiology. Having observed bacilli in the blood of animals that had died from anthrax, Koch demonstrated their pathogenicity by injecting mice with the blood. The injected mice died and the bacilli were present in preparations from their swollen spleens. He was also able to transfer the infection from mouse to mouse and to demonstrate the bacilli in each newly infected mouse. Initially, Koch used blood serum for growing the anthrax bacillus *in vitro*. Later, he developed solid media which allowed isolation of individual bacterial colonies. Using a solid medium, he was eventually able to isolate the tubercle bacillus from the tissues of an experimental animal in which he had demonstrated microscopically the presence of the organism. As a result of these observations, Koch formulated certain principles for proving that a specific microorganism caused a particular disease (Box 1.1). Pasteur's germ theory of disease and Koch's postulates are the two cornerstones on which microbiology is based and without which this branch of biology could not have advanced.

By the end of the nineteenth century a number of important infectious diseases had been confirmed as bacterial in origin. Both Pasteur and Koch contributed to the identification and confirmation of the causal agent of anthrax. Pasteur demonstrated that fowl cholera, malignant oedema and suppurative lesions were each associated with a specific bacterial infection. The causative organisms of tuberculosis and typhoid fever were recognized by Koch and his associates. Other bacterial agents responsible for serious infectious diseases including glanders, gas gangrene, diphtheria and dysentery were isolated by laboratory scientists in Europe, North America and Japan.

The basic technical approaches, pioneered by Pasteur and Koch, failed to shed light on the causes of such serious infectious diseases as rabies, smallpox, foot-and-mouth disease and rinderpest. Despite the absence of specific knowledge

about the aetiology of these diseases, successful vaccines were introduced both for smallpox, by Edward Jenner in the late eighteenth century, and for rabies, by Pasteur and his associates in the latter half of the nineteenth century. The development by Pasteur's co-worker, Charles Chamberland, of the porcelain filter to produce bacteriologically-sterile water for use in culture media, eventually facilitated isolation of the filterable agents which caused viral diseases. Remarkably, the technique was first used to elucidate the cause of a plant viral disease, tobacco mosaic disease.

BOX 1.1 Koch's postulates

- The pathogenic microorganism must be present in every case of the disease but absent from healthy animals
- The suspected microorganism must be isolated and grown in pure culture
- The same disease must occur when the isolated microorganism is injected into healthy susceptible animals
- The same microorganisms must be isolated again from the injected animals which developed disease

Dmitri Ivanovsky, a Russian scientist, reported in 1892 that it was possible to transmit tobacco mosaic disease from diseased to healthy plants using filtered leaf extract as inoculum. The filters used by Ivanovsky were Chamberland porcelain filters designed to remove bacteria from drinking water. In 1898, Martinus Beijerinck, unaware of the work of Ivanovsky, also demonstrated the filterability of the agent of tobacco mosaic disease. Moreover, he realized that the disease could not be due to a toxin as the filtered sap from infected plants could be used for serial transmission of the disease without loss of potency. In the same year, Loeffler and Frosch identified the first filterable agent from animals, the virus of foot-and-mouth disease. Yellow fever virus, a filterable agent pathogenic for humans, was described by Walter Reed and his team in 1901. Ellerman and Bang, in 1908, demonstrated the oncogenic potential of a filterable agent, the cause of avian leukosis. In 1915, Frederick Twort observed that bacteria were susceptible to a filterable agent, and two years later Felix d'Herelle made a similar observation. D'Herelle named these viruses 'bacteriophages' and developed a technique for establishing their concentration in active preparations. Bacteriophages have proved to be particularly useful in studies on viral replication and bacterial genetics.

Initially, the only method available for recovering large quantities of virus was through infecting susceptible animals. In 1913 Steinhardt and his colleagues

succeeded in growing vaccinia virus in explants of guinea-pig cornea embedded in clotted plasma. Some 20 years later, Furth and Sturmia used mice as a host species for propagating viruses, while Woodruff and Goodpasture were successful in propagating fowlpox virus on the chorioallantoic membrane of embryonated eggs. A major advance was made in the early 1950s with the development of single cell cultures. Factors critical in this development included the availability of antibiotics to control bacterial contamination, and the use of trypsin to obtain cell suspensions from embryonic or adult tissue. The separated cells could then be grown as monolayers on glass surfaces. Continuous cell lines, capable of multiplying indefinitely, provided a reliable source of cells for virus cultivation.

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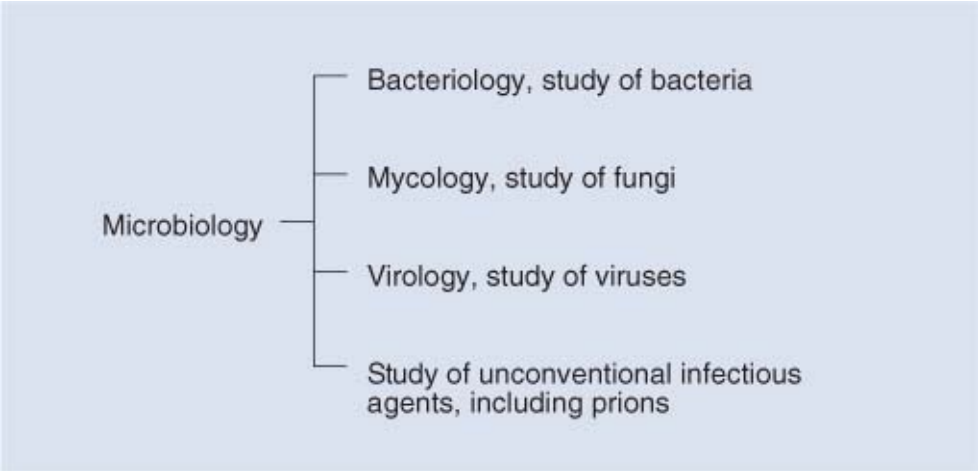
Figure 1.2 Subdivisions of microbiology, a subject which has areas of common interest with pathology, immunology, pharmacology, medicine and therapeutics.



In 1887 Buist observed vaccinia virus using a light microscope. However, because of the limited resolving power of this type of microscopy, the structure of the virus was not discernible. In 1939 Kausche and his co-workers employed the newly-developed electron microscope and a metal shadowing technique to identify tobacco mosaic virus in purified preparations. Ultrastructural studies of viruses were greatly expanded and enhanced in the 1950s by the development of negative staining and methods for cutting ultrathin sections. X-ray diffraction methods have been applied to viruses since the 1930s, when it was discovered that simple viruses could be crystallized. The first complete high-resolution structure of a crystalline virus, tomato bushy stunt virus, was obtained by Harrison and his co-workers in 1978. Computer analysis of the diffraction patterns obtained by such studies has contributed to knowledge of the molecular structure of viruses.

The crystallization of tobacco mosaic virus (TMV) by Stanley in 1935 provided a boost to the analysis of the chemical composition of viruses. In 1937 Bawden and Pirie showed that TMV contained nucleic acid as well as proteins, and helped to promote the idea that viruses consisted of nucleic acid contained within a protein coat. Having elucidated the structure of DNA and observed the limited coding capacity of viral nucleic acid, Watson and Crick in 1956 suggested that viral nucleic acid was surrounded by a shell of identical protein subunits. In 1962 Lwoff and his colleagues proposed a universal system on which the modern classification of viruses is based. The method of classification proposed was based on the following criteria: (1) the type of nucleic acid; (2) the symmetry of the virus; (3) the presence or absence of an envelope; (4) the diameter of the nucleocapsid (helical viruses) or the number of capsomers (icosahedral viruses). The discovery of the enzyme reverse transcriptase in 1970 by Temin and Baltimore helped to elucidate retrovirus replication and provided an essential tool for producing complementary DNA (cDNA). This ushered in the recombinant DNA revolution. The study of retroviruses has made a substantial contribution to the advancement of basic research in neoplasia and the role of oncogenes in the emergence of malignant tumours.

During the past century, major developments have taken place in microbiological concepts, techniques and applications. Modern microbiology encompasses the study of bacteria, fungi, viruses and other microscopic and submicroscopic organisms (Fig. 1.2). In veterinary microbiology, emphasis is placed on those microorganisms associated with infectious diseases of animals. Immunology, the study of host responses to infectious agents, is a discipline

closely related to microbiology and is sometimes considered a distinct but cognate subject.

Further reading

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Chapter 2

Subdivisions, classification and morphological characterization of infectious a gents

Living cells, the smallest units capable of independent existence, can be divided into two sharply differentiated groups, eukaryotes and prokaryotes. The main differentiating features of eukaryotic and prokaryotic cells are presented in Table 2.1. Eukaryotes possess true nuclei which contain chromosomes, and individual cells replicate by mitosis. In addition, a typical eukaryotic cell contains organelles such as mitochondria, a Golgi apparatus, lysosomes and relatively large ribosomes. Organisms in the domains *Archaea* and *Bacteria*, which are less complex than eukaryotic organisms, are prokaryotes which lack true membrane-bound nuclei. Their genetic information is contained in a single circular chromosome. In some prokaryotic cells such as bacteria, extrachromosomal DNA in the form of plasmids encodes for certain characteristics of the organism. Although the origin of life is a much debated subject, it is probable that primitive microorganisms originated from ancestral life forms several billion years ago. The degree of relatedness among microorganisms can be assessed by comparison of their ribosomal ribonucleic acid (rRNA). There is some evidence that all organisms developed from a group of primitive cells rather than from a single organism (Doolittle, 1999). Prokaryotes are considered as one branch of the phylogenetic tree and eukaryotes as the second branch (Fig. 2.1). Lateral as well as horizontal transfer of genetic material probably occurred in the course of evolutionary development, with some bacterial genes incorporated into members of the *Archaea* and perhaps with some prokaryotic genes incorporated into eukaryotes. This lateral gene transfer may explain how complex eukaryotic cells acquired some of their genes and organelles. The endosymbiosis hypothesis proposes that at some stage in their early development, eukaryotic cells became primitive phagocytes and acquired particular bacterial cell types which enhanced their respiratory activity (de Duve, 1996). It is proposed that the engulfed bacteria provided extra energy through this enhanced respiration to the host cell and eventually evolved into mitochondria. A similar phenomenon may account

for the development of chloroplasts in plant cells. The cytoplasmic membrane is the site of respiratory or photosynthetic energy generation in prokaryotes, unlike eukaryotes in which these activities occur in the membranes of mitochondria and chloroplasts.

Microscopical techniques

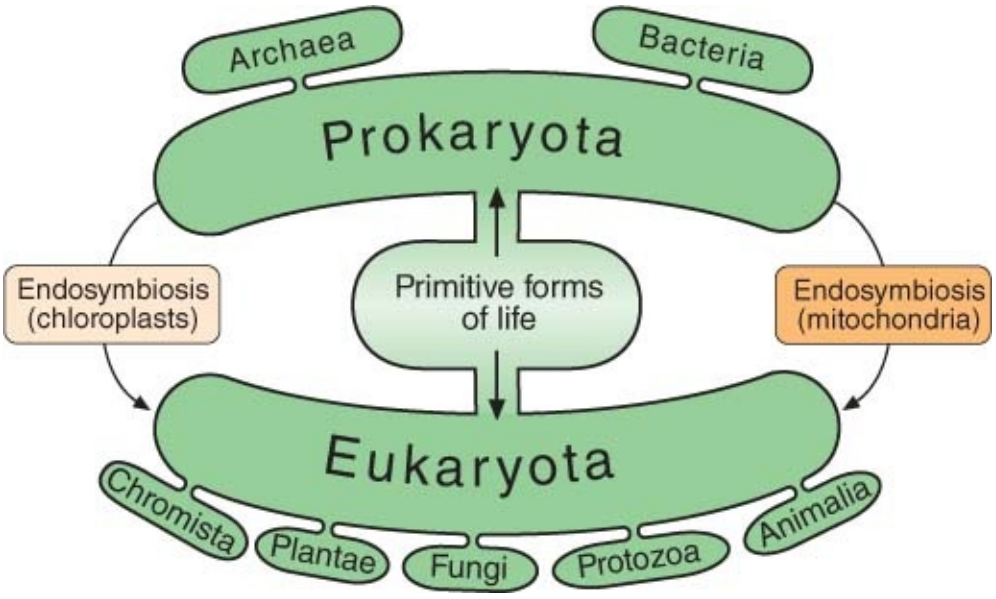
A number of different microscopical methods are employed for examining microorganisms. These include bright-field, dark-field, phase-contrast and electron microscopy. Table 2.2 summarizes common techniques employed for the examination of microorganisms and the particular types of microorganisms for which the techniques are appropriate. Units of measurement employed in microscopy are indicated in Table 2.3.

The maximum magnification obtainable by bright-field microscopy, using oil-immersion objectives, is approximately 1,000×. With bright-field microscopy, suitably stained bacteria as small as 0.2 µm in size can be visualized. With dark-field microscopy, the scattering of light by fine microorganisms such as spirochaetes suspended in liquid allows them to be observed against a dark background. In common with dark-field techniques, phase-contrast microscopy can be used to examine unstained specimens. This procedure is more appropriate for research purposes than for routine diagnostic microbiology.

Table 2.1 Comparative features of prokaryotic and eukaryotic cells.

Feature	Prokaryotic cell	Eukaryotic cell
Size of individual cells	Usually less than 5 µm in greatest dimension	Typically greater than 5 µm
Genetic material	Not separated from cytoplasm	Nucleus separated from cytoplasm by a nuclear membrane
Characteristics of chromosomes	Usually single and circular	Multiple and linear
Mitochondria	Absent	Present
Golgi apparatus	Absent	Present
Endoplasmic reticulum	Absent	Present
Location of ribosomes	Dispersed throughout cytoplasm	Dispersed throughout cytoplasm and also attached to endoplasmic reticulum
Cell division	Binary fission	Mitosis

Figure 2.1 The evolutionary relationships of living organisms. Endosymbiosis is one of the postulated mechanisms whereby eukaryotic cells acquired mitochondria or chloroplasts by incorporation of prokaryotic cells.



In transmission electron microscopy, beams of electrons are used in place of visible light to visualize small structures such as viruses. Specimens, placed on grids, are negatively stained with electron-dense compounds such as potassium phosphotungstate and viewed as magnified images on a fluorescent screen. Magnifications greater than 100,000 × are possible with modern instruments. Scanning electron microscopy is used to obtain three-dimensional views of microorganisms when coated with a thin film of heavy metal. With this technique a wide range of magnifications up to 100,000 × is feasible.

Table 2.2 Microscopical techniques used in microbiology.

Technique	Comments
Bright-field microscopy	Used for demonstrating the morphology and size of stained bacteria and fungi; staining affinity may allow preliminary classification of bacteria and the morphology of fungal structures permits identification of the genus
Phase-contrast microscopy	Used for examining unstained cells in suspension
Dark-field microscopy	Used for examining unstained bacteria such as spirochaetes in suspension
Fluorescence microscopy	Used for identifying microorganisms with specific antibodies conjugated with fluorochromes
Transmission electron microscopy	Used for demonstrating viruses in biological material and for identifying ultrastructural details of bacterial, fungal and mammalian cells
Scanning electron microscopy	Used for demonstrating the three-dimensional structure of microorganisms

Table 2.3 Units of measurement used in microbiology.

Unit	Abbreviation	Comments
Millimetre	mm	One thousandth of a metre (10^{-3}m). Bacterial and fungal colony sizes are usually measured in mm. When growing on a suitable medium, bacterial colonies range in size from 0.5mm to 5mm
Micrometre (micron)	µm	One thousandth of a millimetre (10^{-6} m). Used for measuring the size of bacterial and fungal cells. Most bacteria range in size from 0.5 µm to 5 µm. A small number of bacteria may exceed 20 µm in length

Nanometre	nm	One thousandth of a micrometre (10^{-9} m). Used for expressing the size of viruses. Most viruses of veterinary importance range in size from 20 nm to 300 nm

Pathogenic microorganisms

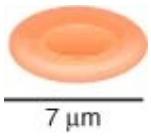
Most microorganisms found in nature are not harmful to humans, animals or plants. Indeed, many bacteria and fungi make an important contribution to biological activities which take place in soil, in water and in the alimentary tract of animals and humans. Those microorganisms that can cause disease in animals or humans are referred to as pathogenic microorganisms.

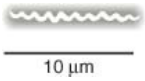
Bacteria

Microorganisms belonging to the domain *Archaea* (formerly *Archaebacteria*) are not associated with diseases of domestic animals. Organisms (bacteria) belonging to the domain *Bacteria* (formerly *Eubacteria*) include many pathogens of veterinary importance.

Bacteria are unicellular and are smaller and less complex than eukaryotic cells such as mammalian red blood cells (Table 2.4). They usually have rigid cell walls containing a peptidoglycan layer, multiply by binary fission and exhibit considerable morphological diversity. They occur as rods, cocci and helical forms and occasionally as branching filaments. Despite their morphological diversity, most bacteria are between 0.5 µm and 5 µm in length. Motile bacteria possess flagella by which they can move through liquid media. The majority of bacteria can grow on suitable inert media; some require special growth supplements and particular atmospheric conditions for growth. Two groups of small bacteria, rickettsiae and chlamydiae, which are unable to multiply on inert media, require living cells for *in vitro* growth. Cyanobacteria, formerly referred to as blue-green algae, utilize chlorophyll for some metabolic pathways. Unlike algae, which store chlorophyll in organelles referred to as chloroplasts, cyanobacteria have chlorophyll distributed inside their cell membranes.

Table 2.4 A comparison of the morphology and size of bacterial cells relative to a mammalian red blood cell.

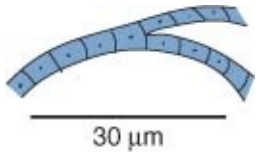
Cell	Morphology / size	Comments
Red blood cell		Readily seen using conventional light microscopy

Bacillus		Rod-shaped cells, usually stained by the Gram method. Using bright-field microscopy, a magnification of 1,000 × is required to observe most bacterial cells
Coccus		Spherical cells, often occurring in chains or in grape-like clusters
Spirochaete		Thin, helical bacteria. Dark-field microscopy (without staining) or special staining methods are required to demonstrate these unusual microorganisms

Fungi

Yeasts, moulds and mushrooms belong to a large group of non-photosynthetic eukaryotes termed fungi. Fungi may be either unicellular or multicellular. Multicellular fungi produce filamentous microscopic structures called moulds; yeasts, which are unicellular, have a spherical or ovoid shape and multiply by budding. In moulds, the cells are cylindrical and attached end to end, forming branched hyphae (Table 2.5). A notable feature of fungi is their ability to secrete potent enzymes that can digest organic matter. When moisture is present and other environmental conditions are favourable, fungi can degrade a wide variety of organic substrates. A small number of yeasts and moulds are pathogenic for humans and animals. Some fungi invade tissues whereas others produce toxic substances called mycotoxins which, if present on crops or in stored food such as grain or nuts, can cause disease in animals and humans.

Table 2.5 A comparison of the morphology and size of a bacterial cell and two fungal forms.

Structure	Morphology / size	Comments
Bacterial cell		
Coccus		Often occur in chains or grape-like clusters
Fungal forms		
Yeast		Reproduce by budding
Mould		Branched structures (hyphae) composed of many cells

Algae

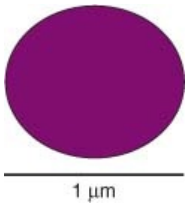
A morphologically and physiologically diverse group of organisms, algae are

usually considered plant-like because they contain chlorophyll. Many algae are free-living in water; others grow on the surfaces of rocks and on other structures in the environment. Some algae produce pigments which impart distinct coloration to water surfaces containing algal blooms. When water temperatures are high, algal growth may be marked, leading to the production of toxins that can accumulate in shellfish or in water containing algal blooms.

Viruses

Unlike bacteria and fungi, viruses are not cells. A virus particle or virion consists of nucleic acid, either DNA or RNA, enclosed in a protein coat called a capsid. In addition, some viruses are surrounded by envelopes. Viruses are much smaller than bacteria, and typically range in size from 20 nm to 300 nm in diameter (Table 2.6). Despite their simple structure, viruses occur in many shapes. Some are spherical, others are brick-shaped or bullet-shaped and a few have an elongated appearance. Because they lack the structures and enzymes necessary for metabolism and independent reproduction, viruses can multiply only within living cells. Both prokaryotic and eukaryotic cells are susceptible to infection by viruses. Those viruses that invade bacterial cells are called bacteriophages. Pathogenic viruses which infect humans and animals can cause serious disease by invading and destroying host cells. A small number of viruses are aetiologically implicated in the development of malignant tumours in humans and animals.

Table 2.6 A comparison of a bacterial cell and a large and a small virus.[a]

Structure	Morphology / size	Comments
Bacterial cell		
Coccus		Readily seen at magnification of 1,000 ×
Viruses		
Poxvirus		Viruses cannot be seen using conventional bright-field microscopy
Parvovirus		Electron microscopy at a magnification of up to 100,000 × is used to demonstrate viruses in clinical specimens or in laboratory preparations

[a], not drawn to scale.

Prions

Infectious particles that are smaller than viruses have been implicated in the neurological diseases of animals and humans that are termed transmissible spongiform encephalopathies. These particles, called prions, are distinct from viruses and appear to be devoid of nucleic acid. Prions seem to be composed of an abnormally-folded protein capable of inducing conformational changes in homologous normal host cell protein. Following the induced changes, structurally-altered abnormal protein accumulates in and damages long-lived cells such as neurons. Genetic factors seem to influence the susceptibility of humans and animals to prion diseases. Prions exhibit remarkable resistance to physical and chemical inactivation procedures.

Biological classification and nomenclature

Microscopic living organisms were formerly classified on the basis of phenotypic expression including morphology and distinct attributes reflecting unique metabolic properties. Increasingly, classification methods for microorganisms have come to rely heavily on genotypic analysis. In recent years this has led to changes in the classification and nomenclature of microorganisms.

The practice and science of orderly classification of organisms into hierarchical units termed taxa (singular taxon) is known as taxonomy. There are three interrelated parts to taxonomy: identification, nomenclature and classification. Taxonomy is important in microbiology because (1) it permits accurate identification of organisms; (2) it provides precise names that permit efficient communication; (3) it groups similar organisms in a way that allows predictions to be made and hypotheses to be framed with reasonable confidence regarding members of the same group. Most organisms are grouped according to their genotypic and phenotypic characteristics. Traditionally, great emphasis was placed on anatomical or morphological similarities but this has increasingly been replaced by a polyphasic approach facilitated by the availability of highly sophisticated methods of identification and the inclusion of additional criteria for the description of new species. Examples of phenotypic characteristics used in taxonomy include morphology, metabolism, physiology, cell chemistry (particularly fatty acid composition in the case of bacteria) and motility. DNA

profiling, DNA–DNA hybridization, multilocus sequence typing (MLST) and percentage of guanine plus cytosine in an organism's DNA (GC ratio) are examples of the genetic methods used. Complementing these two types of analyses is phylogenetic analysis, which attempts to create a framework of evolutionary relationships. The exponential growth in the availability of genetic sequencing data has permitted taxonomy to increasingly reflect phylogenetic relationships among microorganisms.

The basic taxonomic unit or group is the species. Similar species are grouped into genera, which in turn are placed in families. Several levels or ranks are used in this classification, with higher ranks including individual groups based on the shared properties of these groups. The levels in ascending order are species, genus, family, order, class, phylum and kingdom or domain (Fig. 2.2). Traditionally, biologists have grouped organisms into five kingdoms: animals, plants, fungi, protists and bacteria. However, analysis of small subunit ribosomal RNA gene sequences has suggested that cellular life has evolved along three primary lineages. The three resulting groups are called domains and are usually placed above the kingdom level. Two of the domains, *Bacteria* and *Archaea*, are exclusively microbial and prokaryotic, while the third domain, *Eukarya*, contains the eukaryotes.

In terms of sexually reproducing higher organisms, a species is defined as a group or population composed of similar individuals that are capable of interbreeding naturally and are reproductively isolated from other groups. However, the definition of what constitutes a species poses particular problems in microbiology. Members of the *Bacteria* and *Archaea* do not undergo true reproduction. As a result, bacteria tend to be defined operationally or in subjective terms as a collection of strains that share many similar properties but differ significantly from other strains. It is possible to be more precise by using a definition based on genetic data with a species expected to share 70% or greater binding in standardized DNA–DNA hybridization studies and/or over 97% gene-sequence identity for 16S ribosomal RNA (rRNA). Recently a threshold range of 98.7 to 99% sequence similarity has been recommended as the point at which DNA–DNA reassociation experiments should be required for testing the genomic uniqueness of a novel isolate (Stackebrandt and Ebers, 2006). Further debate and refinement regarding the definition of a bacterial species is on-going.

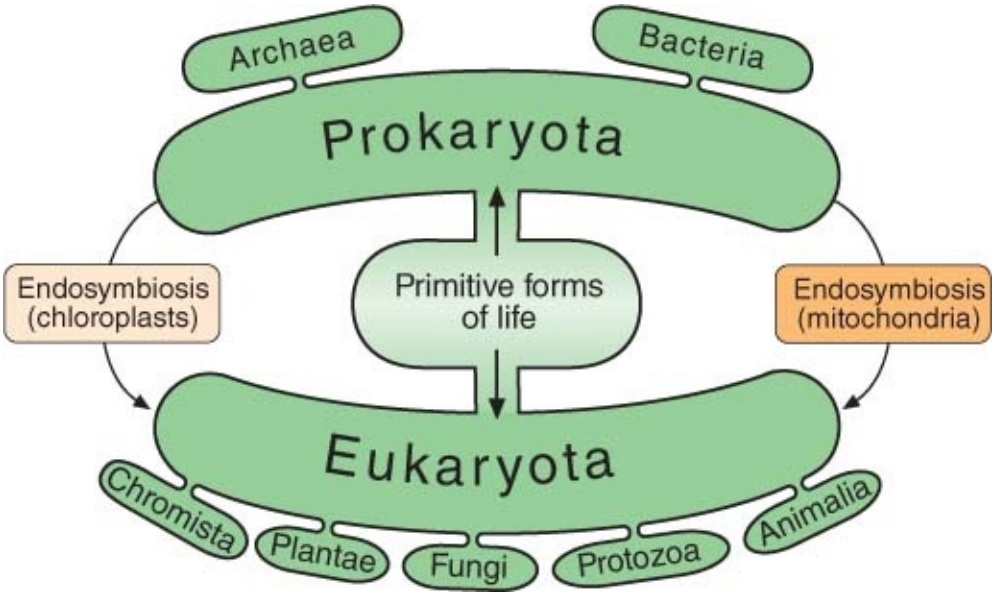
Microorganisms are generally named according to the binomial system devised by the Swedish botanist Carolus Linnaeus in the eighteenth century. The names are Latin or Latinized Greek derivations and are printed in italics. There

are two parts, a capitalized generic name and a specific epithet. For example, the bacterium that causes anthrax in humans and animals is termed *Bacillus anthracis, Bacillus* being the generic name and *anthracis* the specific name. The naming of species and higher groups of bacteria is regulated by the Bacteriological Code – *The International Code of Nomenclature of Bacteria. The International Journal of Systematic and Evolutionary Microbiology* is the official publication for recording taxonomical changes of *Bacteria* and *Archaea*. Websites providing listings of approved names include List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr) and Bacterial Nomenclature Up-to-Date (http://www.dsmz.de/bactnom/bactname.htm).

Viruses pose particular taxonomic problems in that they are regarded as subcellular, non-living, infectious entities and are often ignored or considered alongside their host species by scientists dealing with the global taxonomy of organisms. However, virologists are agreed that viruses should be considered as a separate group of organisms regardless of their host species, and have established a free-standing virus taxonomy derived from classical Linnean systematics but with rules unique to the discipline of virology. The system is operated by the International Committee on Taxonomy of Viruses (ICTV) with the latest information on viral taxonomy published periodically in report form (Fauquet *et al*., 2005) or available electronically at http://ictvonline.org/virusTaxonomy.asp. A number of points must be borne in mind when considering the taxonomy of viruses: (1) it is considered unlikely that viruses evolved from a single original protovirus and, as a result, the highest level recognized is that of order; (2) some viruses frequently undergo genetic recombination and reassortment resulting in chimeric organisms with polyphyletic genomes; (3) some viruses infect both vertebrate and invertebrate hosts, evolving differently in the different host species; (4) some viruses integrate into the genome of their host but can switch between horizontal and vertical transmission by moving in and out of the host cell genome, which may result in the incorporation of host genes into the viral genome. As a result, it is inevitable that the classification system will at times appear artificial and give rise to misfits. A non-systematic, polythetic, hierarchical system of classification is used for viruses. In essence a virus species is defined by a consensus group of properties without any one of these properties being essential. Viruses are generally grouped in families based on virion morphology and nucleic acid type. Further subdivision of pathogenic animal viruses frequently relates to the species

of host affected and to the clinical disease which is produced. Each viral genus contains a type species, which is defined as the virus species responsible for the original creation of the genus and whose name is linked to the use of the genus name. Virus species names are commonly abbreviated, for example BPIV-3 for bovine parainfluenza virus 3. In presenting taxonomic descriptions of viruses, several informal categories, collectively known as the order of presentation, are used based on the composition and structure of the viral genome, including genome polarity and reverse transcription: double-stranded DNA viruses, single-stranded DNA viruses, DNA and RNA reverse transcribing viruses, double-stranded RNA viruses, negative-stranded single-stranded RNA viruses and positive-stranded single-stranded RNA viruses. In addition there is a category of unassigned viruses and one for subviral agents including viroids, satellites and prions.

Figure 2.2 Example of hierarchical levels of taxonomy using the bacterium *Escherichia coli*. Note that many of the levels are deliberately incomplete to simplify the diagram.



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Chapter 3

Infection and immunity

Infectious disease is a major cause of morbidity and mortality in avian and mammalian species. Individual body systems supply the host's respiratory, nutritional and sensory needs, and the immune system is uniquely equipped to provide defence against microbial or parasitic infection irrespective of the source or the route of transmission. Some microorganisms cause opportunistic infections in domestic animals; other infectious agents, termed pathogenic microorganisms, are capable of causing serious infection if they gain entry into the body. The immune system is composed of an array of structures, cells and secretions which offer defence not only against opportunistic infections but also against pathogenic microorganisms which can cause life-threatening infections in susceptible animals.

The first barriers to infection that offer rapid, protective responses are components of innate immunity. These components include anatomical structures such as the skin and mucous membranes, inhibitory secretions, antimicrobial factors and phagocytic cells (Fig. 3.1). If an infectious agent enters the tissues, material from this invading pathogen can be presented to lymphocytes by phagocytic cells such as macrophages. These lymphocytes then undergo functional changes, proliferate and secrete soluble factors which promote the involvement of other cells of the immune system in an attempt to contain the infection. This response on the part of lymphocytes is referred to as an adaptive immune response. Moreover, following an encounter with a microbial pathogen, the body's immune system learns from the experience by responding in a specific manner to the pathogen and by 'remembering' the interaction. Immunological memory resides in some lymphocytes that are produced in the course of a response to an infectious agent, and these memory cells react quickly to subsequent invasion by the same agent. The immune system, therefore, has components that function as innate, non-specific barriers to infectious agents, and components that exhibit specificity combined with immunological memory. It provides protection against a vast array of actual or

potential pathogens present in the immediate environment of animals. Immune responses, however, are not confined to infectious agents and responses to innocuous substances such as pollens, foreign proteins and some therapeutic drugs, can cause potentially destructive hypersensitivity reactions. Although the primary activity of the immune system is usually considered to be associated with protection against infectious agents, it has a defined role in immune surveillance for the detection of neo-plastic tissue changes and, in some instances, elimination of such undesirable mutated cells or neoplastic cells by immune mechanisms.

Soon after birth the external surfaces of the body, extensive portions of the alimentary tract and regions of the respiratory and urinary tracts become colonized by bacteria. The host and colonizing bacteria live in a relatively peaceful state of coexistence, with microorganisms restricted to parts of the body where they can be tolerated and microbial invasion of tissues can be prevented by natural antibacterial defence mechanisms. Bacteria that colonize tissues of the body without producing disease constitute part of the normal flora. This harmonious relationship between animals and their environment can be reinforced by good management systems, optimal nutrition, adequate floor space and effective disease control programmes (Fig. 3.2). Negative factors that can tilt the balance in favour of potential or actual pathogens include overcrowding, uncontrolled environmental temperature, nutritional imbalances and absence of a well designed and implemented disease control programme. Even if bacteria, fungi or viruses succeed in entering the tissues and causing infection, disease is not an inevitable outcome. Characteristics of the infectious agent, environmental influences and the susceptibility of the infected animal usually determine the outcome of infection. If infection is not quickly eliminated, clinical disease or subclinical infection is the likely result (Fig. 3.3). Characteristics of individual species of pathogenic bacteria can strongly influence their ability to overcome host defences and produce disease. Structural, metabolic and other features of bacteria that promote disease development are presented in Table 3.1.

Figure 3.1 Cells, secretions and other elements of innate and adaptive immunity which contribute to protection against infectious agents. IL: Interleukin; IFN-γ: interferon-γ; TNF: tumour necrosis factor.

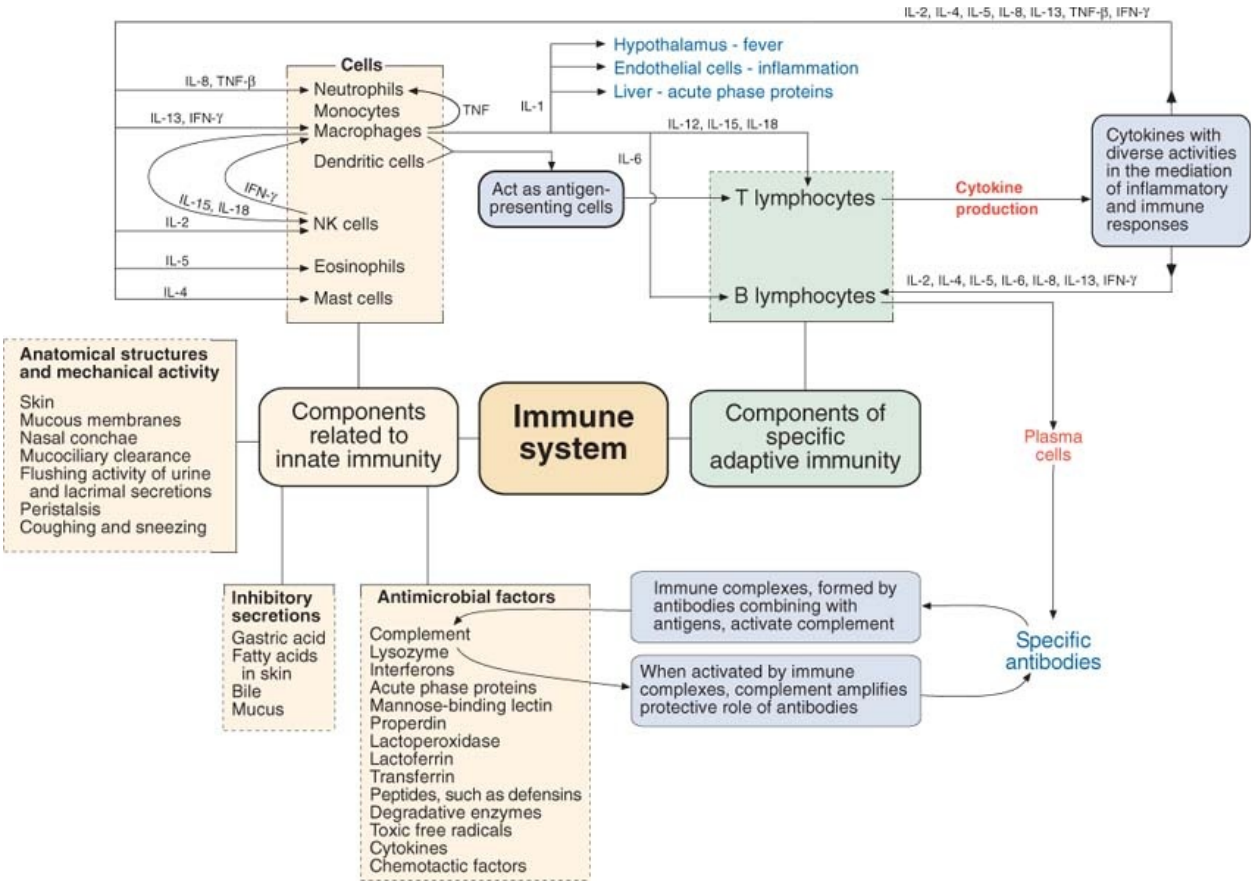
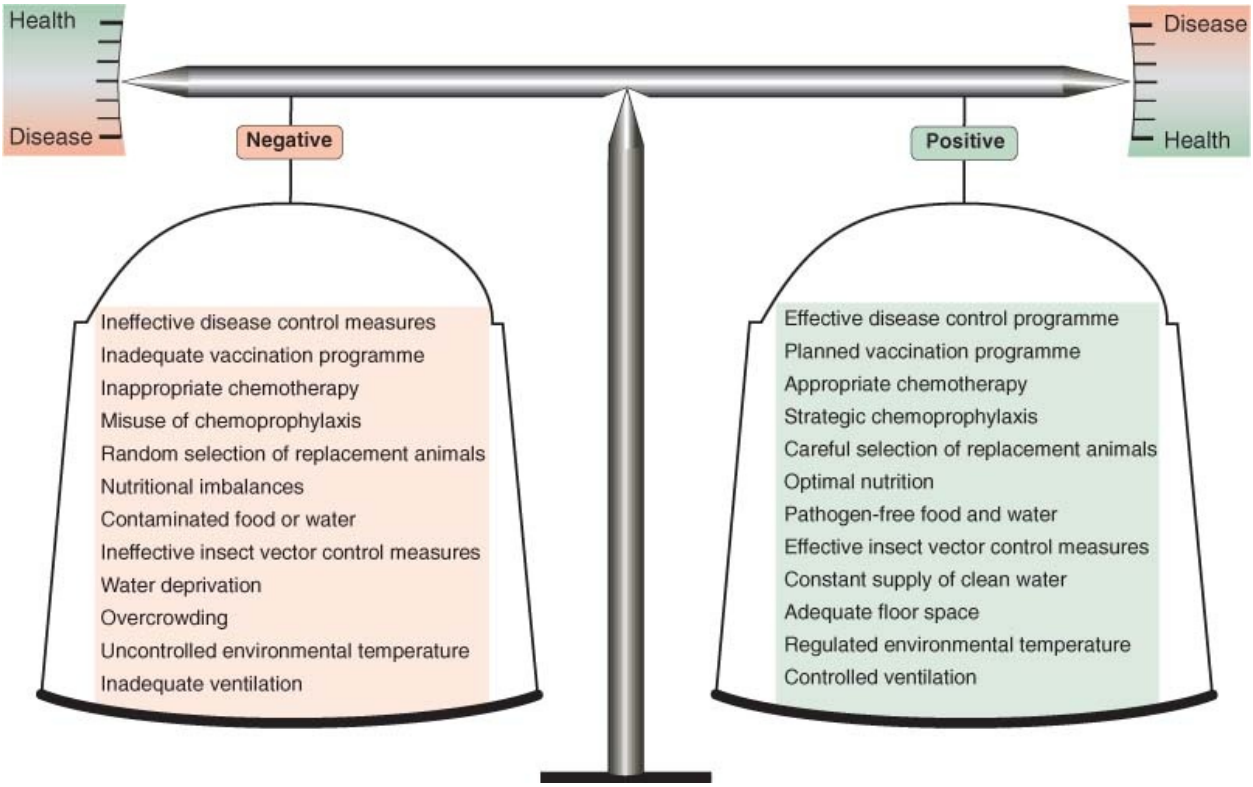


Figure 3.2 The influence of disease control programmes, environmental conditions and other factors on the health status of animal populations. The balance between positive factors that promote health, and negative factors that predispose to disease can determine the health and welfare status of animal populations.

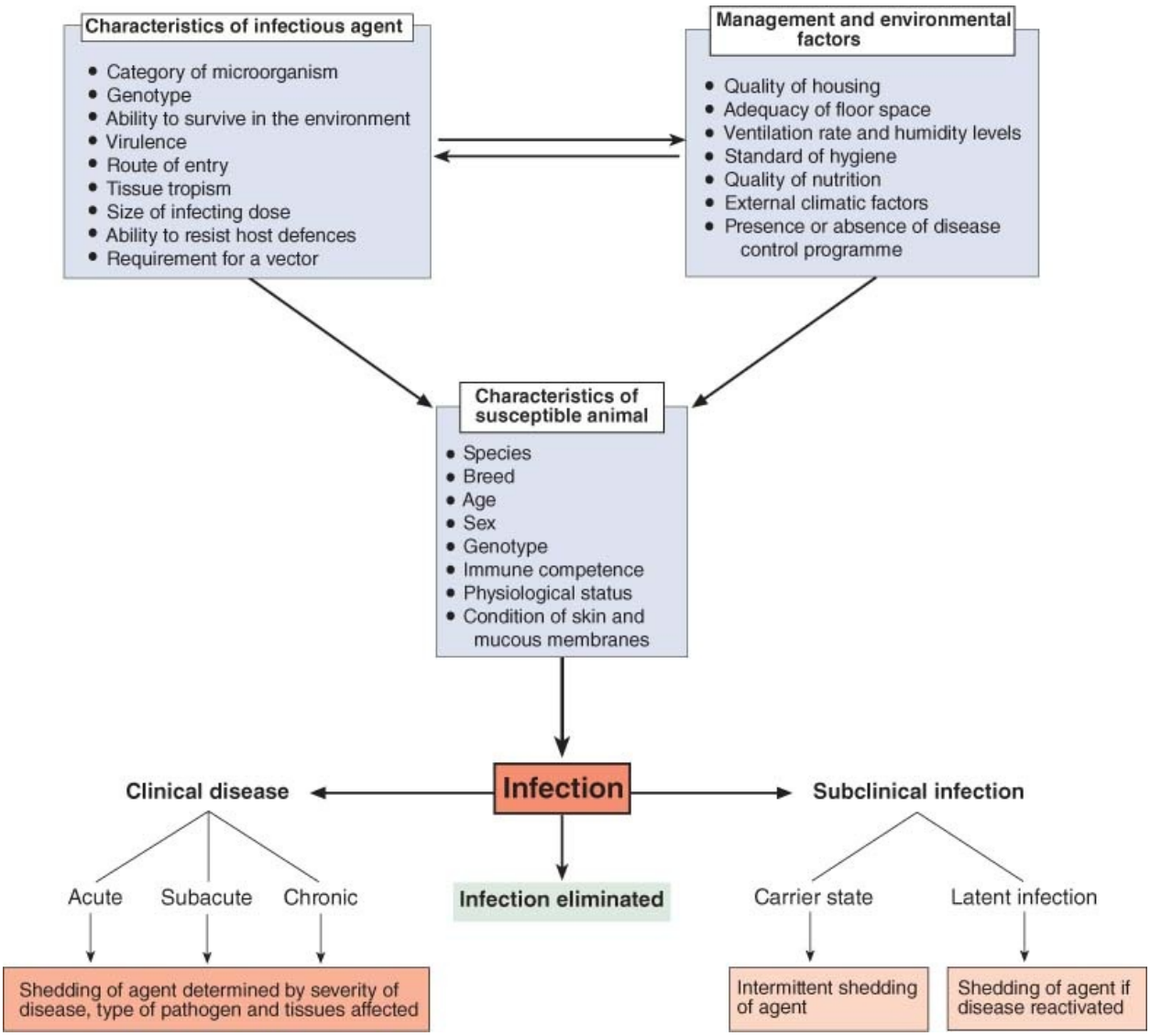


Normal flora

Soon after birth, neonatal animals are exposed through contact, ingestion or inhalation to microorganisms present on the dam. Bacteria, yeasts and eventually other microorganisms from the animal's immediate environment may colonize particular sites on the skin and regions of the alimentary, respiratory or urogenital tracts. Microorganisms that compete successfully for particular sites gradually form a stable normal flora. Different regions of the body may have a distinctive resident flora suggesting that regional colonization may reflect a selective advantage on the part of successful microorganisms. The ability to survive acidic conditions in the alimentary tract or tolerance for some naturally occurring antimicrobial factors confers particular survival capabilities on some resident flora. Adherence to host cells or synthesis of metabolic substances antagonistic to competitors may enhance colonization of the skin, mucous membranes or parts of the alimentary tract by some bacteria and yeasts. There is evidence that the normal flora can compete with and sometimes prevent establishment of pathogenic microorganisms. This may be achieved by competition for nutrients, by formation of inhibitory substances, or by attachment to receptors on cell surfaces, thereby preventing colonization by

invading pathogens. Although the normal flora is not directly associated with non-specific immunity, their competitive role can be considered beneficial for the host. In addition, normal flora may gently challenge the naïve immune system of young animals at an early age, thereby preparing them for subsequent encounters with virulent pathogens. As the animal matures, the normal flora may play a vital part in digestion, especially in ruminants. In some species, the normal flora may contribute to synthesis of the B vitamins and vitamin K. Prolonged therapy with antimicrobial drugs may interfere with normal intestinal microorganisms, permitting the survival and proliferation of organisms resistant to the drugs used. This can lead to the emergence of resistant strains of bacteria which may replace the normal flora and lead to digestive upsets and disease. In the absence of resident microorganisms in the alimentary tract, overgrowth of the pathogenic yeast *Candida albicans* can occur leading to tissue invasion.

Figure 3.3 Factors which influence host-pathogen interactions and the possible outcome of a primary infection in a surviving animal.



Comparative aspects of innate and adaptive immunity

During embryological development, myeloid and lymphoid cells arise from a pluripotent stem cell in the bone marrow (Fig. 3.4, Table 3.2). Myeloid cells, along with natural killer (NK) cells, are part of the innate immune defences. From the bloodstream, monocytes migrate to tissues where they become either fixed or free macrophages. Among the polymorphonuclear leukocytes, neutrophils play the most prominent role in combating pyogenic bacterial infections. Polymorphonuclear leukocytes move into tissues from the bloodstream in response to release of soluble factors from damaged host cells. In

addition, soluble factors in the blood or in body fluids attract inflammatory cells to developing lesions. Two types of lymphocytes referred to as T lymphocytes and B lymphocytes arise from a lymphoid stem cell in the bone marrow (Fig. 3.5). Following maturation in appropriate tissues, these specialized cells, through their secretions or direct involvement, constitute adaptive immunity.

Table 3.1 Structural, metabolic and other features of bacteria which promote disease development.

Bacterial characteristics / example	**Comments**
Presence of capsule / *Bacillus anthracis*	Can resist phagocytosis and multiply uninhibited in tissues
Intracellular replication / *Brucella abortus*	The ability to survive within macrophages is an important virulence feature of brucellae
Presence of high lipid content and mycolic acid in cell wall / *Mycobacterium bovis*	The presence of a complex, lipid-rich cell wall containing mycolic acid, which imparts resistance to environmental factors, detergents and disinfectants, renders mycobacteria resistant to intracellular killing by macrophages
Exotoxin production / *Clostridium tetani*	Production of a potent neurotoxin, tetanospasmin, is responsible for the clinical signs of tetanus
Endotoxin production / *Escherichia coli*	Endotoxin acts as a pyrogen causing fever; it also causes intravascular coagulation and hypotensive shock
Tropism for specific tissue / *Moraxella bovis*	Attachment to the conjunctiva of cattle leads to keratoconjunctivitis
Localization and replication in sites with limited immunological responses / *Leptospira interrogans* serovars	These spirochaetes localize in the renal tubules and are shed in the urine of infected animals
Synergistic bacterial interaction / *Fusobacterium necrophorum* and *Arcanobacterium pyogenes*	In ruminant foot lesions, *Arcanobacterium pyogenes* produces a growth factor for *Fusobacterium necrophorum*. Production of leukotoxin by *F. necrophorum* facilitates survival of *A. pyogenes* at the site of infection

Innate immunity and adaptive immunity are compared in Table 3.3. Innate resistance to infection is demonstrable in both vertebrates and invertebrates. Physical barriers, mechanical action, physiological factors, soluble antimicrobial substances and phagocytic cells contribute to this natural resistance to infection characterized by a rapid response but without a ‘memory’ for the pathogens encountered. The contribution of anatomical structures and mechanical activity to innate protection against infection are presented in Table 3.4. Other factors that can alter susceptibility to microbial pathogens are presented in Box 3.1. Adaptive immunity, a response to infection which is confined to vertebrates, requires induction either through infection or vaccination. The T and B lymphocytes which participate in this specific response release soluble factors called cytokines. These low molecular weight regulatory proteins or glycoproteins act as chemical messengers between cells and are produced by many cell types, especially by subsets of T lymphocytes. Antibodies, which have a high specificity for the infectious agents that induced their formation are produced by plasma cells which arise from B lymphocytes (Fig. 3.6).

Innate immune responses could be considered as the first line of defence against opportunistic pathogens whereas specific immunity, although relatively

slow in developing, ultimately produces an effective response to a wide range of virulent microorganisms. Because of the production of memory cells, secondary responses involving B and T lymphocytes are more rapid than primary responses. A comparison of T lymphocytes and B lymphocytes and their roles in adaptive immune responses is presented in Table 3.5.

Recognition of pathogens

The recognition of microbial pathogens by macrophages and other phagocytic cells relies on germline encoded molecules termed pattern recognition receptors (PRRs). Unlike the recognition system that operates specifically in adaptive immunity, these molecules do not undergo somatic mutation. These receptors, present on cells of the innate immune system, recognize conserved microbial structures referred to as pathogen-associated microbial patterns (PAMPs). Typical examples of conserved structures include lipoteichoic acid of Gram-positive bacteria, lipopoly-saccharide of Gram-negative bacteria and fungal β-glucan. Recognition of these conserved structures enables the host to identify a wide variety of microorganisms by means of a limited set of receptors. Expression of these receptors can be influenced or regulated by cytokines and other immune-modulating agents including microbial components. In addition to pathogen recognition, PRRs also influence the response mediated by macrophages and, to a large extent, determine the nature of the immune response towards the invading microorganism. These receptors may be present on cell membranes, free in serum or located intracellularly. Serum-derived PRRs include collectins, pentraxins and the complement components C1q and C3. By binding to microorganisms, complement components facilitate recognition and attachment of pathogens to opsonic receptors on phagocytic cells. Following the production of specific antibodies after the induction of adaptive immunity, microorganisms coated with antibodies bind to Fc receptors on phagocytes, providing another form of opsonic recognition for cells of the immune system. Direct recognition of microorganisms without the involvement of opsonizing molecules can occur with C-type lectins, leucinerich proteins, scavenger receptors and integrins. Although the binding and engulfment of microorganisms can be mediated by many different PRRs, generation of the appropriate responses has been attributed to a family of receptors, the toll-like receptors (TLRs). These TLRs have characteristics resembling interleukin-1 (IL-1) receptor. By acting in combinations, TLRs can distinguish various microbial

pathogens and initiate signalling cascades similar to those induced by the IL-1 receptor that results in the activation of nuclear factor kB (NF-κB) present in the cytoplasm. Activated NF-κB moves from the cytoplasm to the nucleus, binds to transcription initiation sites and increases the transcription of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β and IL-12. In addition to the macrophage receptors involved in the recognition of pathogens, there are many other surface receptors for cytokines and chemokines together with receptors involved in migration, adhesion and antigen presentation to T lymphocytes.

Figure 3.4 Origin, differentiation and maturation of cells produced during haematopoiesis in mammals. Extracellular growth factors and differentiation factors strongly influence the lineage of haematopoietic cells. CFU: colony-forming unit; Epo: erythropoietin; GM-CSF: granulocyte-monocyte colony-stimulating factor; G-CSF: granulocyte colony-stimulating factor; IL: interleukin; IFN-γ: interferon-γ; M-CSF: monocyte colony-stimulating factor; SCF: stem cell factor; SDF-1: stromal-derived factor-1; TNF-α : tumour necrosis factor-α; Tpo: thrombopoietin.

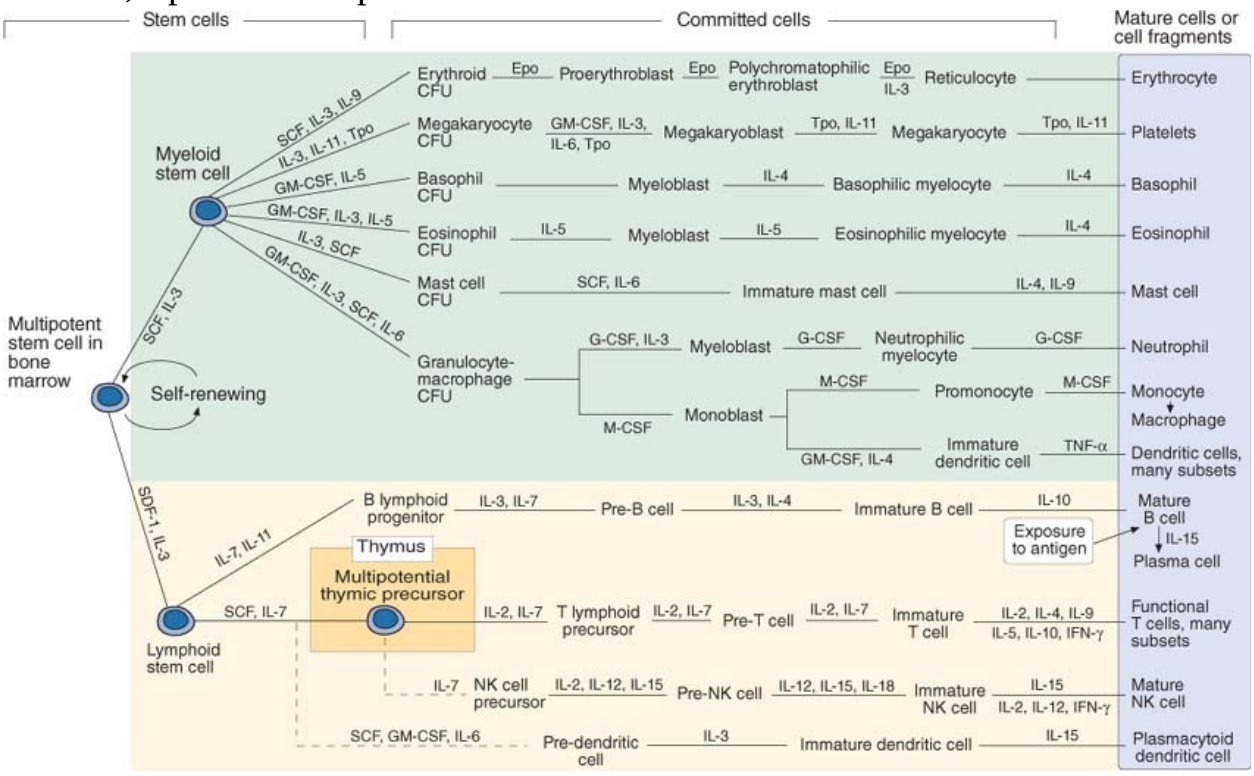


Table 3.2 Origin, lineage, distribution and other characteristics of cells produced during haematopoiesis.

Cells or formed elements	Origin	Lineage	Morphology	Distribution	Comments
Basophils	Bone marrow	Myeloid	Lobed nuclei, with large metachromatic cytoplasmic granules	Blood	Non-phagocytic circulating granulocytes, with structural and functional similarities to mast cells; the least common leukocyte observed in blood smears, less than 1% of circulating white blood cells; their granules, which stain deeply with basic dyes, contain pharmacologically active substances including histamine, proteases and cytokines; express high affinity receptors for IgE; participate in immediate-type hypersensitivity reactions; increased vascular permeability and vasodilation follow release of mediators from basophils; binding of C3a and C5a to basophil receptors results in degranulation of these cells
B lymphocytes	Bone marrow	Lymphoid	Round or slightly indented condensed nuclei	Blood and tissues	In mammals these cells mature either in the bone marrow or in gut-associated lymphoid tissue; the site of maturation of B lymphocytes in birds is the bursa of Fabricius; found mainly in lymphoid follicles and in secondary lymphoid tissues; B lymphocytes express membrane-bound antibody and after interacting with antigen they differentiate into antibody-secreting plasma cells and memory cells; as the only cell type capable of producing antibody, B cells have a central role in humoral immune responses
Dendritic cells	Bone marrow	Some arise from myeloid stem cells, others may derive from lymphoid stem cells	Large mononuclear cells with long thin processes resembling dendrites of nerve cells	Found in skin, most organs, lymphoid tissue, blood and lymph	Specialized group of antigen-presenting cells, characterized by thin membranous projections, which present antigen to T helper cells; these cells, which derive from either lymphoid or mononuclear lineages, are present in the skin and spleen and in close association with mucosal epithelia; many subsets are described: Langerhans dendritic cells, interstitial dendritic cells, interdigitating dendritic cells and plasmacytoid dendritic cells; a distinct group of these cells, follicular dendritic cells, present antigen to B lymphocytes
Eosinophils	Bone marrow	Myeloid	Bilobed nuclei with large cytoplasmic granules which have an affinity for acidic dyes	Blood and tissues	Motile granulocytes with some phagocytic activity which have a protective role against some metazoan parasites; present in large numbers in infiltrates of immediate-type hypersensitivity reactions
Erythrocytes	Bone marrow	Myeloid	Non-nucleated flattened, bi-concave cells	Blood	The most abundant cells in the blood with a lifespan of 120 days in humans and up to 140 days in domestic animals; erythrocyte production is controlled by the hormone erythropoietin which is produced by cortical and medullary kidney cells; a receptor for C3b and C4b, present on erythrocytes, promotes clearance of immune complexes

Macrophages	Bone marrow	Myeloid	Large mononuclear cells; the nuclei have irregular outlines	Present in tissues throughout the body. Some reside in particular organs, others move through the tissues	Tissue-based mononuclear phagocytes which derive from blood monocytes; named according to their location: alveolar macrophages in the lung, Kupffer cells in the liver, mesangial cell in the kidney; when activated by interferon-γ secreted by T cells or NK cells, macrophages become actively phagocytic and can destroy ingested microbial pathogens; can act as antigen-presenting cells and have a central role in innate and adaptive immune responses
Mast cells	Bone marrow	Myeloid	Mononuclear cells with metachromatic cytoplasmic granules	Connective tissue near blood vessels and nerves, the lamina propria of mucosal tissue	Contain numerous mediator-filled granules, rich in histamine and heparin; express high affinity receptors for IgE; antigen-induced cross-linking of IgE, bound to mast cell Fc receptors, causes degranulation of mast cells and release of mediators, resulting in an immediate-type hypersensitivity reaction; binding of C3a and C5a to mast cell receptors results in degranulation of these cells
Monocytes	Bone marrow	Myeloid	Large mononuclear cells with kidney-shaped nuclei	Blood	Motile, mononuclear phagocytic cells which circulate briefly in the bloodstream before differentiating into tissue macrophages
Natural killer cells	Bone marrow	Lymphoid	Large granular mononuclear cells	Blood and peripheral tissues	Large granular cytotoxic lymphocytes, distinct from T lymphocytes and B lymphocytes, which contribute to innate immune responses by destroying virus-infected cells; activate macrophages by secreting interferon-γ and also destroy tumour cells; do not possess clonally distributed antigen receptors like immunoglobulins or T cell receptors; because NK cells have receptors for the Fc portion of IgG, they can participate in antibody-dependent cell-mediated cytotoxicity, with resultant destruction of target cells with antibody bound to their surfaces

Cells or formed elements	Origin	Lineage	Morphology	Distribution	Comments
Neutrophils	Bone marrow	Myeloid	Multilobed nuclei with pale pink cytoplasmic granules	Blood, migrate into tissues in response to chemotactic stimuli	Short-lived, circulating motile phagocytic cells which engulf and destroy bacterial pathogens; their granules contain a wide range of degradative enzymes including acid hydrolases, elastase and lysozyme; engulfed microbial pathogens are digested by lysosomal enzymes
Plasma cells	Bone marrow	Lymphoid	Basophilic cells with prominent endoplasmic reticulum and eccentric cartwheel-shaped nuclei	Connective tissue and secondary lymphoid organs including the spleen, lymphoid aggregates and lymph nodes	When mature B lymphocytes interact with appropriate antigenic material, they differentiate into plasma cells; these terminally differentiated antibody-secreting cells have a lifespan of up to 2 weeks
Platelets	Bone marrow, from megakaryocytes	Myeloid	Cytoplasmic fragments	Blood	Small cytoplasmic fragments produced by megakaryocytes under the influence of thrombopoietin; adhesion of platelets to the sub-endothelium of blood vessels initiates blood clotting; during inflammatory responses, platelet numbers increase under the influence of IL-6, which seems to induce thrombopoietin production; receptor for C1q present on platelets
T lymphocytes	Arise from lymphoid stem cells in the bone marrow which mature in the thymus	Lymphoid	Round or slightly indented condensed nuclei	Blood and tissues	These cells mediate immune responses in adaptive immunity referred to as cell-mediated responses; functional subsets express CD4 or CD8 membrane glycoprotein molecules and T cell receptors; this receptor recognizes antigen bound to self major histocompatibility complex molecules on an antigen-presenting cell; functional subsets include $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes; recently recognized T lymphocytes include T_H17 which secrete inflammatory cytokines, regulatory T cells (T_{REG}), which suppress host immune responses and T_H3 cells which produce transforming growth factor-β and may contribute to differentiation of regulatory T cells

Figure 3.5 Sequential stages in the maturation of mammalian B lymphocytes and T lymphocyte subsets from a lymphoid stem cell in the bone marrow. Following encounter with antigen, effector cells and memory cells are formed. APC: antigen-presenting cell; TCR: T cell receptor.

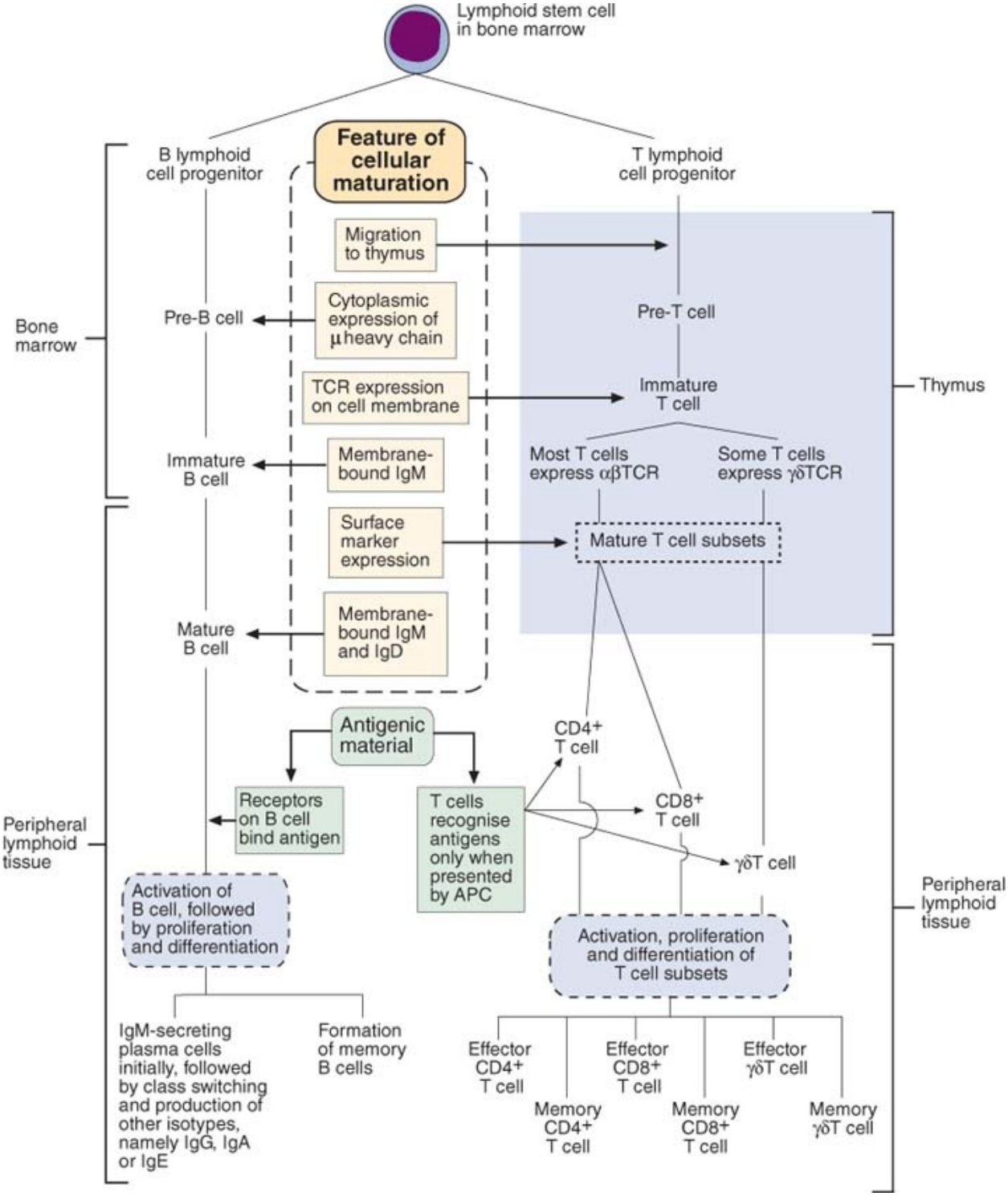


Table 3.3 Comparative features of innate and adaptive immunity.

Feature	Innate immunity	Adaptive immunity
Occurrence	Ancient form of protection present in all members of the animal kingdom	Evolved in vertebrates much later than the innate immune system
Induction	Present at birth and functions without any previous encounter with infectious agents	Develops full capability in response to antigenic challenge postnatally
Rate of response	Rapid response to infection, minutes to hours	Relatively slow; protective immunity may require up to 7 days to develop
Physical barriers	Skin, mucous membranes, mucociliary clearance, nasal conchae	Not applicable
Mechanical action	Flushing activity of tears and urine, peristalsis, coughing and sneezing	Not applicable
Physiological influences	Low pH values on skin, gastric acidity, bile, mucus	Not applicable
Specificity	Relatively non-specific; the same cells and molecules can react with determinants on a range of infectious agents	Highly specific; each responding cell is genetically programmed to respond to a single determinant on an infectious agent
Recognition of infectious agents	Infectious agents are recognized by pattern recognition receptors on phagocytes, on epithelial cells, in serum or located intracellularly	Antigen-recognition molecules consist of membrane-bound immunoglobulins on B lymphocytes and T cell receptors on T lymphocytes
Nature, number and operation of recognition molecules	Pattern recognition receptors recognize conserved microbial structures such as lipoteichoic acid and lipopolysaccharide. There are probably a few hundred receptors for pathogens	Immunoglobulins recognize determinants on foreign antigenic material or infectious agents whether bound to the original material or in a soluble form. T cell receptors recognize peptides bound to MHC molecules on host cells. A vast number of receptors for antigenic material exist on T lymphocytes and B lymphocytes
Immunological memory	Absent; the response does not increase after repeated exposure to the same infectious agent	Immunological memory is present and on repeated exposure the response becomes faster and stronger with enhanced effectiveness
Frequency of undesirable responses	Undesirable responses resulting in tissue damage are uncommon	Tissue damage resulting from undesirable responses occurs periodically with allergic reactions and autoimmune diseases
Contribution to body defences	First line of defence against opportunistic pathogens; offers limited protection against virulent microorganisms but is essential for initiating protective adaptive immune responses	Produces a long-lasting protective response to a wide range of virulent microorganisms; reinfection with the same pathogen results in a heightened immune response on the second or subsequent encounter
Participating cells	Polymorphonuclear leukocytes, monocytes, macrophages, natural killer cells, dendritic cells, mast cells and epithelial cells	B lymphocytes; T lymphocytes in association with antigen-presenting cells
Principal soluble factors	Complement, lysozyme, interferons, acute phase proteins, degradative enzymes, cytokines and antimicrobial peptides	Cytokines produced by T lymphocytes and also cytotoxic mediators; antibodies secreted by plasma cells

Table 3.4 Contributions of anatomical structures and mechanical activity to innate protection against infection.

Structure, mechanical activity	Comments
Skin	Offers mechanical protection against bacterial and fungal invasion. Desquamation of stratified epithelium removes adherent microorganisms
Mucous membranes	Because they are covered by a layer of mucus which entraps microorganisms, these membranes provide protection against entry of opportunistic pathogens. In addition, mucus contains antibacterial substances such as lysozyme. Shedding of enterocytes carries adherent bacteria to the exterior
Mucociliary clearance	Pulmonary airways are coated with mucus, and trapped particles are propelled towards the pharynx by ciliated epithelial cells

Nasal conchae	Act as baffles, deflecting particles in inhaled air on to mucus-coated surfaces
Flushing activity of urine and lacrimal secretions	Dislodge bacteria from tissue surface
Peristalsis	Propels intestinal contents and associated microorganisms towards the exterior
Coughing and sneezing	These reflex reactions expel particles from the upper respiratory tract

Box 3.1 Factors that can limit colonization by pathogenic bacteria or alter susceptibility to microbial pathogens.

- Competition for nutrients and attachment sites on host cells occurs between normal flora and bacterial or fungal pathogens
- Normal body temperature can render some species of animals resistant to particular pathogens
- Some species of animals are innately resistant to specific microbial pathogens

Cells involved in innate immune responses

From a pluripotent stem cell in the bone marrow, two important groups of cells arise: those that belong to the myeloid series and those that belong to the lymphoid series. Neutrophils, eosinophils and basophils, described as polymorphonuclear cells, arise from myeloblasts, whereas cells of the monocyte-macrophage lineage arise from monoblasts (Fig. 3.4). Although many cell types are capable of engulfing particles, two cell types, macrophages and neutrophils, are the phagocytic cells of greatest relevance to innate immunity.

Figure 3.6 Differentiation and maturation of cells of the immune system which participate in cell-mediated and humoral immunity. In birds, lymphoid stem cells which migrate to the cloacal bursa differentiate into B cells.

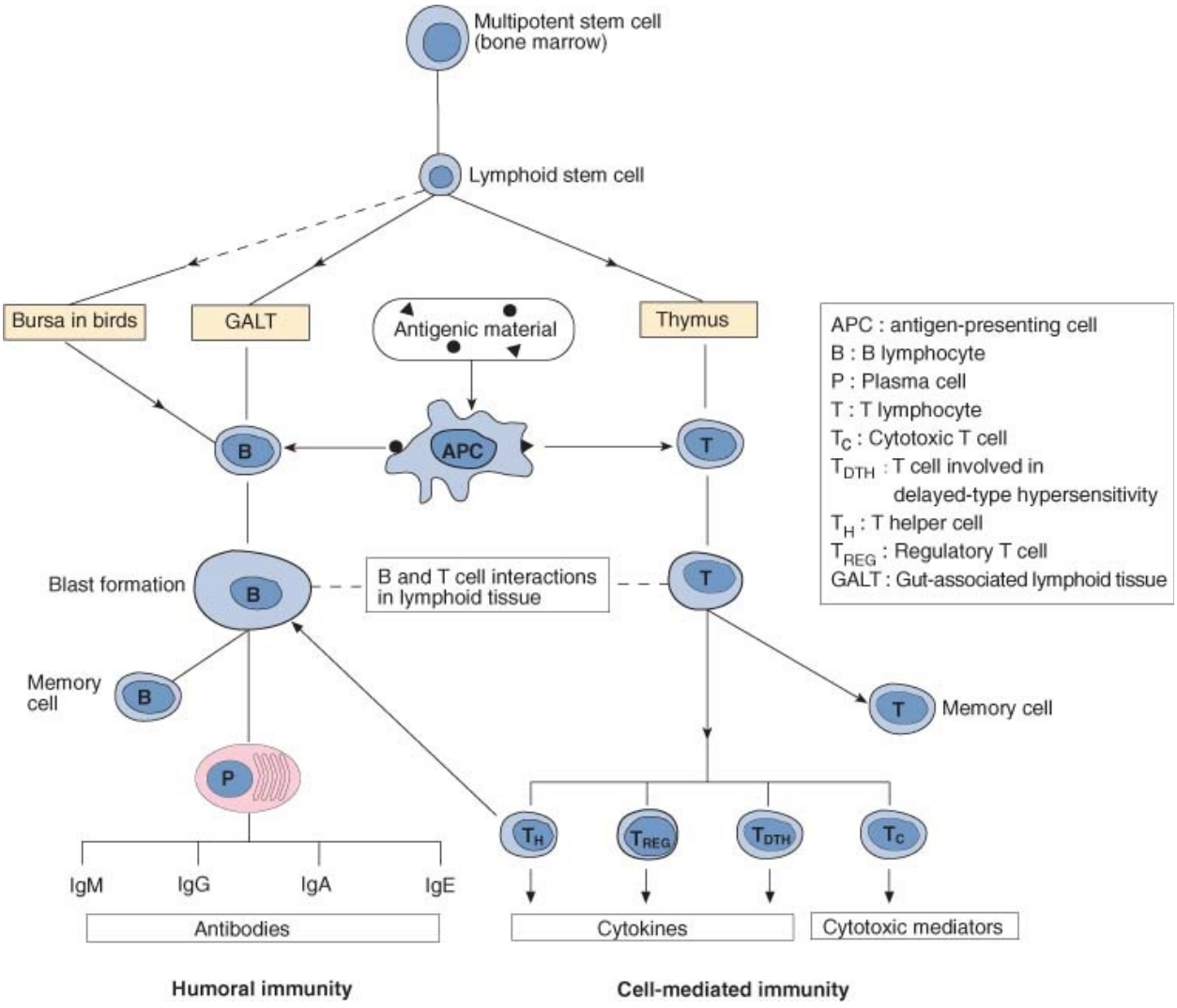


Table 3.5 Comparative features of T lymphocytes and B lymphocytes and their roles in adaptive immune responses.

Feature	T lymphocyte	B lymphocyte
Origin	Bone marrow	Bone marrow
Site of maturation	Thymus	Bursa of Fabricius in birds; bone marrow and gut-associated lymphoid tissue in mammals
Antigen receptors	T cell receptors	Membrane-bound immunoglobulins. Following interaction with antigen, B cells differentiate into plasma cells which produce antibody
Soluble factors produced	Cytokines	Antibodies
Protective role	Subsets of T lymphocytes participate in a wide range of cell-mediated immune responses	Antibodies, which have a protective role against many infectious agents, are the effector molecules of humoral immunity
Participation in hypersensitivity reactions	Participate in type IV reaction	Participate in types I, II and III reactions
Contribution to the development of immunological memory	Memory T cells produced	Memory B cells produced

Neutrophils are formed in the bone marrow and move to the bloodstream and later into the tissues. During their life span of only a few days, they are capable of rapid response to invading microorganisms, especially bacteria. Monocytes and macrophages respond slowly to bacterial invasion but they are better equipped to engulf and destroy invading pathogens, especially those microorganisms that can multiply intracellularly.

Neutrophils

When pyogenic bacteria invade the tissues, neutrophils are the first cells to arrive at the site of inflammation. Damaged endothelial cells at the site express adhesive proteins which bind neutrophils. Rolling and arrest of neutrophils on the endothelium are mediated by successive interactions with selectins and β - integrins that can overcome hydrodynamic force (Ferrante, 2005). Adhesion is followed by diapedesis when the neutrophils pass through the endothelial cell junctions and, stimulated by chemotactic factors such as C5a, migrate to the site of tissue invasion. Chemotaxis is followed by adherence to the pathogens and then phagocytosis. However, in the absence of opsonins, phagocytosis of many bacteria is ineffective.

Mononuclear phagocytes

Although they share a common progenitor cell with neutrophils, monocytes and macrophages are different in many respects. Circulating monocytes move to tissues and become resident tissue macrophages (Fig. 3.4). Tissue macrophages occur throughout the body and have different names and functions depending on the tissue: alveolar macrophages in the lungs, Kupffer cells in the liver, microglial cells in the brain. Unlike neutrophils, macrophages are long-lived cells that are better equipped to internalize and eventually destroy microorganisms. Macrophages have many important functions such as phagocytosis, antigen presentation to T cells to initiate specific immune responses and secretion of cytokines to activate lymphocytes and promote inflammatory responses.

Dendritic cells

A number of cell types which can display antigen in a form that can be recognized by T lymphocytes are referred to as antigen-presenting cells.

Although many cells that express either MHC class I or MHC class II molecules can present peptides to T lymphocytes and could be considered as antigen-presenting cells (APCs), by convention the term APCs is reserved for cells that display peptides to $CD4^+$ T cells (known as T helper lymphocytes) in the context of the MHC class molecules. Cells that present peptides in association with MHC class I molecules to $CD8^+$ T cells (also known as cytotoxic lymphocytes) are referred to as target cells. Antigen-presenting cells (namely macrophages, dendritic cells and B lymphocytes) are a heterogeneous population of leukocytes that link innate and adaptive immune responses and play a central role in the functional activity of T helper cells. Dendritic cells and macrophages are of major importance in this regard, and other cell types can have a functional role in antigen presentation for short intervals during acute inflammatory responses. Because they constitutively express a high level of MHC class II molecules and also have co-stimulatory activity, dendritic cells are considered the most effective APCs.

Dendritic cells are a specialized group of MHC class II-expressing leukocytes which are found in most tissues of the body. These cells derive from either lymphoid or mononuclear phagocyte lineages and are present in the skin, lymph nodes and spleen and in close association with mucosal epithelia. Functionally, dendritic cells can be divided into cells which process and present antigen to T lymphocytes and a separate group of antigen-presenting cells that passively present antigenic material in the form of immune complexes to B lymphocytes in lymphoid follicles. The latter cells are referred to as follicular dendritic cells. Apart from follicular dendritic cells which interact with B lymphocytes, there are at least four subsets of dendritic cells which promote the activation and differentiation of T lymphocytes. These subsets include Langerhans dendritic cells (located in the epidermis), interstitial dendritic cells, interdigitating dendritic cells and plasmacytoid dendritic cells. Although their exact function may vary with their anatomical location, their overall properties confer on them the ability to initiate and direct $CD4^+$ T cell responses. Dendritic cells are extremely effective at taking up and processing complex antigens for MHC class II-restricted presentation. They are capable of expressing a wide range of co-stimulatory molecules and can secrete soluble mediators that promote the activation and differentiation of T lymphocytes. Because dendritic cells are especially sensitive to signals from pathogens and damaged tissue, they can act as critical cells linking innate immune responses and adaptive immunity. They can recognize threats to the body from infectious agents and can signal the

immune system to detect and respond in an appropriate manner. Langerhans cells found in the epidermis and derived from a myeloid precursor typify tissue-resident dendritic cells. These cells are relatively immature, quiescent cells which monitor their environment by endocytosis and phagocytosis. If exposed to products of pathogenic microorganisms such as lipopolysaccharide flagellin, bacterial DNA or viral nucleic acid which bind to appropriate TLRs, these APCs become activated. Antigenic peptides derived from processing of ingested antigenic material are complexed with MHC class II molecules and then transferred as stable peptide)MHC class II complexes to the cell surface where they can remain for long periods. Accompanying these activities, dendritic cells become motile and migrate to draining lymph nodes. Activation of dendritic cells also results in the expression of co-stimulatory signals required for full activation of T lymphocytes. Dendritic cells are versatile antigen-presenting cells which occur in many forms and perform diverse functions. In peripheral tissues, they act as sentinels, sampling their environment and then transferring antigenic material to lymphoid organs for presentation to T lymphocytes.

A distinct group of dendritic cells, referred to as follicular dendritic cells, have characteristics that distinguish them in both lineage and function from dendritic cells which present antigenic material to T lymphocytes. Follicular dendritic cells are morphologically similar in many respects to other dendritic cells. However, these cells do not appear to arise from myeloid or lymphoid sources and do not express MHC class II molecules. Accordingly, they do not function as APCs for T helper cell activation. Another distinguishing feature of these dendritic cells is their localization in B cell follicles, hence their assigned name. Follicular dendritic cells present intact antigen or antigen complexed with antibodies to B cells, sometimes with complement components attached to the complexes. It has been suggested that retention of immune complexes on the surface of follicular dendritic cells may contribute to B cell maturation and may have a role in maintaining memory B lymphocytes.

Natural killer cells

Up to 15% of mammalian blood lymphocytes which are neither T lymphocytes nor B lymphocytes belong to a distinct population of cells, referred to as natural killer cells (NK cells). These large granular lymphocytes exhibit cytotoxicity for other cells but lack the type of receptors present on T cells and B cells. Although NK cell precursors can be isolated from the thymus, this lymphoid organ does

not have a critical role in NK cell development. Of particular importance in NK cell development is the cytokine IL-15 (Fig. 3.4). NK cells and cytotoxic T lymphocytes ($CD8^+$ T cells) share some common features. Both cell types have a lymphoid lineage, are cytotoxic and they secrete the pro-inflammatory cytokine, interferon-gamma (IFN-γ). However, in many other respects, these two cytotoxic cell types differ greatly. NK cells are found in the circulation and in blood filtering organs such as the spleen and liver. These cells exhibit natural cytotoxicity against tumour cells and virus-infected cells. During viral infections, NK cells are recruited and activated by cytokines secreted by infected cells or myeloid cells. Type I interferons, IL-12 and IL-15 are of special importance in the stimulation of NK cell production and in the activation of these cells. Despite lacking antigen-specific receptors, NK cells can recognize tumour cells or virus-infected cells. Potential target cells can be recognized in two different ways. An NK cell uses an NK-cell receptor to detect abnormalities such as unusual surface antigens or a reduction of normal surface molecules called major histocompatibility molecules (MHC class I). In some viral infections, infected cells display viral antigens on their surfaces which in turn induce an antibody response, and antiviral antibodies bind to the displayed viral antigens. Because NK cells have receptors for bound antibodies, they can attach to the antibodies on virus-infected cells and lyse them. This cytotoxic reaction is called antibody)dependent celhmediated cytotoxicity (ADCC). In this reaction, antibody bound to the virus-infected cell identifies such a cell for destruction by NK cells.

Destruction of target cells by NK cells shares some common features with cytotoxic T lymphocytes. The cytoplasm of NK cells contains numerous granules which store perforin and granzymes. On contact with a target cell, the contents of the granules are released on to the surface of the target cell, and in the presence of high extracellular calcium levels perforin induces small lesions in the target cell surface. These allow entry of the granzymes, which are serine proteases, into the target cell. The serine proteases cleave and activate cellular caspases, and this results in target cell death by apoptosis.

Involvement of NK cells is evident at an early stage in viral infections together with production of type I interferons. These two components of innate immune responses offer considerable short-term protection against viral pathogens before adaptive immune responses develop. NK cells do not develop immunological memory.

Complement

The complement system consists of approximately 30 serum and membrane proteins which can mediate or contribute to a variety of immune reactions including promotion of inflammatory responses, chemotaxis, opsonization and destruction of cell membranes and of enveloped viruses. The soluble proteins that constitute the complement system are synthesized at various sites throughout the body. However, the liver is a major site of synthesis of some components; monocytes, macrophages and epithelial cells of the gastrointestinal and genitourinary tracts also produce appreciable amounts of individual components. Most of the membrane-bound proteins are synthesized in the cells on which they are expressed. The majority of complement components circulate as functionally inactive forms as proenzymes until proteolytic cleavage removes an inhibitory fragment and exposes the active site of the molecule. The complement system can be activated by microorganisms in the absence of antibody as part of the innate immune response to microbial infection and also by antibodies attached to pathogens as part of the adaptive immune response to infectious agents. In evolutionary terms, the complement system is regarded as a very ancient defence mechanism that predates the development of the adaptive immune system.

Complement components are designated by numerals from C1 to C9. The number assigned to each complement component refers to the order in which these individual proteins were discovered rather than to their position in the activation sequence. The biochemical reaction sequence is C1-C4-C2-C3-C5-C6-C7-C8-C9. Letter symbols, in upper case letters, identify factors that interact with complement components, and lower case letters after a complement component are used to designate peptide fragments formed by activation of that component. A bar over the activated component indicates a fragment that possesses enzymatic activity following cleavage. Examples of such activation may relate to an individual component such as C4b or a complex such as $C\overline{4b}$, $\overline{2a}$, the latter referred to as C3 convertase. Activated complement components or complex become covalently attached to cell surfaces where activation occurs, thereby ensuring that activation is confined to defined sites. A small number of activated complement components leads to the production of large numbers of effector molecules. For this reason the complement system is effectively regulated by a number of circulating and cell membrane proteins.

Complement activation

There are three pathways of complement activation, two initiated by microorganisms without the requirement for antibody, called the alternative and lectin pathways, and a third initiated by immune complexes, called the classical pathway (Fig. 3.7). Components involved in the classical, alternative and lectin pathways of complement activation are listed in Table 3.6. The three activation pathways converge on a common terminal pathway that leads to the formation of the membrane attack complex which produces transmembrane pores in the membranes of target cells resulting in osmotic lysis of these cells.

Figure 3.7 Activation of complement through the classical, alternative and lectin pathways. The formation of immune complexes (antibody bound to antigen) initiates the classical pathway. Microbial surfaces can activate the alternative pathway and microbial carbohydrates can activate the lectin pathway. These two activation methods proceed without the requirement for antibody.

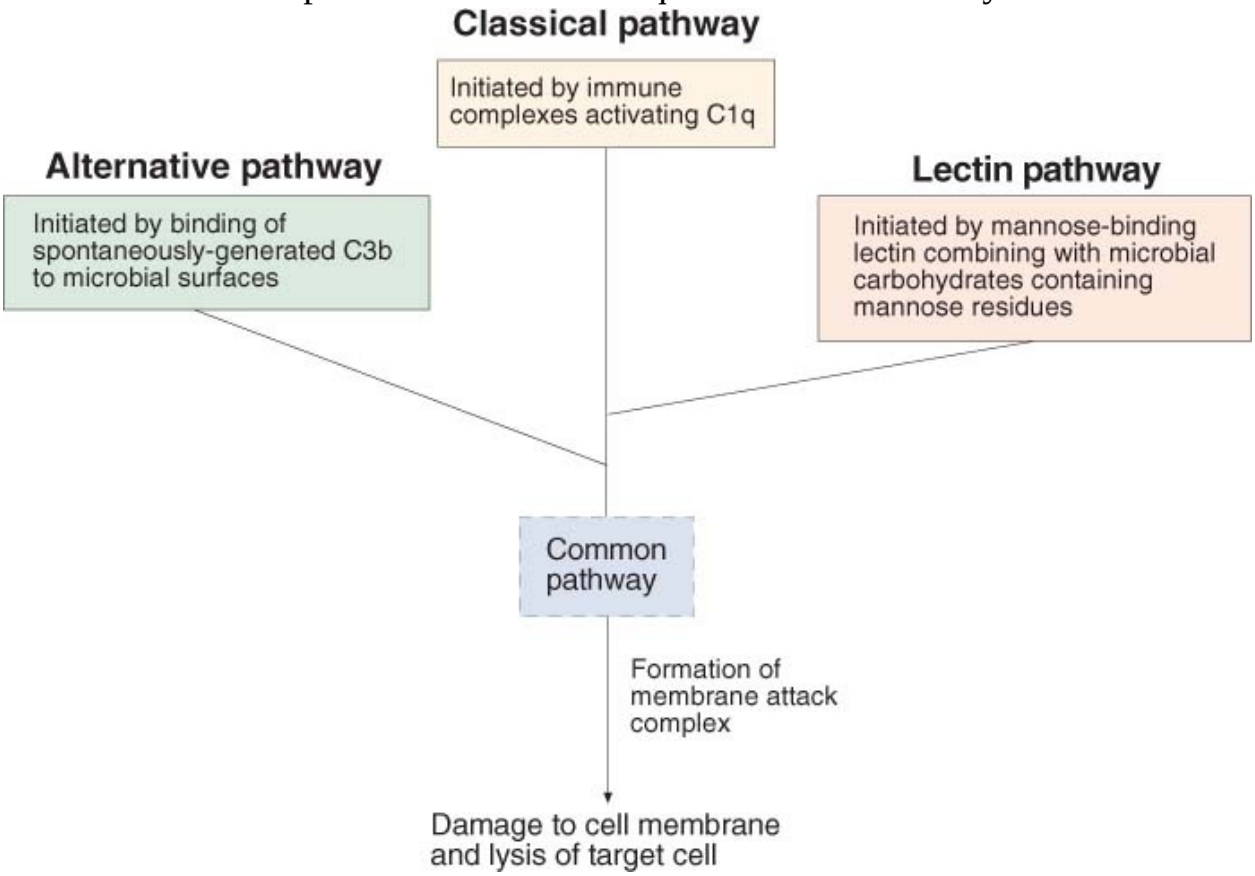


Table 3.6 Components involved in the classical, alternative and lectin pathways of complement activation.

Component	Role

Classical pathway	
C1q	Binds through its globular head groups to the Fc region of antibody in an immune complex and causes the autocatalytic activation of C1r
C1r	Activated C1r cleaves C1s
C1s	When activated, C1s has serine protease activity and this enzyme has two substrates, C4 and C2. When activated, the trimolecular complex of C1q, r and s is designated $C\overline{1}$
C4	Cleavage of C4 by C1s produces two fragments, C4b which binds to $C\overline{1}$ and another fragment, C4a
C2	Binds to C4b and is cleaved by C1s into C2a, which binds to the complex, and another fragment C2b. The combination of $C\overline{4b}, \overline{2a}$ (C3 convertase), cleaves C3 into C3b which binds to the complex and also releases the fragment C3a
C3 (common to the three pathways)	When acted on by C3 convertase, C3 is cleaved into C3b which binds to C4b, 2a and a small fragment, C3a is released. The complex of $C\overline{4b}, \overline{2a}, \overline{3b}$ forms C5 convertase
Alternative pathway	
C3	The spontaneous hydrolysis of C3 to C3b and the binding of C3b to microbial surfaces initiates activation of the alternative pathway
Factor B	This factor binds to C3b and activates factor D
Factor D	When factor D acts on bound factor B, the latter is cleaved into the fragments Ba and Bb; Bb binds to the complex, forming $C\overline{3b}, \overline{Bb}$ (C3 convertase)
Properdin	The complex, $C\overline{3b}, \overline{Bb}$, is stabilized by the binding of properdin
Lectin pathway	
Mannose-binding lectin (MBL)	Binds to mannose residues on glycoproteins or carbohydrates on the surface of microorganisms; this molecule has activity similar to C1q
MBL-associated serine proteases (MASP), MASP-1 and MASP-2	Following binding of MBL to mannose residues, MASP-1 and MASP-2 become autocatalytically activated and behave like C1 in the classical pathway, cleaving C4
Terminal pathway	
C5	The complex $C\overline{4b}, \overline{2a}, \overline{3b}$ (C5 convertase) in the classical pathway, acts on C5 cleaving it into C5b which binds to the complex with the release of the fragment C5a; in the alternative pathway $C\overline{4b}, \overline{Bb}, C\overline{3b}$ forms C5 convertase
C6	Binds to C5b and interacts with C7
C7	Binds to the $C\overline{5b}, \overline{6}$ complex and creates a binding site for C8
C8	Binds to the $C\overline{5b}, \overline{6}, \overline{7}$ complex
C9	When C9 binds to the $C\overline{5b}, \overline{6}, \overline{7}, \overline{8}$ complex, it forms the membrane attack complex

Classical pathway

Activation of the classical complement pathway is usually associated with immune complex formation involving IgM or IgG antibodies. Formation of an antigen-antibody complex induces conformational change in the Fc portion of the antibody molecule bound to antigen, exposing a binding site for the C1 component of the complement system. In plasma, C1 is a large multicomponent complex, composed of a single molecule of the recognition molecule C1q, two molecules of the enzymatic unit C1r and two molecules of the enzymatic unit C1s. The three subunits of C1, namely C1q, C1r and C1s, associate with each

other in a calcium-dependent complex. Binding of C1q to the Fc portion of the antibody results in conformational changes in the C1q molecule which in turn induces conformational alternations in the C1r proenzyme, causing autocatalytic activation of the molecule. Activated C1r cleaves C1s which exhibits serine protease activity. In the activated $C\overline{1}$ complex, C1s cleaves the next component of the classical pathway, C4 into C4a and C4b. The C4b fragment attaches to the target surface area in the vicinity of C1 and the C2 proenzyme then attaches to the exposed binding site on C4b in the presence of magnesium ions. When C2 binds to the complex, it is then cleaved by the adjacent C1s giving rise to C2a and the smaller fragment C2b diffuses away. The resulting $C\overline{4b}$, $\overline{2a}$ complex is called C3 convertase because C2a in the complex, with its serine protease activity, cleaves C3, the most abundant of the complement proteins. Cleavage of C3 releases a small, biologically active peptide, C3a, and the other fragment, C3b, binds to the activating $C\overline{4b}$, $\overline{2a}$ complex creating a new enzyme $C\overline{4b}$, $\overline{2a}$, $\overline{3b}$, referred to as C5 convertase. The next component, C5, attaches to C3b in the C5 convertase complex and is cleaved by $C\overline{2a}$ into C5b which attaches to the complex and C5a which diffuses away (Fig. 3.8).

The membrane attack or terminal pathway of complement activation involves $C\overline{5b}$, C6, C7, C8 and C9 which interact sequentially, forming a macromolecular structure called the membrane attack complex. This complex forms a pore in the membrane of the target cell which allows the free movement of water and ions, resulting in osmotic lysis of the target cell. Formation of the membrane attack complex involves binding of C6 by $C\overline{5b}$. A newly exposed binding site in C6 enables the $C\overline{5b}$, $\overline{6}$ complex to bind C7. Attachment of C7 to the complex results in conformational change in the trimolecular $C\overline{5b}$, $\overline{6}$, $\overline{7}$ complex, its release from the convertase and the formation of a binding site for C8. Binding of C8 to the complex induces a conformational change in C8 and this component interacts with the plasma membrane of the target cell. The $C\overline{5b}$, $\overline{6}$, $\overline{7}$, $\overline{8}$ complex creates a small pore in the target cell membrane. The final step in the formation of the membrane attack complex is the binding and polymerization of C9 to the $C\overline{5b}$, $\overline{6}$, $\overline{7}$, $\overline{8}$ complex. When C9 binds to the $C\overline{5b}$, $\overline{6}$, $\overline{7}$, $\overline{8}$ complex, it undergoes major conformational change. The formation of this terminal complex exposes additional binding sites for C9 thereby facilitating completion of the membrane attack complex which becomes inserted into the cell membrane forming a pore, clearly visible by electron microscopy. Pore formation in the membrane of erythrocytes leads to osmotic lysis. Nucleated cells can resist killing by a combination of ion pump activity and membrane repair. However, membrane

attack complex-induced lesions can sometimes cause apoptosis in nucleated target cells.

A feature of complement activation is the initiation of a cascade of proteolytic events. A single C3 conver-tase complex can generate hundreds of C3b molecules, thereby amplifying this step of the sequence. As a consequence of activation, complement components, fragments and complexes play a central role in innate immune responses to infectious agents; they also amplify the protective role of specific antibodies to pathogenic microorganisms.

Lectin pathway

The lectin pathway, which is activated by microbial carbohydrates, differs from the classical pathway in the initial recognition and activation steps. Lectins are proteins that recognize and bind to specific carbohydrate targets, and mannose-binding lectin is a high molecular weight serum lectin that binds to mannose and N-acetylglucosamine present in the cell walls of diverse pathogens including bacteria, fungi and protozoa and on the envelopes of some viruses. The enzymatic activity of this method of complement activation resides in mannose-binding lectin-associated serine proteases, MASP-1 and MASP-2 (Fig. 3.8). When mannose-binding lectin binds to the carbohydrate residues on the surface of pathogens, it induces auto-catalytic activation of MASP-1 and MASP-2, a consequence of conformational changes resulting from particle binding. The serine protease activity arising from this activation cleaves C4 and C2 in a manner similar to $C\bar{1}$ in the classical pathway as MASP-1 and MASP-2 structurally resemble C1r and C1s and their biochemical activities are also similar. Activation of C4 and C2 without the requirement for specific antibody represents an important innate defence mechanism comparable to the alternative pathway but retains close links with the classical pathway of activation. Recent evidence indicates that ficolins, a family of lectin-like plasma proteins, can replace mannose-binding lectin and also form complement-activating complexes with MASP-1 and MASP-2 (Morgan, 2005).

Alternative pathway

Activation of the alternative pathway can occur efficiently only at cell surfaces, typically microbial cell walls. This alternative pathway of complement activation also provides an antibody-independent method of initiating conversion of C3 to C3b. Normally, C3 in plasma is being continuously cleaved at a low rate,

generating C3b in a process referred to as C3 ‘tickover’. This spontaneous hydrolysis of C3 occurs because it contains an unstable thioester bond. For activation of the alternative pathway, four plasma proteins are required: C3, factor B, factor D and properdin. When C3b is generated in the fluid phase, two inhibitors, factors H and I, inactivate most of its activity. Factor H binds C3b and recruits factor I which has protease activity and cleaves C3b, abolishing its convertase function (Fig. 3.9). However, when C3b is bound to a cell surface, it first interacts with factor B and then with factor D and the resulting complex is stabilized by properdin. Under these conditions, factors H and I have less inhibitory activity on C3b.

Figure 3.8 Outline of the pathways that lead to activation of complement. The usual method of activation of the classical pathway follows binding of C1q to the Fc portion of IgM or IgG antibody involved in the formation of an immune complex. Activation of the alternative pathway, which proceeds without the requirement for immune complex formation, results from the binding of spontaneously generated C3b to microbial surfaces. The lectin pathway, also an antibody-independent route of activation, follows the binding of mannose-binding lectin to mannose residues on the surface of microorganisms. The complement components from C5 to C9, which are activated sequentially, constitute the terminal pathway. MASP, mannose-binding lectin-associated serine protease.

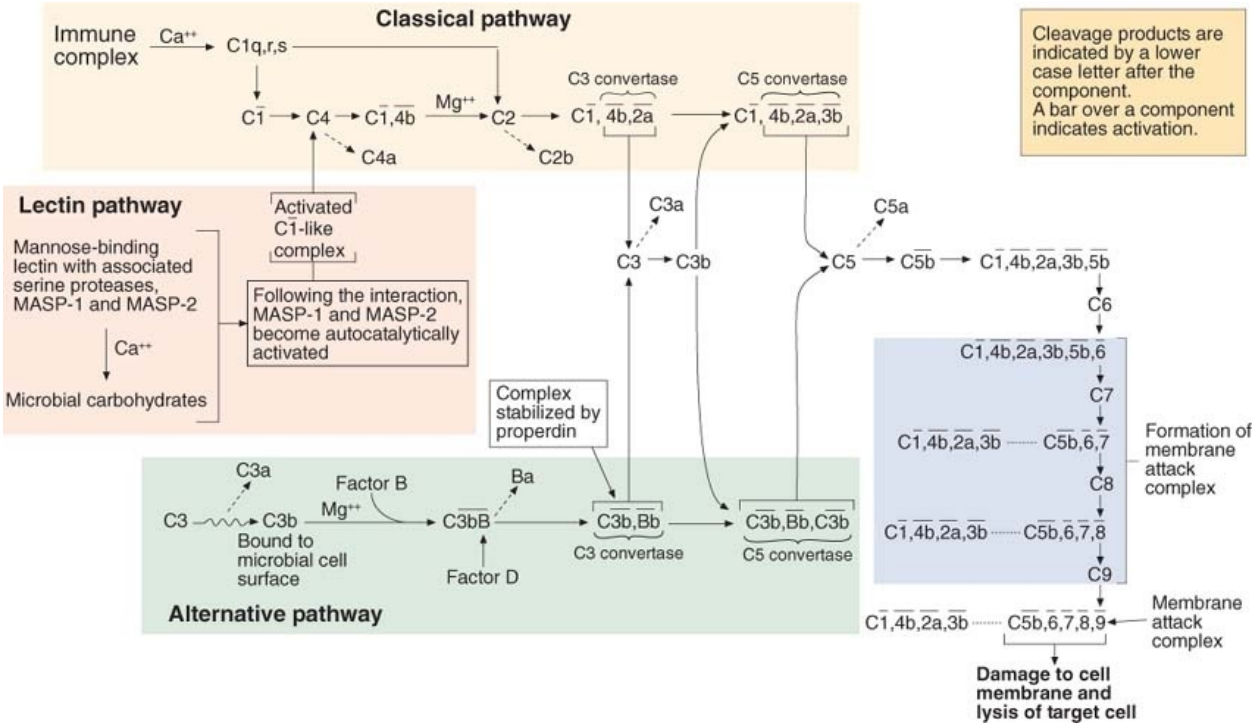


Table 3.7 Molecules derived from the immune system, particles of microbial, parasitic and mammalian origin and chemicals which are capable of activating the complement pathways.

Pathway	Usual method of activation	Other activating particles or substances
Classical pathway	Immune complexes formed by IgM or IgG antibodies combined with antigen	Components of damaged cells, C-reactive protein, some viruses, bacterial lipopolysaccharide, nucleic acids, dextran sulphate
Lectin pathway	Mannose-binding lectin attached to mannose and *N*-acetylglucosamine in the cell walls of bacteria, fungi and protozoa and in the envelopes of viruses	Some viruses, especially retroviruses
Alternative pathway	Interaction of spontaneously generated C3b with microbial surfaces	Some viruses and virus-infected cells, fungal cell walls, pathogenic protozoa, immune complexes composed of IgA and IgE bound to antigen, agarose, dextran sulphate

The membranes of most mammalian cells have a high level of sialic acid which contributes to the rapid inactivation of bound C3b on host cells. Unlike mammalian cells, microbial surfaces such as bacterial cells, fungal cells and some viral envelopes have low levels of sialic acid and, accordingly, C3b bound to such surfaces remains active for a longer time. When C3b attaches to the surface of a microbial cell, it binds factor B in a magnesium-dependent reaction; after it is bound, factor B is cleaved by factor D which has serine protease activity, generating a fragment called Bb which remains attached to C3b with the release of a smaller fragment, Ba (Fig. 3.8). The $C\overline{3b}$, $\overline{Bb}$ complex, which has C3 convertase activity, is analogous to the $C\overline{4b}$,$\overline{2a}$ complex in the classical pathway. The plasma protein properdin contributes to the survival of $C\overline{3b}$, $\overline{Bb}$ by binding and stabilizing the complex. Some of the C3b molecules, generated by the alternative pathway C3 convertase, bind to the convertase itself and this results in the formation of a $C\overline{3b}$, $\overline{Bb}$, $C\overline{3b}$ complex, which functions as the alternative pathway C5 convertase. Cleavage of C5 into $C\overline{5b}$, which remains associated with the convertase, and C5a which diffuses away, is the last proteolytic step in the alternative pathway. Incorporation of $C\overline{5b}$ into the membrane attack complex occurs by the same sequence of reactions as in the classical pathway.

Apart from the well recognized methods of activating the complement pathways, additional activator substances include viruses, components of bacterial and fungal cells and a number of chemicals (Table 3.7).

Regulation of complement activation

Many elements of the complement system are capable of damaging host cells as well as foreign cells and microorganisms. To minimize damage to self, complement activation is tightly controlled at many stages by regulatory plasma proteins and by membrane-bound protein widely distributed on blood cells,

endothelial cells and other cell types. Another mechanism of regulation in all complement pathways is the generation of highly labile components that undergo rapid inactivation if they are not stabilized by other components. In the activation pathways, regulators block or regulate the enzymes that cause amplification. The first step of the classical pathway is regulated by C1 inhibitor, a serine protease inhibitor present in plasma. The C1 inhibitor forms a complex with C1r and C1s, causing them to dissociate from C1q, thereby preventing further activation of C4 or C2.

Control of C3 convertase is provided by factor H in the alternative pathway and by C4-binding protein in the classical pathway (Fig. 3.9). On the membrane of cells, decay-accelerating factor accelerates the decay of C3 convertase. Membrane cofactor protein acts as a cofactor for the cleavage of C4b and C3b by factor I. The membrane attack complex is regulated by inhibitors present in plasma and on cell membranes. A plasma protein, called S protein, and another plasma protein, clusterin, contribute to the regulation of the membrane attack complex by preventing the complex $C\overline{5b}$, $\overline{6}$, $\overline{7}$ from becoming inserted into the membrane of nearby cells. Binding of C8 to the complex of $C\overline{5b}$, $\overline{6}$, $\overline{7}$, in the fluid phase, blocks membrane binding and prevents formation of the membrane attack complex. Complement-mediated lysis of cells is more effective if the source of complement is from one species and the cells being lysed are from a different species. If the complement and the target cells are from the same species, inhibition occurs. The basis of this inhibition is that a membrane protein, homologous restriction factor or CD59, can block formation of the membrane attack complex by binding to C8 and preventing assembly of poly-C9 and its insertion into the membrane of the target cell. Regulatory proteins of the complement system, their distribution, the pathway affected and functions are presented in Table 3.8.

Figure 3.9 Proteins (in red) which, either alone or in association with cofactors, regulate complement activation. DAF, decay accelerating factor. MASP, mannose-binding lectin-associated serine protease.

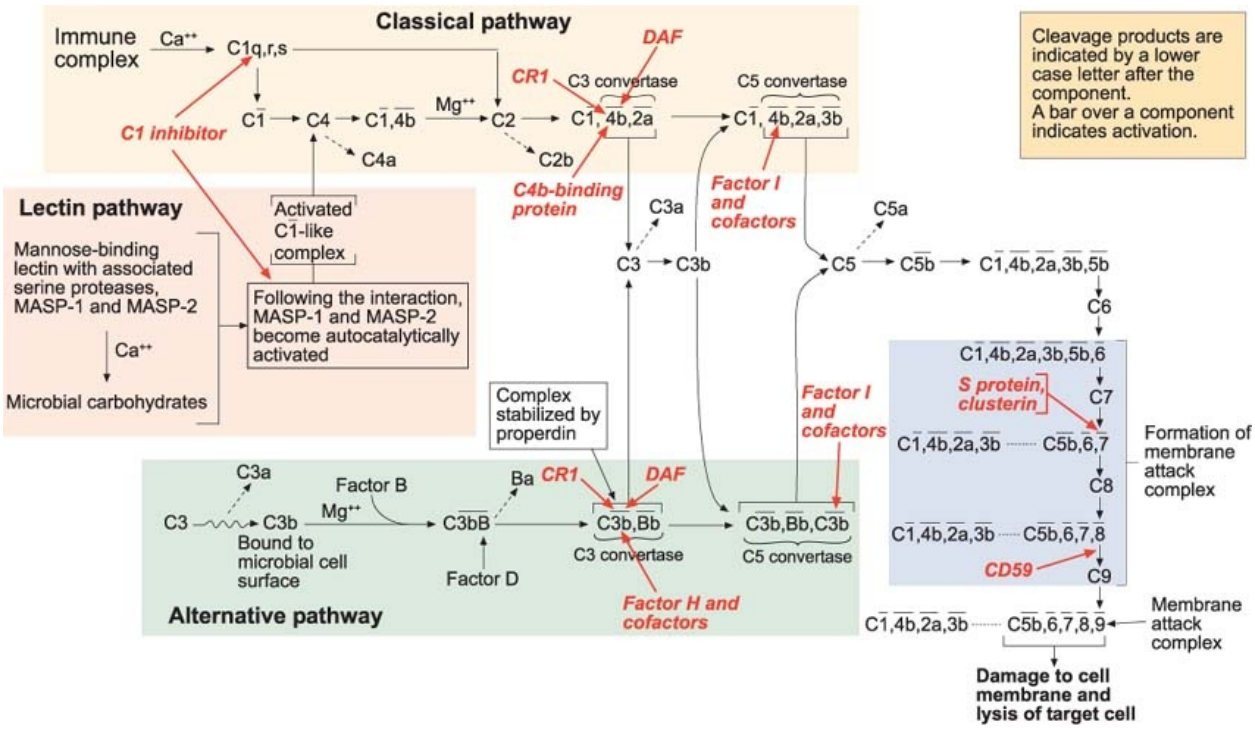


Table 3.8 Regulatory proteins of the complement system.

Regulatory protein	Distribution	Pathway affected	Function
C1 inhibitor	Plasma	Classical and lectin pathways	Inhibits the enzymatic activity of C1r and C1s; removes MASP enzymes from the MBL complex
C4-binding protein	Plasma	Classical and lectin pathways	Blocks formation of C3 convertase by binding C4b; cofactor for cleavage of C4b by factor I
Factor H	Plasma	Alternative pathway	Blocks formation of C3 convertase by binding C3b; cofactor for cleavage of C3b by factor I
Factor I	Plasma	Classical, alternative and lectin pathways	In the presence of an appropriate cofactor, cleaves C4b and C3b, thereby inactivating C3 convertase and C5 convertase
Membrane cofactor protein	Membrane of white blood cells, epithelial cells, endothelial cells	Classical, alternative and lectin pathways	Cofactor for factor I-mediated cleavage of C4b and C3b
Decay-accelerating factor	Membrane of blood cells, epithelial cells, endothelial cells	Classical, alternative and lectin pathways	Accelerates dissociation of C4b,2a and C3b,Bb
S protein and clusterin	Plasma	Act on the terminal pathway	Bind soluble C5b,6,7 and prevent its insertion into the lipid bilayer of the target cell
CD59 (protectin)	Blood cells, epithelial cells, endothelial cells	Acts on the terminal pathway	Blocks binding of C9 and prevents formation of the membrane attack complex
CR1 (CD35)	Neutrophils, mononuclear phagocytes, eosinophils, follicular dendritic cells, lymphocytes	Classical, alternative and lectin pathways	Blocks formation of C3 convertase by binding C3b or C4b. Cofactor for factor I-catalysed cleavage of C3b or C4b

Role of complement in host defences

Through its diverse interactions with components of innate immunity and also with humoral immune responses, the complement system initiates and amplifies inflammatory reactions, promotes phagocytosis of microorganisms and induces lysis of microbial, protozoal and mammalian cells. In addition, cleavage products of complement components activate B lymphocytes and enhance antibody production.

Through its activation, the complement system contributes to the development of inflammatory reactions and forms one of the major defence systems of the body, not only through its involvement in innate immune responses but also through its role in adaptive immune responses. The classical pathway of complement activation links innate and adaptive immune mechanisms. Binding of C1q to the Fc portion of IgM or IgG already combined with antigen activates complement proteins involved in the classical pathway, amplifies the protective role of specific antibodies, promotes the attraction of phagocytic cells by generating a number of chemotactic peptides and opsonizes microbial pathogens. Formation of the membrane attack complex on the surface of microbial cells and on enveloped virus particles results in lysis of susceptible target cells or viruses.

Table 3.9 Receptors for complement components, their cellular distribution and functions.

Receptor	Complement component or fragment bound	Cellular distribution of receptor	Functions
C1qR	C1q	Polymorphonuclear leukocytes, monocytes, macrophages, endothelial cells, platelets	Binds collagenous tail of C1q and promotes phagocytosis
C1qRp (CD93)	C1q	Endothelial cells, monocytes, macrophages, neutrophils	Binds to globular heads of C1q and enhances phagocytosis
CR1 (CD35)	C3b, C4b	B cells, erythrocytes, monocytes, macrophages, eosinophils, follicular dendritic cells	Promotes cleavage of C3b and C4b; blocks formation of C3 convertase, promotes clearance of immune complexes
CR2 (CD21)	iC3b[a], C3d[b]	B cells, follicular dendritic cells, some T cells	Promotes activation of B cells and antibody production
CR3 (CD11b/CD18)	iC3b, ICAM-1[c]	Monocytes, macrophages, neutrophils, NK cells, follicular dendritic cells	Binds cell adhesion molecules, enhancing leukocyte adhesion to endothelium and phagocytosis
CR4 (CD11c/CD18)	iC3b	Monocytes, macrophages, neutrophils, NK cells, dendritic cells	Binds immune complexes and promotes phagocytosis
CRIg	C3b, iC3b	Kupffer cells	Promotes phagocytosis of particles and pathogens in the bloodstream
C3aR	C3a	Basophils, mast cells, neutrophils, smooth muscle cells	Activates mast cells and basophils inducing degranulation; promotes smooth muscle contraction
C5aR	C5a	Mast cells, basophils, endothelial cells, smooth muscle cells, macrophages, neutrophils	Induces chemotaxis and inflammatory responses; degranulation of mast cells and basophils

a, Inactivated C3b; b, cleavage fragment of C3b; c, intercellular adhesion molecule-1.

In recent years it has become apparent that immune complexes with bound C3b are highly efficient in stimulating B cells. These observations arose from studies in complement-depleted mice and complement-deficient mice in which antibody responses to antigenic material were markedly reduced. A number of mechanisms contribute, either directly or indirectly, to these stimulatory effects of complement fragments on B cells. Type 2 complement receptors (CR2) stimulate humoral immune responses by enhancing B cell activation by antigen and by promoting the trapping of immune complexes in germinal centres. When immature B cells bind antigenic material through the B cell receptor and simultaneously attach to bound C3d through CR2, this dual binding promotes B cell maturation. While in lymph nodes, mature B cells interact with opsonized antigenic material, become activated and proliferate. Follicular dendritic cells in lymphoid organs capture antigenic material through attached C3 fragments and present it to activated B cells which proliferate and differentiate into plasma cells and memory cells. The importance of C3d in the generation of an efficient antibody response is now being utilized in immunization studies where C3d coupled to antigen acts as a molecular adjuvant.

The binding of complement components and their fragments to cell receptors mediates many of the biological activities of the complement system. Although complement receptors are present on many cell types, they are especially prominent on neutrophils, monocytes, macrophages, follicular dendritic cells, mast cells and basophils (Table 3.9). Accordingly, opsonization, binding to phagocytic cells and phagocytosis feature prominently in the functions of these cell receptors. Degranulation of mast cells and basophils by the small cleavage fragments of C3 and C5, namely C3a and C5a, results in the release of vasoactive amines including histamine and 5-hydroxytryptamine which cause smooth muscle contraction and increased vascular permeability and contribute to the inflammatory response. Because of their ability to release vasoactive amines from mast cells and basophils and to mimic anaphylactic reactions, C3a and C5a are sometimes referred to as anaphylatoxins. The activity of these anaphylatoxins is curtailed by a plasma enzyme, carboxypeptidase-N, which cleaves the carboxy terminal amino acid arginine from both of these fragments, rendering C3a inactive and decreasing the activity of C5a.

Binding of C3b to immune complexes composed of virions and antibodies opsonizes the complex and promotes phagocytosis. Complement binding can interfere with the ability of a virus to interact with the membrane of a target cell, thereby preventing viral entry into the cell. Many enveloped viruses can be lysed by the membrane attack complex which damages the envelope and leads to disintegration of the nucleocapsid. The composition of the cell wall influences the susceptibility of bacteria to lysis by the membrane attack complex. The thick peptidoglycan layer of the Gram-positive bacteria confers resistance to the terminal complement components. Although there are notable exceptions, most Gram-negative bacteria are susceptible to complement-mediated lysis.

The number of immunological reactions in which the complement system participates sets it apart from other components of innate immunity. In addition, complement has a central role in the promotion of antibody responses to infectious agents and in amplifying the protective role of antibody against microbial pathogens. Deficiencies of complement components, which are reviewed in Chapter 4, render affected humans and animals susceptible to a range of microbial infections. Many immunological reactions which are essential for defence against microbial pathogens are mediated by complement components, fragments or complexes (Table 3.10).

Characteristics of complement components and complement levels in different species of animals

The labile nature of complement has been known since the time of its discovery, more than 100 years ago. Heating fresh serum at 56°C for 30 min inactivates complement. The ability of the complement system of different species to lyse red blood cells sensitized with antibody shows wide variation. Bovine, ovine, canine, feline and porcine sera have moderate to low levels of haemolytic activity. Human serum has a comparatively high level of complement and guinea pig serum has an exceptionally high level of complement activity. To ensure the optimal participation of complement components in serological tests, diluents should supply adequate concentrations of calcium and magnesium ions.

The antimicrobial activity of cellular and soluble components of innate immunity

Early recognition of invading microbial pathogens is essential for the mobilization of innate defences. Many microbial pathogens have distinctive macromolecules on their surfaces which are not present in mammals. These distinctive molecular structures, which are called pathogen-associated molecular patterns, can be recognized by cellular and soluble receptors of the innate immune system (Table 3.11). The cell types that participate in innate immune responses are not equally effective against bacterial, fungal and viral pathogens. Neutrophils are particularly effective against bacteria and fungi. NK cells have an important role in the recognition and destruction of virus-nfected cells. The ability of interferons to inhibit viral replication at the early stages of virus infections before adaptive immune responses develop is of particular importance in antiviral immunity. When activated, the complement system can lyse microbial pathogens or promote their destruction through phagocytosis or by the initiation of inflammatory responses. Cellular and soluble components of innate immunity and the categories of microbial pathogens affected by their antimicrobial activity are presented in Table 3.12.

Adaptive immunity

At a particular stage in its development, the foetus acquires the ability to recognize foreign antigenic material and to respond to infectious agents

encountered *in utero*. Newborn animals, transferred from a sterile intrauterine environment to a world abounding in microorganisms, have an innate ability to resist invasion by many environmental organisms. Without colostral protection, however, neonatal animals are susceptible to many enteric and respiratory pathogens. As an animal matures, its immune system develops in tandem with other anatomical and physiological alterations. Within weeks of birth, most young animals are immunologically competent and, if challenged by infectious agents, they can respond in an appropriate manner to prevent or limit tissue invasion.

The immune system can distinguish ‘self’ components from foreign material such as cells or soluble substances introduced into the body. This recognition of ‘self’ and tolerance to its own tissue antigens occurs during embryological development. In exceptional circumstances, some individual animals produce an immune response against their own tissues and this condition is termed autoimmunity.

Lymphocytes can interact with foreign material through surface receptors. On B cells, the receptors are membrane-bound immunoglobulins. In contrast, T cell receptors are not immunoglobulins and can only react with antigen in association with other molecules. Lymphocyte receptors can recognize a diverse range of foreign molecules including the components or products of bacteria, viruses, fungi, protozoa and helminth parasites. These foreign substances are collectively referred to as antigens. An antigen can be defined as any substance capable of binding specifically to components of the immune system such as specific antibodies or T cell receptors. An immunogen is any agent or substance capable of inducing an immune response. This differentiation of antigens and immunogens is necessary because some low-molecular-weight compounds, referred to as haptens, which include breakdown products of some antibiotics, cannot induce immune responses unless coupled with large molecules such as proteins. Haptens, however, can bind to components of the immune system specifically produced against them. For a substance to be immunogenic, it must have certain characteristics. These include foreignness, high molecular weight, chemical complexity and biodegradability. In general, compounds that have molecular weights less than 1,000 Da are not immunogenic, whereas those with molecular weights between 1,000 and 6,000 Da may be immunogenic in some instances. Compounds with molecular weights greater than 6,000 Da are usually immunogenic. Proteins are highly immunogenic and carbohydrates moderately immunogenic, whereas lipids and nucleic acids are usually poor immunogens.

Table 3.10 Complement-dependent immunological reactions which contribute to host defences.

Immunological reaction	Complement component, fragment or complex	Comments
Activation and chemotaxis of phagocytic cells and other cell types	C3a, C5a, $C\overline{5b}, \overline{6}, \overline{7}$	C5a is a potent chemotactic factor for neutrophils, monocytes and macrophages; C3a exerts its chemotactic effect on monocytes and macrophages; C3a and C5a activate eosinophils and basophils
Complement-mediated cytolysis of bacterial, protozoal and mammalian cells	$C\overline{5b}, \overline{6}, \overline{7}, \overline{8}, \overline{9}$	Formation of the membrane attack complex on the surface of susceptible target cells results in osmotic lysis of these cells. Because of their thick peptidoglycan layer, Gram-positive bacteria are generally resistant to complement-mediated lysis. Most Gramnegative bacteria are susceptible to complement-mediated lysis
Degranulation of mast cells and basophils	C3a, C5a	Mast cells and basophils have receptors for C3a and C5a. Binding of these fragments to their receptors causes degranulation of mast cells and basophils with release of histamine, other pharmacologically active mediators and cytokines. Released products which induce contraction of smooth muscle and increased vascular permeability facilitate fluid containing antibody reaching the site and promote an influx of phagocytic cells
Limiting viral infectivity:		
Viral neutralization	C3b	Binding of C3b to immune complexes composed of virions and antibody enhances virus neutralization
Virus opsonisation	C3b	Attachment of C3b to virus particles promotes phagocytosis and activates phagocytes
Lysis of virions	$C\overline{5b}, \overline{6}, \overline{7}, \overline{8}, \overline{9}$	Formation of the membrane attack complex can lyse enveloped viruses
Opsonization of bacteria	C3b, C4b, iC3b, C1q	Fragments of C3 and also C4 which attach to pathogenic microorganisms facilitate engulfment by phagocytes. C3b is the major opsonin of the complement system; iC3b, C4b and C1q also have opsonizing activity. Phagocytic cells express receptors for C3b, iC3b, C4b and C1q, and antigenic material coated with these fragments is readily phagocytosed
Release of hydrolytic enzymes from neutrophils	C5a	Binding of C5a to its receptor on neutrophils results in cell activation with release of cytoplasmic granules and proinflammatory molecules; other changes include increased adhesive properties of these activated cells
Role in the activation and maturation of B cells	C3d	When C3d, a cleavage product of C3b, binds to antigen, it can interact with B cells through CR2 receptors. B cells can bind antigen through their immunoglobulin receptors and simultaneously bind C3d through their CR2 receptors. The combined signals arising from this dual binding promote B-cell activation and maturation
Solubilization and clearance of immune complexes from the circulation	C3b	Immune complex formation frequently occurs in the circulation. Deposition of immune complexes in capillary beds can cause strong inflammatory responses. Binding of C3b to immune complexes facilitates their attachment to red blood cells through the CR1 receptor. In the liver and spleen, the immune complexes are released from red blood cells and engulfed and destroyed by macrophages

Table 3.11 Pattern recognition receptors for microbial pathogens.

Receptors	Distribution	Structure recognized	Comments
Cell surface			
Toll-like receptors	Unequally distributed among antigen-presenting cells including monocytes, macrophages and dendritic cells; also present on neutrophils, endothelial cells and some types of epithelial cells	Components of bacterial, viral, fungal and protozoan pathogens	These receptors can interact with a wide variety of microbial substances including lipopolysaccharide of Gram-negative bacteria, peptidoglycans of Gram-positive bacteria, bacterial DNA and viral RNA
Type 3 and other complement receptors	Monocytes, macrophages and neutrophils	Complement-coated microorganisms	Phagocytosis is facilitated by these cell surface receptors
CD14	Monocytes, macrophages and neutrophils	Lipopolysaccharide, lipoteichoic acid, lipoprotein and peptidoglycan	Induces cytokine production in responding cells
C-type lectins	Macrophages	Carbohydrates with mannose residues	Receptor binds to terminal mannose and fucose residues of glycoproteins and glycolipids, typically part of microbial cell walls
Scavenger receptors	Macrophages	A range of Gram-positive and Gram-negative bacteria, lipopolysaccharide, lipoteichoic acid and bacterial DNA	Contribute to clearance of microorganisms from tissues; promote phagocytosis
Receptor for *N*-formylmethionyl peptides	Neutrophils, macrophages	Bacterial proteins	Enables neutrophils and macrophages to detect and respond to the presence of bacterial protein
Receptor for β-glucans	Macrophages	Fungal pathogens, β-glucans, zymosan	Promotes phagocytosis of fungal pathogens
Intracellular			
Bactericidal permeability-increasing protein	Primary granules of neutrophils	Gram-negative bacteria, lipopolysaccharide	Exerts a bactericidal effect on Gram-negative bacteria
Caspase-recruitment domain proteins	Monocytes, macrophages, epithelial and other cell types	Lipopolysaccharide and bacterial muramyldipeptide	Originally termed nucleotide-binding oligomerization domain proteins (NOD), this family of proteins shares some common features with toll-like receptors and CD14. They are considered to be intracellular sensors of pathogen-associated molecular patterns
Protein kinase R	Macrophages	Viral double-stranded RNA	Induces type I interferon production which protects against viral infection
Soluble			
C-reactive protein	Acute phase protein synthesized in the liver	Phosphorylcholine	Promotes complement activation with resultant opsonization of target microorganisms
Soluble CD14	Produced by monocytes and macrophages	Lipopolysaccharide, lipoprotein, lipoteichoic acid and peptidoglycan	Induces production of pro-inflammatory cytokines
Lipopolysaccharide-binding protein	Acute phase protein synthesized in the liver	Lipopolysaccharide and lipoteichoic acid	Activates macrophages and promotes secretion of pro-inflammatory cytokines
Mannose-binding lectin	Acute phase protein synthesized in the liver	Mannose-containing bacterial and fungal polymers	Promotes complement activation and phagocytosis of bacterial and fungal pathogens
Serum amyloid-P	Acute phase protein synthesized in the liver	Components of Gram-positive bacteria, fungi and some protozoa	Promotes complement activation, opsonization and phagocytosis
Surfactant protein A	Acute phase protein synthesized by pulmonary epithelium	Haemagglutinin molecules of influenza viruses	By binding to the viral haemagglutinin molecules, virus infectivity is reduced

Table 3.12 Cellular and soluble components of innate immunity and their

antimicrobial spectra.

Component	Microbial pathogens			Comments
	Bacteria	Viruses	Fungi	
Cells				
Neutrophils	+++	+	++	Short-lived phagocytic cells which produce a range of antimicrobial substances; can engulf and destroy a range of Gram-positive bacteria, Gram-negative bacteria, yeasts and fungal spores
Macrophages	+++	++	++	Long-lived phagocytic cells which produce antimicrobial substances and cytokines. In addition to engulfment of microbial pathogens they can act as antigen-presenting cells for T cells
Dendritic cells	++	+++	++	As antigen-presenting cells for T cells and also through cytokine secretion, dendritic cells play a central role in the initiation of adaptive immune responses
NK cells	+	+++	+	These large granular lymphocytes, which do not recognize specific antigen, exhibit cytotoxic activity for virus-infected cells and tumour cells; IFN-γ secreted by these cells activates macrophages
Secretions				
Interferons α and β	+	++	+	These cytokines, produced by leukocytes and fibroblasts, inhibit viral replication, stimulate $CD4^+$ T_H1 cells and increase the cytotoxic activity of NK cells
Complement	++	+	+	The complement system consists of a large number of plasma proteins which, when activated by immune complexes or microorganisms, can generate proteolytic enzymes which lyse microbial pathogens or promote their destruction through phagocytosis or by initiation of inflammatory responses
Lysozyme	++	–	–	This highly cationic protein enzymatically cleaves peptidoglycans, structural components of the walls of Gram-positive bacteria; present in lacrimal secretions, sweat, saliva, serum and mucus
C-reactive protein	++	–	+	Acute phase protein synthesized in the liver, promotes activation of complement and opsonization of bacterial and fungal pathogens
Transferrin	++	–	–	Transferrin, an iron-binding protein present in serum, competes with bacteria for iron. Iron is essential for the growth of many bacteria and also influences their expression of important virulence genes
Lactoferrin	++	–	–	This iron-binding protein, present in exocrine secretions including milk and saliva, is a member of the transferrin family. It is released by degranulating neutrophils and sequesters iron, thereby inhibiting growth of pathogenic bacteria
Defensins	++	+	+	These cationic antimicrobial peptides contribute to natural protection of mucosal surfaces; α defensins are present in the primary granules of neutrophils and Paneth cells in the crypt regions of the small intestine and in epithelium of the respiratory and reproductive tracts; β defensins are produced by epithelial cells in the oral mucosa, in the trachea and bronchi, in salivary glands and in the skin. Some α defensins have antiviral activity
Cathelicidins	++	–	+	A large family of antimicrobial peptides present in the secondary granules of neutrophils. Some members have direct effects on bacterial and fungal pathogens; the α-helical peptide called LL-37 binds lipopolysaccharide and induces chemotaxis
Dermcidin	+	–	+	This antimicrobial peptide is produced in sweat glands

Component	Microbial pathogens			Comments
	Bacteria	Viruses	Fungi	
Phospholipase A_2	++	–	–	In common with a number of antimicrobial innate defence molecules, phospholipase A_2 is produced in intestinal Paneth cells and primary granules of neutrophils. This enzyme has activity against streptococcal and staphylococcal bacteria
Mannose-binding lectin	++	–	+	The lectin pathway of complement activation is triggered by the plasma protein mannose-binding lectin (MBL) which binds to mannose and *N*-acetylglucosamine in bacterial and fungal cell walls. When MBL, which is structurally similar to C1q, binds to target cells, a conformational change occurs in the molecule. Following interaction with MBL-associated serine proteases (MASPs) complement activation is initiated with opsonization of the pathogens recognized by MBL
Serum amyloid-P	++	–	+	This acute phase protein, which is synthesized in the liver, binds to components of Gram-positive bacteria, fungi and some protozoa promoting activation of complement, opsonization and phagocytosis
Surfactant protein A	–	+	–	Although one of the acute phase proteins, surfactant protein A is synthesized by pulmonary epithelium. By binding to the haemagglutinin molecules of influenza viruses, this protein reduces the infectivity of these viruses
Lipopolysaccharide-binding protein	++	–	–	When lipopolysaccharide is released by Gram-negative bacteria, it is recognized and bound by lipopolysaccharide-binding protein present in the blood. This acute phase protein, which is produced mainly in the liver, is also produced by epithelial cells in the intestinal and respiratory tracts. Following binding of lipopolysaccharide by lipopolysaccharide-binding protein, this toxic bacterial complex is transferred to either soluble CD14 or the membrane form of CD14 expressed on macrophages and neutrophils. Binding of lipopolysaccharide to the membrane form of CD14 activates macrophages and promotes secretion of pro-inflammatory cytokines
Soluble CD14	++	–	–	This molecule, which binds lipopolysaccharide produced during growth or following death of Gram-negative bacteria, induces production of pro-inflammatory cytokines by monocytes and macrophages

Infectious agents are composed of structures containing molecules of great complexity. Accordingly, an individual bacterium can have a vast array of complex surface antigens which a lymphocyte receptor can recognize. The lymphocyte receptor can recognize only a small portion of a complex molecule and this small part of the molecule is referred to as an antigenic determinant or epitope. Complex antigens consist of a mosaic of individual epitopes and, when similar determinants are present on different infectious agents, cross-reactions may occur in serological test procedures involving these infectious agents.

Structure and organization of lymphoid tissue

In contrast to the innate immune system, which uses a limited number of genetically encoded receptors such as PRRs and TLRs, the adaptive immune

system utilizes subsets of lymphocytes, which have the ability to generate a large number of antigen-specific cell surface receptors by random gene rearrangement. Lymphoid structures are divided into primary and secondary lymphoid organs. In mammals, the primary lymphoid organs are the thymus, where specialized lymphoid differentiation takes place, and the bone marrow, the origin of lymphoid stem cells and also the location in which specialized lymphocyte subset differentiation occurs. Thymus-derived lymphocytes, T lymphocytes, develop T cell receptors for recognizing antigen and collectively these cells are responsible for cell-mediated immune responses to infectious agents, delayed-type hypersensitivity reactions and promotion of humoral immune responses. Bone-marrow-derived lymphocytes, B lymphocytes, possess special antigen receptors, B-cell receptors, and, following interaction with antigens of appropriate specificity, B cells differentiate into plasma cells which produce specific antibody. As a consequence of this interaction, some B cells form memory cells which retain the ability to recognize antigen and respond rapidly to a second or subsequent encounter with the same antigen. In avian species, T lymphocyte development takes place in the thymus but B lymphocyte differentiation occurs in the cloacal bursa, formerly called the bursa of Fabricius. When naïve lymphocytes have completed their differentiation in primary lymphoid organs, they migrate to secondary lymphoid organs.

The secondary lymphoid organs include the spleen, peripheral and mesenteric lymph nodes, mucosa-associated lymphoid tissue including the tonsils, and gut-associated lymphoid tissue such as Peyer’s patches. Because of their location and activity these lymphoid organs fulfil different roles. The mucosa-associated lymphoid tissue limits the progress of infectious agents which enter the body through mucosal surfaces. Peripheral lymph nodes collect and offer protection against pathogens detected in body fluids. The spleen and, to a lesser extent, the Kupffer cells in the liver remove blood-borne infectious agents, degenerating cells and foreign antigenic material. Although morphologically different, lymphoid organs serve a common defence purpose. Because they are positioned at sites where antigen-presenting cells can sample blood or body fluids and present microbial pathogens or their products to T and B lymphocytes, secondary lymphoid organs have a central role in the initiation of specific immune responses. In military terms, they are akin to strategically located garrisons, detecting and responding in a specific manner to microbial invasion.

Lymphocytes

All lymphocytes arise from a common stem cell in the bone marrow (Cumano and Godin, 2007). On the basis of function and cell membrane components, lymphocytes can be broadly subdivided into three major populations namely B cells, T cells and natural killer cells (Fig. 3.4). Before T or B lymphocytes encounter antigen, they are referred to as naïve or unprimed cells. Interaction with antigen induces change; these cells enlarge and develop into lymphoblasts. Ultimately, lymphoblasts differentiate into effector cells or memory cells. The name assigned to lymphocytes reflects the tissue or organ in which they mature after leaving the bone marrow or, in the case of natural killer cells, their immunological activity. Thus, the designation T cell relates to lymphocytes that mature in the thymus, and the name B cell relates to lymphocytes that mature in the avian cloacal bursa or its equivalent in mammals, the bone marrow. Additional sites in mammals in which B lymphocyte maturation may take place include Peyer's patches or other gut-associated lymphoid tissues.

Although lymphocytes are morphologically heterogeneous, B and T cells can be differentiated by their antigen receptors and by characteristics of their surface markers. Up to 70% of lymphocytes in peripheral blood are T cells. The primary role of B lymphocytes is antibody production. Each B cell is genetically programmed to express a surface receptor for a particular antigen. When stimulated by antigen for which they have receptors, B cells differentiate into plasma cells which produce large amounts of specific antibody. In addition, B lymphocytes can process antigen and present it to T cells, thereby initiating or enhancing specific immune responses. Mature T lymphocytes express characteristic markers which can be used for their identification. These cells, which comprise functionally distinct populations, are involved in the activation of many cell types including B lymphocytes, macrophages and other cells involved in inflammatory responses. Subsets of T cells express different markers and, through the release of cytokines, T lymphocytes are responsible for the activation and control of many specific immune responses. One subset of T cells can recognize and kill host cells infected with viruses, tumour cells and foreign cells such as tissue grafts. Lymphocytes which are distinct from T and B lymphocytes, referred to as natural killer (NK) cells, have some characteristics in common with T cells but do not exhibit antigenic specificity. These large granular lymphocytes are part of the innate immune system and do not express clonally distributed antigen receptors such as immunoglobulin receptors or T cell receptors.

The only cells in the body capable of recognizing antigenic determinants in a

highly specific manner are T and B lymphocytes. In addition, these lymphocytes produce memory cells following an encounter with antigenic material. Accordingly, specificity and memory are two fundamental features of adaptive immune responses. As a consequence of immunological memory, a more effective immune response occurs on second or subsequent encounters with an infectious agent than on primary exposure. This contrasts with innate immune responses which do not alter following repeated exposure to the same infectious agent.

Subsets of lymphocytes and their contribution to adaptive immunity

Adaptive immune responses occur in two phases, recognition of the infectious agent's surface antigens and the development of specific immune responses aimed at clearing the microbial pathogen from the tissues. In the first phase, selection of lymphocytes involves the recognition of antigen with subsequent clonal expansion of T and B lymphocytes which have surface receptors for the antigen. In the second phase, differentiation of the lymphocytes into effector cells and memory cells results in the development of humoral and cell-mediated responses. These specific responses lead to the neutralization or elimination of the infectious agent and the development of memory cells which can respond rapidly to second or subsequent encounters with the microbial pathogen.

Subsets of T lymphocytes

Lymphocytes are heterogeneous, phenotypically, functionally and morphologically. There are two well defined subpopulations of T cells, T helper (T_H) cells and cytotoxic T cells (T_C). In recent years, two additional subsets of T cells, T helper 17 (T_H17) and regulatory T cells (T_{REG}), have been identified (DeFranco *et al.*, 2007). Subsets of T lymphocytes can be distinguished by their expression of membrane molecules recognized by monoclonal antibodies. Unlike polyclonal antibodies which can react with a wide range of antigenic determinants, monoclonal antibodies react with a particular determinant. Cell surface molecules present on lymphocytes and other cell types which can be distinguished by monoclonal antibodies are given the designation of cluster of differentiation (CD). Although several hundred CD markers have been described, common CD molecules are used to distinguish functional lymphocyte

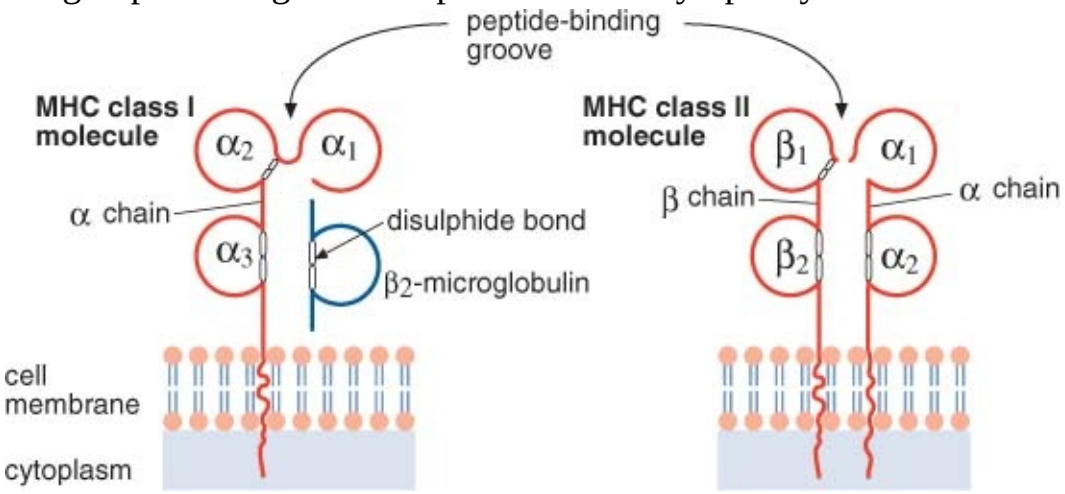
subpopulations. Thus, subsets of T lymphocytes with helper or cytotoxic activity can be distinguished by the presence or absence of CD4 or CD8 membrane glycoproteins. Most $CD4^+$ T cells are T helper cells; lymphocytes which are $CD8^+$ generally function as cytotoxic T cells. Lymphocytes with regulatory activity are identified by the presence of both CD4 and CD25 markers on their membranes. These T_{REG} cells tend to suppress immune responses and unlike T_H cells which promote immune reactivity, act as cellular regulators of the immune system. All T cells express antigen-binding T cell receptors which functionally resemble membrane-bound antibodies on B lymphocytes but differ from them structurally. The T cell antigen receptor is a heterodimer composed of an αβ or γδ polypeptide pair associated with the CD3 molecular complex. Most T lymphocytes express the αβ T cell receptor (TCR); some express the γδ TCR (Fig. 3.5). The receptors that interact with peptide antigens which are processed and presented on the surface of antigen-presenting cells are clonally distributed on $CD4^+$ and $CD8^+$ T lymphocytes. Before antigenic material can be recognized by the TCR, it must be bound to major histocompatibility complex molecules on the surface of the antigen-presenting cell. While membrane-bound antibodies on B lymphocytes can recognize native macromolecules such as proteins, lipids, carbohydrates and nucleic acids, T cell receptors recognize antigen only when it is bound to membrane glycoproteins encoded by the major histocompatibility complex. These molecules, which are genetically diverse glycoproteins found on cell membranes, present fragments of antigen on the cell surface where it can be recognized by the TCR. Before antigens can be recognized by T cells, they must be processed or degraded and the determinants identified by the TCRs are fragments of the original antigenic material. Processing of antigen refers to degradation of the antigenic material into peptide fragments. The major histocompatibility complex (MHC) is a collection of genes arranged within a continuous stretch of DNA encoding three classes of molecules including the peptide-binding molecules recognized by T lymphocytes. MHC class I genes encode glycoproteins expressed on the surface of most nucleated cells. The major function of class I gene products is the presentation of antigen to cytotoxic T lymphocytes, $CD8^+$ cells. MHC class II genes encode glycoproteins expressed primarily on antigen-presenting cells such as macrophages, dendritic cells and B cells. These antigen-presenting cells present processed antigenic peptides to T helper cells, $CD4^+$ T cells (Snyder, 2007). MHC class III genes encode secreted molecules with immune functions such as cytokines and components of the

complement system.

MHC class I and class II molecules bind peptides derived from different sources. While MHC class I molecules bind peptides derived from cytosolic proteins, referred to as endogenous proteins, MHC class II molecules bind to peptides derived from extracellular proteins which have been taken into the cell by phagocytosis or endocytosis. The structures of MHC class I and MHC class II molecules are illustrated in Fig. 3.10. Generally, the peptides bound by MHC class I molecules are derived from endogenous intracellular proteins which are digested in the cytosol. These peptides are then transported from the cytosol into the cisternae of the endoplasmic reticulum where they interact with MHC class I molecules. From the endoplasmic reticulum, MHC class I molecules and bound peptides are transported to the surface of the cell for recognition by the TCR of $CD8^+$ T cells. Following recognition of antigen-MHC complexes by $CD8^+$ T cells, these lymphocytes undergo proliferation and differentiation into cytotoxic T cells or into memory cells. Cytotoxic T cells have a vital role in monitoring host cells and eliminating cells which display foreign antigen complexed with MHC class I molecules such as virus-infected cells. T helper cells recognize antigen associated with MHC class II molecules. Following activation by interaction with appropriate antigen-MHC complexes, T_H cells differentiate into effector cells which initiate and promote immune responses. These $CD4^+$ lymphocytes activate macrophages, B lymphocytes, cytotoxic T lymphocytes and other cell types which participate in immune responses. Based on the range of cytokines that they secrete, $CD4^+$ effector cells can be divided into a T_H1 subset and a T_H2 subset. The T_H1 subset secretes IL-2, IL-3, IFN-γ and TNF-β and is responsible for classical cell-mediated functions such as activation of cytotoxic T lymphocytes and delayed-type hypersensitivity reactions. The other subset, T_H2, secretes IL-4, IL-5, IL-6, IL-10 and IL-13. A third subset, T_H17, has been described recently (Miossec *et al.*, 2009). It is likely that a common precursor cell exists that can differentiate into a T helper cell, and it is probable that the cytokine environment in which the antigen-primed T helper cell differentiates determines the subset that develops. Additional factors which influence differentiation of T_H cells include the sites of antigen presentation and type of antigen-presenting cells involved and the cytokines which they produce. Ultimately, cytokine balance is the major influence in determining $CD4^+$ T cell differentiation. Dendritic cells and, to a lesser extent, macrophages are the sources of IL-12 which is essential for the development of $CD4^+$ T_H1 cells.

Interleukin-4 is essential for the development of T_H2 responses. The differentiation of antigen- stimulated T cells into the T_H2 subset is dependent on IL-4, which functions by activating STAT6, a transcription factor which stimulates T_H2 development. The T_H2 subset, which develops in response to allergic diseases and helminth infections, produces IL-4, IL-5, IL-10 and IL-13 and promotes eosinophil and mast cell activation and production of IgE antibodies. There is mutual antagonism between T_H1 and T_H2 pathways of development and differentiation. Through their pattern of cytokine secretion, T helper cell subsets exert a positive influence on lymphocytes which produce cytokines required for their own development; they exert a negative influence on the other subset. Thus, production of IL-4 has a positive influence on T_H2 development and a negative influence on T_H1 development. Likewise, production of IL-12 promotes T_H1 development while depressing T_H2 development. Stimuli other than cytokines which may influence T helper cell differentiation include the amount of antigen presented, the nature of the antigen-presenting cells and the influence of co-stimulatory molecules. Differentiation of $CD4^+$ T cells into the $CD4^+$ T_H1 subset is strongly influenced by infection with many intracellular bacteria such as *Mycobacterium* species and *Listeria monocytogenes* and also by infection with a number of pathogenic protozoa. Appropriate adjuvants preferentially stimulate development of T_H1 helper cells. A feature of many intracellular pathogens and some adjuvants is that they elicit IL-12 production by cells of the innate immune system. Some pathogenic microorganisms which interact with toll-like receptors on macrophages and dendritic cells directly stimulate these cells to secrete IL-12. Other microbial pathogens may indirectly promote IL-12 secretion by stimulating NK cells to produce IFN-γ which, when it acts on macrophages, induces production of IL-12. The major cytokine involved in the induction of cellmediated immunity is IL-12: it binds to receptors on antigen-stimulated $CD4^+$ T cells and activates the transcription factor STAT4 which promotes differentiation of these cells into T_H1 cells. Another transcription factor termed T-bet, which is induced by IFN-γ, augments T_H1 development. Interferons, particularly IFN-γ, also promote T_H1 development not only by stimulating production of IL-12 by macrophages but also by promoting the expression of functional IL-12 receptors on T lymphocytes.

Figure 3.10 Structure of MHC class I and MHC class II molecules showing the peptide-binding groove where peptide fragments of antigens on the surface of antigen-presenting cells are presented to T lymphocytes.



Recent studies indicate that two newly described cytokines, IL-23 and IL-27, part of the interleukin family, contribute to the development and differentiation of T_H1 cells. These two cytokines share some common structural features and functions with IL-12.

Functional role of T lymphocyte subsets in adaptive immune responses

Antibodies produced by activated B lymphocytes can neutralize viruses and bacterial toxins and, through the secretion of IgA on mucosal surfaces, can protect the respiratory and alimentary tracts against microbial attack (Snoeck *et al.*, 2006). The protective role of antibody, however, is limited to extracellular pathogens, and humoral immunity is ineffective against microbial and parasitic pathogens which replicate within phagocytic or non-phagocytic host cells. Protection against intracellular pathogens including viruses, bacteria such as *Mycobacterium bovis* and *Listeria monocytogenes*, and fungal pathogens such as *Histoplasma capsulatum*, is dependent on effective cell-mediated immune responses which involve both T helper cells and cyto-toxic T lymphocytes. If, following intracellular infection by a microbial pathogen, the infected cells are unable to kill the invading infectious agent, the only means of eradication of infection is by destruction of the infected cells. Accordingly, cell-mediated immunity is an essential part of adaptive immune responses to infectious agents. It also has a central role in allograft rejection and anti-tumour immunity.

Production, structure and biological activities of antibodies

When a B cell that has not previously encountered an antigen binds to antigen through its membrane-bound antibody molecules, this binding activates the B cell and causes it to divide rapidly. This activated B cell differentiates into effector cells called plasma cells and also into memory B cells. Plasma cells secrete antibodies which enter the circulation. These antibody-secreting cells, which do not divide, are end-stage cells and usually secrete antibodies for up to 2 weeks. Unlike plasma cells, memory B cells have a long life span and they express the same membrane-bound antibody as their parent B cells. Antibodies are glycoprotein molecules present in the gamma globulin fraction of serum. These molecules are members of the immunoglobulin family of serum proteins. There are five classes or isotypes of immunoglobulins, each with characteristic structural features and with particular immunological activities (Table 3.13). Despite the distinctive immunological features of immunoglobulin classes and their diverse biological activities, they share common structural arrangements and their functional activity can be related to their molecular structure.

Immune responses are initiated when an animal encounters foreign antigenic material, often an infectious agent. Within days, the infected animal responds by producing antibody molecules specific for the antigenic determinants of the infectious agent and by expansion and differentiation of antigen-specific regulatory and effector T lymphocytes. As a consequence of the encounter, lymphocytes with an immunological memory are produced. If challenged later by the same infectious agent, a more rapid and sustained antibody response occurs (Fig. 3.11). A similar enhanced and more effective T cell response usually occurs in secondary immune responses. This is the basis of vaccination which ensures a rapid and usually protective immune response to antigenic material, injected or otherwise administered to susceptible animals. An outline of the principal elements of adaptive immunity and its induction is presented in Fig. 3.12.

Table 3.13 Important features of immunoglobulin isotypes in domestic animals.

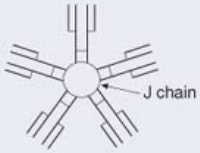
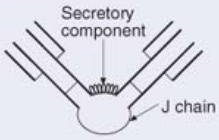
Feature	Isotype				
	IgM	IgG	IgA	IgD	IgE
Molecular weight	900,000	160,000	160,000 (monomer) also occurs as a dimer and trimer	180,000	190,000
Structure of secreted form				Not secreted	
Heavy chains	μ	γ	α	δ	ε
Accessory chains or components	J chain	None	J chain and secretory component	None	None
Distribution	Mostly intravascular	Intravascular and extravascular	Intravascular and secretions	Antigen receptor on naïve B cells	Low levels in blood and extravascularly; binds to mast cells and basophils
Valency	10	2	2 (monomer)	2	2
Role in hypersensitivity reactions	Participates in some type II reactions and may occasionally be involved in type III reactions	Participates in type II and type III reactions	Not involved	Not involved	Participates in type I reactions
Protective role and other activities	The monomeric form acts as an antigen receptor on B cells; in the circulation the pentamer activates complement and agglutinates bacterial cells. As the first antibody produced during infection, IgM has a protective role against bacterial and viral pathogens	Activates complement, has agglutinating, precipitating and opsonizing activity; has a major role in antibacterial and antiviral immunity and in toxin neutralization	Predominant immunoglobulin involved in the protection of mucosal surfaces	Receptor for antigen on the surface of B cells	Plays a central role in type I hypersensitivity reactions; has a protective role against some helminth parasites

Figure 3.11 Primary and secondary antibody responses following natural exposure to an infectious agent or after vaccination. The primary response occurs after an interval of almost 10 days and the predominant immunoglobulin is IgM. The secondary immune response reaches higher levels and lasts longer, and the antibodies produced are mainly IgG.

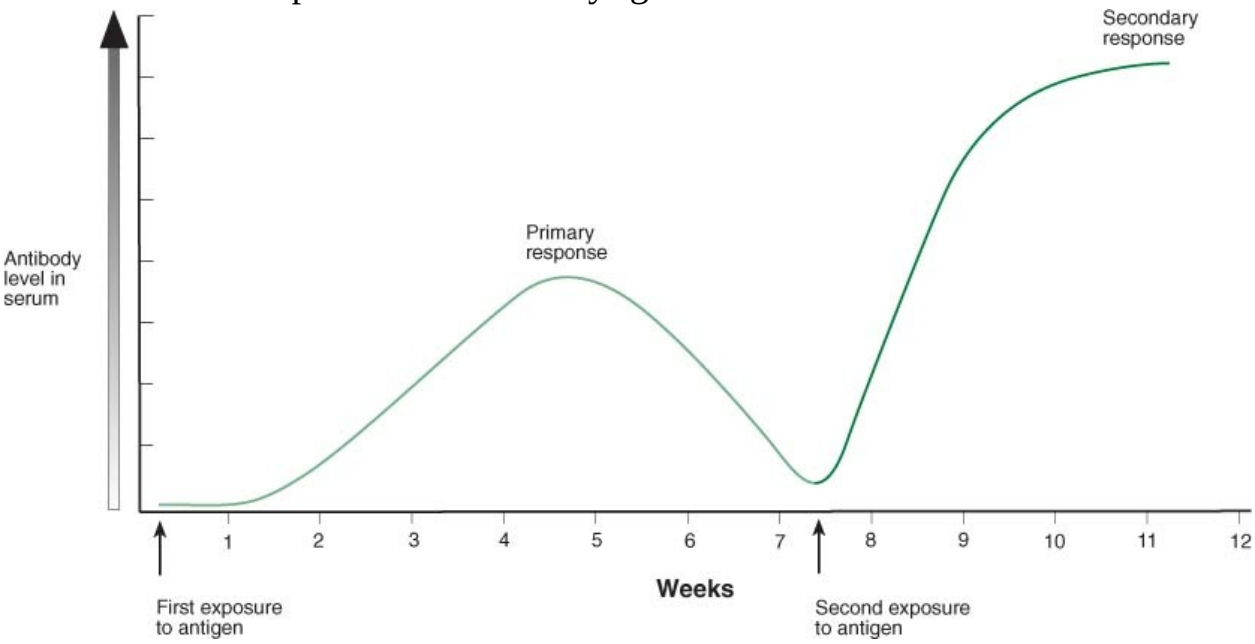
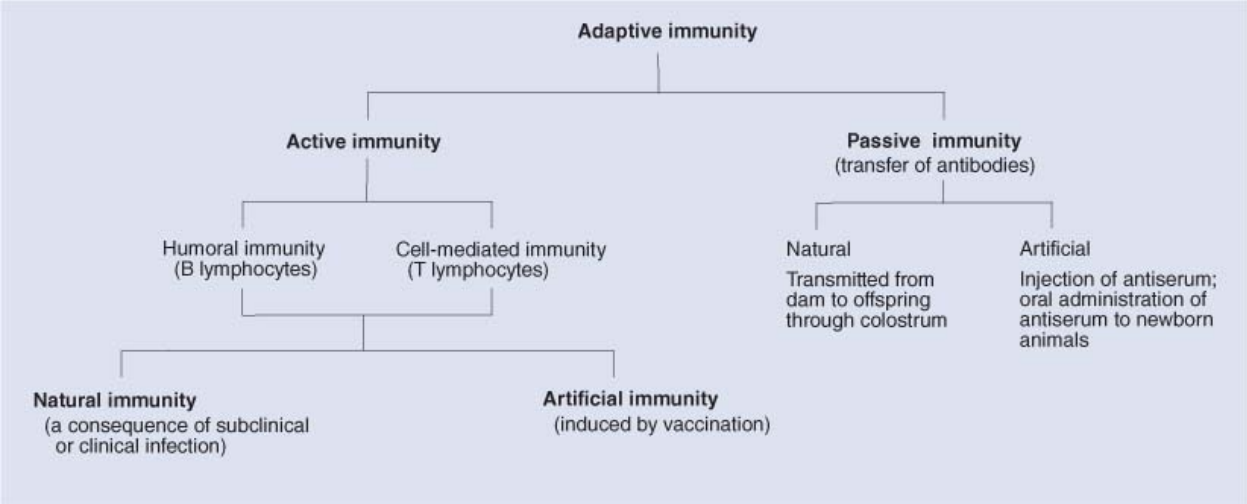


Figure 3.12 An outline of the principal elements of adaptive immunity and the

methods used to confer passive immunity and to induce active immunity.

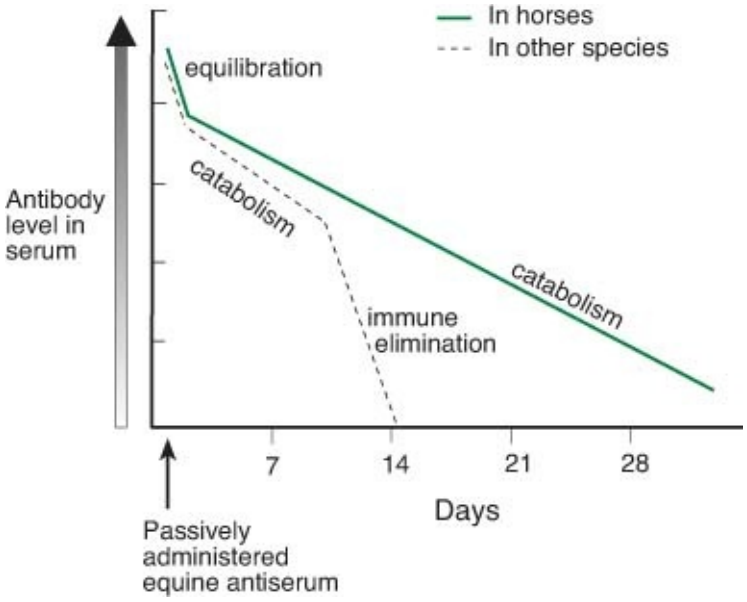


Antibodies produced against infectious agents have the ability to neutralize bacterial toxins and viruses. They can opsonize microbial pathogens for phagocytosis by macrophages and neutrophils. Some antibodies such as IgA, produced locally in the gastrointestinal and respiratory tracts, prevent attachment of pathogens to host cells, thereby hindering colonization and minimizing the likelihood of disease production. This form of local immunity, referred to as mucosal immunity, is of particular importance in young animals. By activating the classical complement pathway, antibodies can initiate responses which lead to lysis of microbial pathogens and opsonization through fixation of C3b on the target membrane to which they have attached. They can also promote inflammatory responses through the generation of cleavage components once the complement system is activated. Antibodies produced by the dam and secreted in colostrum passively protect newborn animals against a wide range of respiratory and enteric pathogens.

Passive immunity refers to the transfer of antibodies from an actively immune animal to a susceptible animal. Antiserum specific for a particular pathogen or toxin can be administered by injection to give immediate short-term protection against infectious agents. Newborn animals can be given antiserum orally to protect them against infection with certain enteropathogens. Following administration of antiserum the duration of passive immunity is shorter in a heterologous species than in a homologous species (Fig. 3.13).

Figure 3.13 The duration of passive immunity following administration of equine antiserum to horses and to other species of animals. The dose of antiserum administered influences the duration of protection in the homologous

species which may be up to 3 weeks when the recommended amount of antiserum is administered.



Immunity to bacteria

Many different species of bacteria are present in different regions of an animal's body, with particularly high concentrations present on the skin and in the alimentary tract. Most of these bacteria are commensals which coexist with the host without inducing adverse effects. Bacteria, yeasts and other microorganisms colonize particular sites on the skin and regions of the alimentary and urogenital tracts. Based on their distribution on or within the body, their interaction with host tissues and their influence on the functioning of body systems, bacteria can be arbitrarily considered as part of the normal flora, opportunistic pathogens or pathogenic bacteria. These bacterial categories are not absolute as they depend not only on the characteristics of the infectious agent but also on the immune status of the host. Bacteria which colonize defined regions of the body without producing disease constitute part of the normal flora. Unlike the normal flora, pathogenic bacteria have the ability to invade tissues, elaborate toxins and produce disease. Even if pathogenic bacteria succeed in entering the tissues and causing infection, disease is not inevitable. Following tissue invasion by the bacteria, innate body defences are rapidly mobilized to contain the infection and eliminate the microbial pathogens. Anatomical structures including the skin and mucous membranes offer physical protection against bacterial invasion, while antimicrobial factors in body fluids and phagocytic cells offer effective

protection against many bacterial pathogens. Antibodies and T lymphocytes, components of specific adaptive immunity, offer more effective and prolonged protection against bacterial pathogens than innate defences. Cell-mediated and antibody-mediated immune responses are required for protective immunity against intracellular pathogens, encapsulated organisms and toxigenic bacteria. Cooperative interactions between innate defences and adaptive immune responses are often required to ensure effective protection against virulent bacterial pathogens.

Innate immunity to bacterial infections

The skin, mucous membranes, mucociliary clearance and inhibitory secretions including gastric acid and bile, and fatty acids in the skin, contribute to protection against pathogenic bacteria. A well-established normal flora in the alimentary tract renders colonization by enteric pathogens more difficult than in neonatal animals or in animals on prolonged antimicrobial therapy. Pathogenic bacteria capable of causing systemic infections must compete with normal commensal bacteria to colonize tissues in sufficient numbers to produce disease. The normal flora can also produce antimicrobial factors such as bacteriocins. Antimicrobial drugs administered to animals with bacterial infections can disrupt the normal flora colonizing mucosal surfaces and facilitate tissue colonization by bacterial pathogens resistant to particular antimicrobial therapy. Viral invasion of epithelial tissues in the respiratory tract or alimentary tract provides an opportunity for tissue invasion by pathogenic bacteria.

A central question relating to the functioning of the immune system is how infectious agents are recognized and distinguished from the vast number of environmental substances encountered by the body. For many years, it had been suggested that innate immunity was non-specific and offered limited protection against infectious agents. As knowledge of the functioning of the immune system progressed, it became evident that components of innate immunity can recognize structures shared by infectious agents which are not present on host cells. Bacteria, fungi and viruses can be recognized by receptors present on host cell membranes, within cells and also by secreted molecules. These receptors, referred to as pattern recognition receptors, bind to structures common to pathogenic microorganisms, and lead to cellular responses that are central to the functioning of the innate immune system. Among the important structures recognized by pattern recognition receptors are peptidoglycans,

lipopolysaccharide, double-stranded RNA and mannose residues. Unlike receptors expressed by cells of specific adaptive immunity, receptors of the innate immune system are encoded in the germline and are not produced by somatic mutation of genes. Consequently, they are distributed on cells of the same lineage. In comparison to the enormous diversity of receptors on lymphocytes for adaptive immune responses, receptors of innate immunity have limited diversity. Because receptors on cells which participate in innate immune responses are encoded in the germline, cells of a particular type, such as macrophages, express identical receptors for pathogenic microorganisms. Accordingly, many cells responding to bacterial infections may recognize the same structures on the invading pathogens. The innate immune system responds in the same manner to repeated encounters with the same microbial pathogens. In contrast, because of the development of immunological memory, adaptive immune responses increase in intensity with successive encounters with the same pathogenic microorganisms.

Receptors used by cells of the innate immune system for the recognition of bacterial pathogens are present on macrophages, neutrophils, dendritic cells, epithelial cells and endothelial cells (Table 3.11). The consequences of recognition of microbial agents by the innate immune system depend on the tissue involved and the recognition system activated. Increased production of antimicrobial peptides, secretion of mediators involved in inflammatory responses, activation of complement and chemotaxis of phagocytes to the site of tissue invasion are typical innate responses to localized bacterial infection. The inflammatory response to the invading bacteria is enhanced by release of cytokines such as IL-1, IL-6 and tumour necrosis factor-α and of acute phase proteins, including C-reactive protein.

Antimicrobial peptides

In addition to acting as a physical barrier to bacterial invasion, epithelial layers of the skin produce antimicrobial peptides which can inhibit bacterial and fungal growth. Antimicrobial peptides, produced by epithelial cells in particular locations and by neutrophils, are concentrated in sites where there is marked bacterial activity. In neutrophils these compounds contribute to the killing of engulfed bacteria. Defensins and cathelicidins are two important groups of antimicrobial peptides with activity against a wide range of bacterial pathogens. The two classes of defensins, α-defensins and β-defensins, have similar

structures and prevent bacterial colonisation at particular sites. α-Defensins are present in the primary granules of neutrophils and are produced by Paneth cells at the base of the crypts of the small intestine. They are also produced in the epithelium of the respiratory and reproductive tracts. β-Defensins are produced by epithelial cells in the oral mucosa, salivary glands and skin and along the respiratory and reproductive tracts. Cathelicidins are present in the granules of neutrophils and also in macrophages, mast cells and pulmonary epithelial cells. Antimicrobial peptides have the ability not only to damage bacterial and fungal cells but also to attract neutrophils and dendritic cells to sites of infection and contribute to the initiation of adaptive immune responses.

Lysozyme

One of many antimicrobial factors present in body fluids, lysozyme is a highly cationic, low molecular weight protein. Lysozyme acts directly on the cell wall of Gram-positive bacteria and enzymatically cleaves the bonds between *N*-acetylglucosamine and *N*-acetylmuramic acid which stabilizes the peptidoglycan layer. The cell walls of Gram-negative bacteria are protected from the action of lysozyme by the outer membrane which masks the underlying peptidoglycan layer. However, lysozyme can cleave the peptidoglycan layer of Gram-negative bacteria if this layer is exposed by the membrane attack complex of the complement system. Lysozyme is present in the granules of neutrophils and is a secretory product of macrophages. It is present in most body fluids, including saliva and tears.

Lactoferrin

The availability of iron is essential for the growth of many pathogenic bacteria including Staphylococcus aureus, Pasteurella multocida and E. coli. Sequestration of iron can therefore have an inhibitory effect on bacterial growth, especially for intracellular bacteria.

Lactoferrin, a member of the transferrin family present in exocrine secretions, is an iron-binding protein released by degranulating neutrophils. This protein sequesters iron from the plasma and tissues. Reduction of the amount of iron available for bacterial pathogens limits their ability to multiply. For some bacteria, such as Pseudomonas aeruginosa, reduced availability of iron interferes with biofilm formation. Gastric pepsin hydrolysis of lactoferrin generates lactoferricin, a peptide with potent antibacterial activity.

Peroxidase activity, present in many mucosal secretions, derives from lactoperoxidase synthesized in exocrine glands and secreted on to mucosal surfaces. Neutrophils also contribute to peroxidase activity through secretion of myeloperoxidase. The protective effect of peroxidases is due to their role in catalysing the peroxidation of halides, particularly chlorine and iodine, thereby generating reactive products with marked antimicrobial activity.

Phagocytic cells

Cells that participate in the recognition, removal and destruction of bacterial invaders are referred to as phagocytes. Neutrophils, monocytes, macrophages and dendritic cells are the principal cell types involved in phagocytosis, a process in which relatively large particles such as bacteria or yeasts are engulfed by these phagocytic cells. Phagocytosis is a receptor-mediated process in which specific recognition of a particle by receptors on a phagocytic cell leads to the engulfment of the particle and fusion of the vesicle containing the engulfed item with specialized intracellular organelles. The stages involved in phagocytosis of pathogenic bacteria include activation, chemotaxis and attachment of the organism followed by ingestion and destruction of the bacteria. Pathogenic bacteria are not uniformly susceptible to phagocytosis. In the absence of opsonins, encapsulated bacteria can resist phagocytosis and multiply uninhibited in tissues.

Neutrophils

The ability to ingest and kill pathogenic bacteria is an essential component of host defence. Neutrophils provide a major defence against a wide range of Grampositive and Gram-negative bacteria. Although they have short half-lives ranging from hours to a few days, these cells not only participate in phagocytosis of pathogenic bacteria but they also produce a range of antimicrobial substances including lysozyme, catheli-cidins and defensins. In response to many types of infections, especially bacterial infections, the bone marrow releases large numbers of neutrophils and these cells are usually the first to arrive at the site of tissue invasion. This transient increase in the number of circulating neutrophils, termed leukocytosis, is often interpreted as an indication of an infectious condition. Although neutrophils may contribute to innate immunity through the production of many important antibacterial substances, the predominant role of these cells is the phagocytosis and destruction of microbial pathogens,

particularly pathogenic bacteria.

Within minutes of tissue injury, there is increased blood flow to the affected area accompanied by increased vascular permeability, release of chemoat-tractants and transendothelial migration of phagocytes to the site. Three families of surface proteins, selectins, integrins and intercellular adhesion molecules, contribute to neutrophil arrival at the site of inflammation. Rolling is mediated by weak binding of glycoproteins on the neutrophil surface to selectins on endothelial cells. Under the influence of chemoattract-ants which bind to receptors on neutrophils, confor-mational changes occur in integrin molecules on the phagocytes which enable them to bind firmly to intercellular adhesion molecules on the surface of endothe-lial cells. Subsequently, in a process called diapedesis, the neutrophils move between the endothelial cells and arrive at the site of bacterial invasion. Before neu-trophils migrate across the endothelium to the site of inflammation, chemokines activate circulating neu-trophils and induce them to bind to the endothelium. Two chemokines, IL-8 and macrophage inflammatory protein 1^2, bind to receptors on neutrophils and increase their affinity for intercellular adhesion molecules on the endothelium. Following transendothe-lial migration, neutrophils are attracted to the site of infection by gradients of chemoattractants.

Complement fragments such as C3a and C5a, leu-kotrienes and bacterial peptides containing N- formyl peptides and locally produced chemokines and cytokines bind to specific receptors on neutrophils and contribute to their directed migration to the damaged tissue. Neutrophils express surface receptors which recognize bacterial pathogens in the blood and tissues. Toll-like receptors, complement receptors and receptors for *N*-formylmethionyl peptides function as receptors for bacterial pathogens which lead to phagocytosis and destruction of invading bacteria. Recognition and adherence of bacteria to neutrophils lead to engulfment of these microbial pathogens and this process is facilitated by a variety of protein molecules especially specific antibody and components of the complement system. Substances that facilitate the ingestion of bacteria by phagocytes are termed opsonins and the process involved is referred to as opsonization. Neutrophils have receptors for C3b and the Fc portion of IgG. Other opsonins include C-reactive proteins and mannose-binding lectin. Neutrophils ingest opsonized bacteria into a vesicle termed a phagosome, where they are destroyed. The sequential steps in phagocytosis involve the formation of pseudopodia which extend around the bacteria. These structures meet and fuse, forming a phagosome. The phagosome fuses with lysosomes, becoming a

phagolysosome containing antimicrobial proteins. Lysosomal enzymes digest the engulfed material which is degraded and subsequently released from the phagocyte. Neutrophils have an antimicrobial armoury stored in two types of granules, primary and secondary. These phagocytic cells also contain other types of granules and secretory vesicles. The contents of primary granules include acid hydrolases, myeloperoxidase, elastase, cathepsin G, defensins, bacterial permeability inducing protein and lysozyme. Secondary granules contain lactoferrin, lysozyme and a number of other components. Killing of engulfed bacteria is accomplished by two major antimicrobial systems, one oxygen independent and the other oxygen dependent. Non-oxidative killing of bacterial pathogens involves defensins, cathepsin G and lysozyme. The oxidative systems of bacterial destruction involve the generation of reactive oxygen species and reactive nitrogen species. Oxygen consumed by phagocytic cells to support the generation of reactive oxygen species is provided by what is referred to as the respiratory burst, characterized by a marked uptake of oxygen. The principal toxic factors generated include superoxide anion, hydrogen peroxide, singlet oxygen, hydroxyl radicals and hypochlorous acid. Reaction of nitric oxide with superoxide generates reactive nitrogen species. Myeloperoxidase is present in high concentrations in primary granules. During phagocytosis, activation of the respiratory burst is accompanied by degranulation with release of hydrogen peroxide and myeloperoxidase into the phagosome. Other toxic factors produced include chloramines, hydroxyl radicals and singlet oxygen. Many antibacterial substances are present in body fluids and tissues but ultimately most pathogenic bacteria are killed by phagocytic cells such as neutrophils.

Macrophages

Unlike neutrophils, which have a short life span but are efficient phagocytic cells, macrophages are long-lived cells. Macrophages are not only phagocytic cells but also provide immune surveillance in tissues and participate as effective cells in adaptive immune responses. Some occur as fixed cells in tissues, others as migratory cells. Tissue macrophages have different names and functions depending on their location: alveolar macrophages in the lungs, Kupffer cells in the liver and microglial cells in the brain. In the liver, Kupffer cells play a central role in clearing bacteria from the bloodstream. Besides killing and clearing bacterial pathogens, macrophages also have a central role in the coordination of other cells and tissues of the immune system. They exert their influence by the secretion of a number of cytokines including IL-1, IL-6, IL-8, IL-18, IFN-α and

TNF-α. Macrophages and their circulating precursors, monocytes, have an array of pattern recognition receptors such as receptors for lipoteichoic acid of Gram-positive bacteria, lipopoly-saccharide of Gram-negative bacteria and β-glucan of fungal pathogens. Phylogenetically, macrophage-type cells are among the oldest mediators of innate immunity. Unlike neutrophils, macrophages continue to differentiate after they leave the bone marrow and they can become activated if stimulated in an appropriate manner. Although macrophages and neutrophils share some common features as phagocytic cells, macrophages occupy a more central role in innate immune responses than neutrophils, and as antigen-presenting cells they contribute to the initiation of adaptive immune responses through the activation of T lymphocytes.

Phagocytosis by macrophages is initiated by binding of microbial pathogens to surface receptors, leading to transmembrane activation signals that result in their engulfment. When bacteria are opsonized by antibody or complement components, phagocytosis is enhanced. After ingestion, maturation of the phagosome occurs through a series of sequential steps. The phagosome acquires hydrolytic enzymes such as cathepsin-D and its pH drops. Late stages of maturation involve fusion with lysosomes which results in the formation of a phagolysosomal structure containing a range of degradative lysosomal hydrolases. The entire process of maturation results in the generation of an antimicrobial environment in which destruction and digestion of the engulfed microbial pathogen takes place. Reactive oxygen intermediates, arising from the respiratory burst of macrophages, contribute to the destruction of ingested bacteria. Superoxide ions, hydroxyl radicals and hydrogen peroxide, generated in the phagolysosomal structure, exert toxic effects on the bacterial pathogens. Inducible nitric oxide synthase is an important enzyme which enhances the antibacterial action of macrophages. This enzyme leads to the production of nitric oxide which, following interaction with superoxide and thiol groups, generates a number of antimicrobial compounds.

Activation of macrophages enhances their antimicrobial capability. Macrophage activation occurs most effectively by the combination of exposure to bacterial products and to IFN-γ acting on specific macrophage receptors. In response to IL-12, IL-15 and IL-18 produced by antigen-bearing dendritic cells, NK cells and T_h1 $CD4^+$ cells secrete IFN-γ which enhances a number of microbiocidal pathways in macrophages, including the respiratory burst and generation of inducible nitric oxide synthase. During adaptive immune responses to intracellular bacteria such as *Mycobacterium bovis* and *Brucella abortus*, the

most important source of IFN-γ is T_H1 $CD4^+$ lymphocytes. Tissue macrophages involved in immune responses to intracellular pathogens may undergo terminal differentiation into multinucleated giant cells, often observed in tuberculous lesions. If intracellular pathogens persist at the site of tissue invasion, recruitment and activation of macrophages and T cells can result in the formation of a granuloma at the site. Granulomas associated with tuberculosis in animals are usually composed of a core of infected and uninfected macrophages, epithelioid cells and multi-nucleated giant cells with accumulations of T cells at the periphery (see Fig 23.2).

Adaptive immune responses to bacterial infections

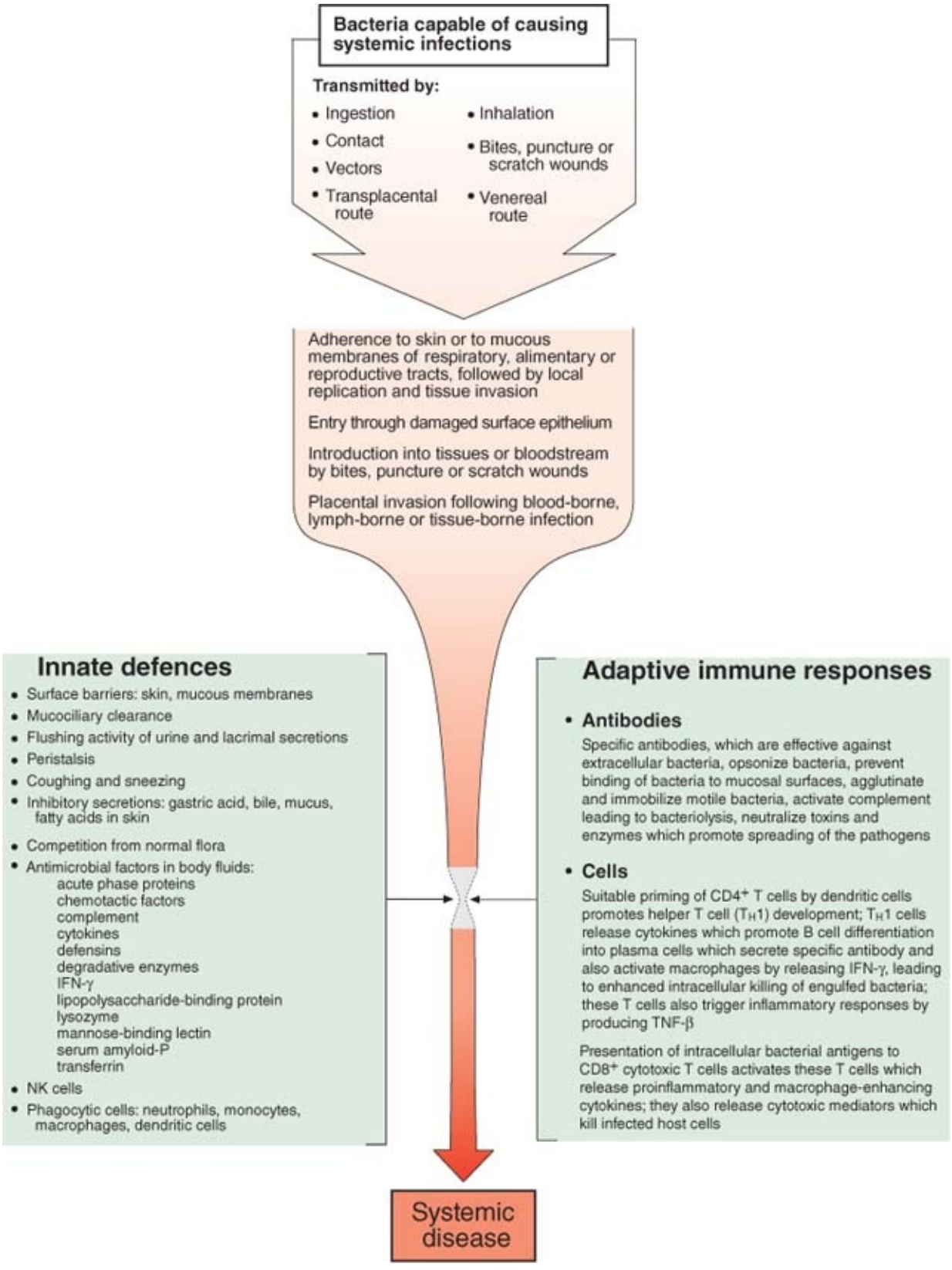
After uptake of bacteria by dendritic cells, peptides of bacterial origin are presented to T cells in association with MHC molecules on the surface of the dendritic cells. Whether they survive intracellularly or exist extracellularly, bacteria can activate $CD4^+$ T_H cells. In addition, some intracellular bacteria can activate $CD8^+$ cytotoxic T lymphocytes, which are MHC class I restricted. Induction of MHC class II restricted T cells follows contact with bacterial antigen from organisms which were exogenous. After binding of bacteria to specific cell surface receptors on macrophages and dendritic cells and engulfment following opsonization or receptor-induced endocytosis, bacterial antigens are degraded in endosomes and digested into peptides. The peptides associate with MHC class II molecules before transfer to the cell surface. Induction of MHC class I-restricted cytotoxic T lymphocytes follows entry of intracellular bacteria followed by antigen processing. Often by escaping from endosomes into the cytoplasm, intracellular bacteria are capable of evading many steps employed by phagocytes in the destruction of bacterial pathogens. MHC class I restricted cytotoxic T lymphocytes kill host cells infected with intracellular bacteria, whereas $CD4^+$ T_H 1 cells release cytokines which activate macrophages and promote bacterial destruction within these phagocytic cells. They also provide support for antibody production by B cells. Functionally distinct subtypes of $CD4^+$ T cells strongly influence immune responses through the secretion of cytokines. Subtype $CD4^+$ T_h1 cells which secrete IFN-γ and TNF-β activate macrophages and promote destruction of engulfed bacteria. This subtype also promotes production of opsonizing antibody. The secretions of $CD4^+$ T_h2 cells include IL-4, IL-5, IL-6, IL-10 and IL-13 which support

production of particular antibody isotypes, some involved in allergic responses. This subtype also plays a central role in the involvement of eosinophils, basophils and mast cells in response to invading parasites, especially helminths. Identified by their secretion of IL-17, T_H17 cells promote antimicrobial peptide production by epithelial cells and contribute to neutrophil participation in local inflammatory responses. Another subtype of $CD4^+$ T cells, referred to as regulatory T cells (T_{REG}), produce IL-10 and TFG-β which suppress host immune responses, especially $CD4^+$ T_H1 responses when effective control of invading microbial pathogens has been achieved.

Cell-mediated immunity is essential for the control of intracellular bacterial infections but humoral immunity has a major protective role against extracellular bacteria. Unless a bacterial pathogen is capable of intracellular growth and survival, antibodies offer effective protection against many bacterial infections. Bacteria can enter the body through ingestion, inhalation, contact, puncture wounds and other routes (Fig. 3.14). Following introduction into the body, attachment to host cells and invasion of tissues may occur. Toxin-induced damage to tissues may result in localized or systemic disease. Bacteria with fimbrial adhesins can adhere to mucosal epithelium or to ciliated epithelium of the upper respiratory tract. Secretory IgA can bind to bacterial adhesins and block bacterial attachment to mucosal surfaces. The presence of a capsule can prevent phagocytosis but, when opsonized by specific antibody and C3b, such encapsulated bacteria can be ingested and destroyed by phagocytic cells. Specific antibodies can exert their protective effects against extracellular bacteria in many different ways. In addition to preventing bacterial attachment to tissues and opsonization of encapsulated bacteria, antibodies can agglutinate and immobilize motile bacteria, activate complement and neutralize bacterial toxins and enzymes which promote their spreading in tissues (Fig. 3.14). Susceptible bacteria with bound IgG or IgM antibody molecules which activate complement can be lysed by the membrane attack complex. Host cells which display on their surfaces peptides derived from intracellular bacteria can be destroyed by NK cells or macrophages in the antibody-dependent cell-mediated cytotoxicity reaction. Antibodies produced by the dam and secreted in colostrum passively protect newborn animals against bacterial toxins such as the neurotoxin of *Clostridium tetani*, which causes tetanus. Many bacterial vaccines used in veterinary medicine confer protection against bacterial infections by inducing production of circulating protective IgG antibodies or memory B cells.

Components of innate and adaptive immune responses which contribute to protection against bacterial pathogens are presented in Table 3.14.

Figure 3.14 Modes of transmission of bacterial pathogens that cause systemic disease. Innate defences and adaptive immune responses against these pathogens are outlined. TNF: tumour necrosis factor; IFN-γ: interferon-γ.


Bacteria capable of causing systemic infections
Transmitted by:
• Ingestion
• Contact
• Vectors
• Transplacental route
• Inhalation
• Bites, puncture or scratch wounds
• Venereal route
Adherence to skin or to mucous membranes of respiratory, alimentary or reproductive tracts, followed by local replication and tissue invasion
Entry through damaged surface epithelium
Introduction into tissues or bloodstream by bites, puncture or scratch wounds
Placental invasion following blood-borne, lymph-borne or tissue-borne infection
Innate defences
• Surface barriers: skin, mucous membranes
• Mucociliary clearance
• Flushing activity of urine and lacrimal secretions
• Peristalsis
• Coughing and sneezing
• Inhibitory secretions: gastric acid, bile, mucus, fatty acids in skin
• Competition from normal flora
• Antimicrobial factors in body fluids:
acute phase proteins
chemotactic factors
complement
cytokines
defensins
degradative enzymes
IFN-γ
lipopolysaccharide-binding protein
lysozyme
mannose-binding lectin
serum amyloid-P
transferrin
• NK cells
• Phagocytic cells: neutrophils, monocytes, macrophages, dendritic cells
Adaptive immune responses
• Antibodies
Specific antibodies, which are effective against extracellular bacteria, opsonize bacteria, prevent binding of bacteria to mucosal surfaces, agglutinate and immobilize motile bacteria, activate complement leading to bacteriolysis, neutralize toxins and enzymes which promote spreading of the pathogens
• Cells
Suitable priming of CD4+ T cells by dendritic cells promotes helper T cell (T_H1) development; T_H1 cells release cytokines which promote B cell differentiation into plasma cells which secrete specific antibody and also activate macrophages by releasing IFN-γ, leading to enhanced intracellular killing of engulfed bacteria; these T cells also trigger inflammatory responses by producing TNF-β
Presentation of intracellular bacterial antigens to CD8+ cytotoxic T cells activates these T cells which release proinflammatory and macrophage-enhancing cytokines; they also release cytotoxic mediators which kill infected host cells
Systemic disease

Immunity to fungi

Of the vast number of fungal species known, only a relatively small number produce disease in humans and animals. Three categories of fungi: moulds, yeasts and dimorphic fungi, can produce infections in susceptible animals. Apart from the dermatophytes, pathogens that cause ringworm in animals and humans, the majority of fungi are saprophytes. Tissue invasion by fungi is usually indicative of immunological incompetence, immunosuppression or, in the case of yeast infections, a consequence of prolonged antibacterial therapy. Innate defence mechanisms offer the first and often the most important protection against many opportunistic fungal invaders. Adaptive immune responses, based on cell-mediated reactions to fungal agents, may strengthen host defences especially against intracellular fungal invaders. Humoral immune responses to fungal agents are specific and useful for diagnostic purposes. Specific antibodies may promote engulfment of fungal structures by neutrophils but, on their own, offer limited protection against invasive *Aspergillus* species and a number of other opportunistic fungal invaders.

Innate immune responses to fungi

Intact skin, with its low pH and secreted fatty acids, and mucosal surfaces with their antimicrobial secretions are major barriers to fungal invasion. Competition from normal commensal microflora on the skin and on mucosal surfaces is important for its inhibitory effect on yeast proliferation in the oral cavity, along the alimentary tract and on other mucosal surfaces. An array of antimicrobial factors in body fluids, phagocytic cells and pathogen-recognition receptors on host cells and soluble pathogen-recognition receptors, provide both protection against fungal invasion and detection of their presence on mucosal surfaces in the respiratory tract, in the alimentary tract and in the urogenital tract.

Aspergillosis, which is primarily a respiratory infection, follows inhalation of spores. Respiratory epithelial cells are the first anatomical barrier to invasion, and mucociliary clearance contributes to local protection. Alveolar macrophages, which typically constitute the first line of phagocytic defence, are joined later by blood monocytes and neutrophils at the site of infection. Natural killer cells contribute to innate defences as infection progresses. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, present in phagocytes, is a requirement for host defence against aspergillosis. Activation of NADPH oxidase generates superoxide anion from oxygen with the production of

additional antimicrobial factors. In neutrophils, activation of NADPH oxidase is linked to antimicrobial proteases in primary granules and results in hyphal damage. Neutrophil-mediated inhibition of fungal spore growth is independent of NADPH oxidase, however, and requires lactoferrin-mediated iron depletion (Segal, 2009).

Cell-associated and soluble pathogen-recognition receptors which identify fungal motifs include tolllike receptors (TLRs), dectin-1, pentraxin-3, mannose-binding lectin and surfactant proteins A and D. Involvement of TLRs induces production of proinflammatory cytokines. Dectin-1 is a receptor for β-glucans, common components of fungal cell walls, and is expressed at high levels in the pulmonary and gastrointestinal tracts. Dectin-1, which recognizes β-glucan motifs, and TLR-2, which recognizes phospholipomannan, stimulate pro-inflammatory cytokine production. These structures, dectin-1 and TLRs, enable host cells to distinguish resting spores from germinating spores and both spore types from hyphae of *Aspergillus fumigatus*. Fungal β-glucans trigger pro-inflammatory responses in macrophages to germinating spores. Gliotoxin, produced by *A. fumigatus*, is a potent inhibitor of macrophage phagocytosis (Richardson, 2005).

Among the pathogen-recognition receptors on host cells, dectin-1, TLR-2 and TLR-4 contribute to fungal recognition. Soluble pathogen-recognition receptors include pentraxin-3, mannose-binding lectin and TLR-9. Dendritic cells recognize fungal structures through pathogen-recognition receptors and stimulate antigen-dependent responses in T helper cells and regulatory T cells (T_{REG}). Mannose-binding lectin can interact with a number of fungal agents including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. Activation of complement by this pathway results in the binding of additional complement components to the target fungal structure and may facilitate phagocytosis or intracellular destruction by phagocytic cells.

Table 3.14 Components of innate and adaptive immune responses that contribute to protection against bacterial pathogens.

Components	Contribution to protection
Soluble factors	
Antibodies	Offer protection against extracellular bacteria and their toxins; opsonize bacteria, prevent their attachment to mucosal surfaces; agglutinate and immobilize motile bacteria; neutralize toxins and enzymes which promote spreading of bacterial pathogens
Antibodies + complement	Activation of complement by IgG or IgM antibodies bound to bacteria leads to bacteriolysis, mediated by the membrane attack complex
Antimicrobial peptides	Defensins and cathelicidins, with activity against a wide range of bacterial pathogens, prevent colonization at particular sites such as mucosal surfaces in the respiratory and alimentary tracts
C-reactive	

protein	Acute phase protein synthesized in the liver promotes complement activation and opsonization of bacterial pathogens
Complement	When activated by immune complexes or microorganisms, this large group of plasma proteins can generate proteolytic enzymes which lyse bacterial pathogens or promote their destruction through opsonization followed by phagocytosis; fragments C3a and C5a have chemotactic activity
Interferon-γ	An important activator of macrophages leading to an increase in phagocytosis and enhanced intracellular killing of bacterial pathogens; promotes development of $CD4^+T_H1$ cells and increased expression of MHC molecules on a range of cells
Lactoferrin	A member of the transferrin family present in exocrine secretions, this iron-binding protein sequesters iron from plasma and tissues and interferes with the multiplication of a number of bacterial pathogens
Lysozyme	A highly cationic protein present in body fluids, this antimicrobial factor cleaves the peptidoglycan layer of Gram-positive bacteria; it can damage the peptidoglycan layer of Gram-negative bacteria exposed by the membrane attack complex of the complement system
Cells	
Neutrophils	Despite their short life span, these circulating cells are efficient phagocytes which have a major role in the ingestion and destruction of bacterial pathogens; production of many antibacterial substances adds to their role in innate defences
Macrophages	These long-1 ived phagocytic cells have a central role in innate defences and also in the initiation of adaptive immune responses where they can act as antigen-presenting cells; some occur as fixed cells in tissues, others as migratory cells; their ability to become activated, through the action of IFN-γ, enhances their antibacterial role, especially against intracellular pathogens
T cell subsets	
$CD4^+T_H1$ cells	Through the release of cytokines, especially IFN-γ, these cells activate macrophages and promote destruction of ingested bacteria and also intracellular bacteria such as mycobacteria and *Listeria* species; promote maturation of precursor T cells to cytotoxic T lymphocytes
Cytotoxic T lymphocytes	Presentation of antigens from intracellular bacteria in association with MHC class I molecules to these $CD8^+$ cytotoxic T lymphocytes activates these effector cells which release cytotoxic mediators which kill infected host cells
Regulatory T cells	These $CD4^+$T cells (T_{REG}) suppress host immune responses, particularly $CD4^+T_H1$ cell responses, when effective control of microbial pathogens has been achieved

Aspects of transmission of fungal pathogens and innate and adaptive immune responses to these agents are illustrated in Fig. 3.15. Components of innate and specific adaptive immunity which contribute to protection against fungal pathogens are presented in Table 3.15.

Adaptive immune responses to fungi

Activation of pathogen-recognition receptors induces maturation of antigen-presenting cells which prime T cell mediated immunity. Interactions between macrophages and lymphocytes are required for the control of intracellular fungal infections, especially for dimorphic fungi. In response to fungal invasion, macrophages release IL-12 which acts on T lymphocytes and NK cells. When stimulated by IL-12, lymphocytes and NK cells release IFN-γ which acts on macrophages, promoting destruction of engulfed fungal structures. In addition, IFN-γ augments TNF-α production by macrophages, which enhances killing of fungal invaders. When dectin-1, on the surface of epithelial cells, binds to β-glucans on fungal structures, activation of pathways which produce cytokines and drive $CD4^+$ T lymphocytes towards the T_H17 phenotype result (Holland and Vinh, 2009). Subsequently, T_H17 lymphocytes elaborate IL-17 and IL-22 which

promote antimicrobial peptide production by epithelial cells. Although IL-17-producing $CD4^+$ T cells stimulate production of specific myelopoietic growth factors, cytokines and chemokines which promote neutrophil recruitment, in some instances IL-17 can depress host defences in experimental models. Regulatory T cells induce tolerance and decrease allergic responses to fungal antigens.

Resistance to most fungal agents is dependent on T cell-mediated immunity, particularly $CD4^+$ T_H1 cells secreting INF-γ, with the participation of dendritic cells. Unlike T_H1 cells, T_H2 cells produce IL-4, IL-5 and IL-13 and, rather than promoting protection, are associated with allergic reactions to fungal antigens. Although specific antibodies may opsonize fungal structures in host tissues and contribute to their clearance by neutrophils, protective immunity is not usually associated with humoral immune responses.

Immunity to viruses

Viral invasion of host cells may result in direct interference with cellular functions and, in addition, may induce deleterious host responses. Because of the complex interactions between viruses and their hosts, clinical signs of disease may not be evident and the form and severity of disease is influenced by host susceptibility, by the virulence of the virus and by other characteristics of the virus including routes of transmission, tissue preferences and dissemination within the host. The diversity evident among viruses that affect mammalian and avian species limits the value of direct comparison of viral pathogens. Likewise, immune mechanisms which apply to viral agents are usually valid only for individual virus families or for viruses that share common biological characteristics.

All viruses are obligate intracellular pathogens. Despite their dependence on host cells for replication, viruses display enormous diversity. They have evolved highly sophisticated mechanisms for cellular invasion, replication and evasion of immune responses. Innate immune responses to viral infections include the induction of type I interferons and activation of NK cells. Cytotoxic T cells and neutralizing antibodies are major adaptive immune responses which confer long-term protection against individual viruses. The high mutation rate among viruses, especially RNA viruses and retroviruses, and the ability of viruses to evade host immunity are reasons why long-lasting protection against reinfection

is difficult to achieve for some viruses.

Innate immunity to viral infections

The skin and mucous membranes offer the first line of defence against viral invasion. If these defences are breached, early innate immune responses begin with the release of soluble mediators of inflammation which include cytokines, chemokines and complement fragments. These soluble mediators regulate cell movement and cell recruitment to sites of inflammation, cell adhesion and cell activation. In addition, some mediators may induce apoptosis of infected cells. The host's immune response is aimed at the complete elimination of the viral pathogen and many released mediators have potent antiviral activity. Factors that influence the outcome of a viral infection include the immune status and age of the host, the general health of the animal and intercurrent infections. Among the viral factors that influence the outcome are strain virulence, size of infecting dose and the route of entry into the host.

Figure 3.15 Modes of transmission of fungal pathogens which cause systemic disease. Innate defences and adaptive immune responses against these pathogens are outlined. IFN-γ: interferon-γ.

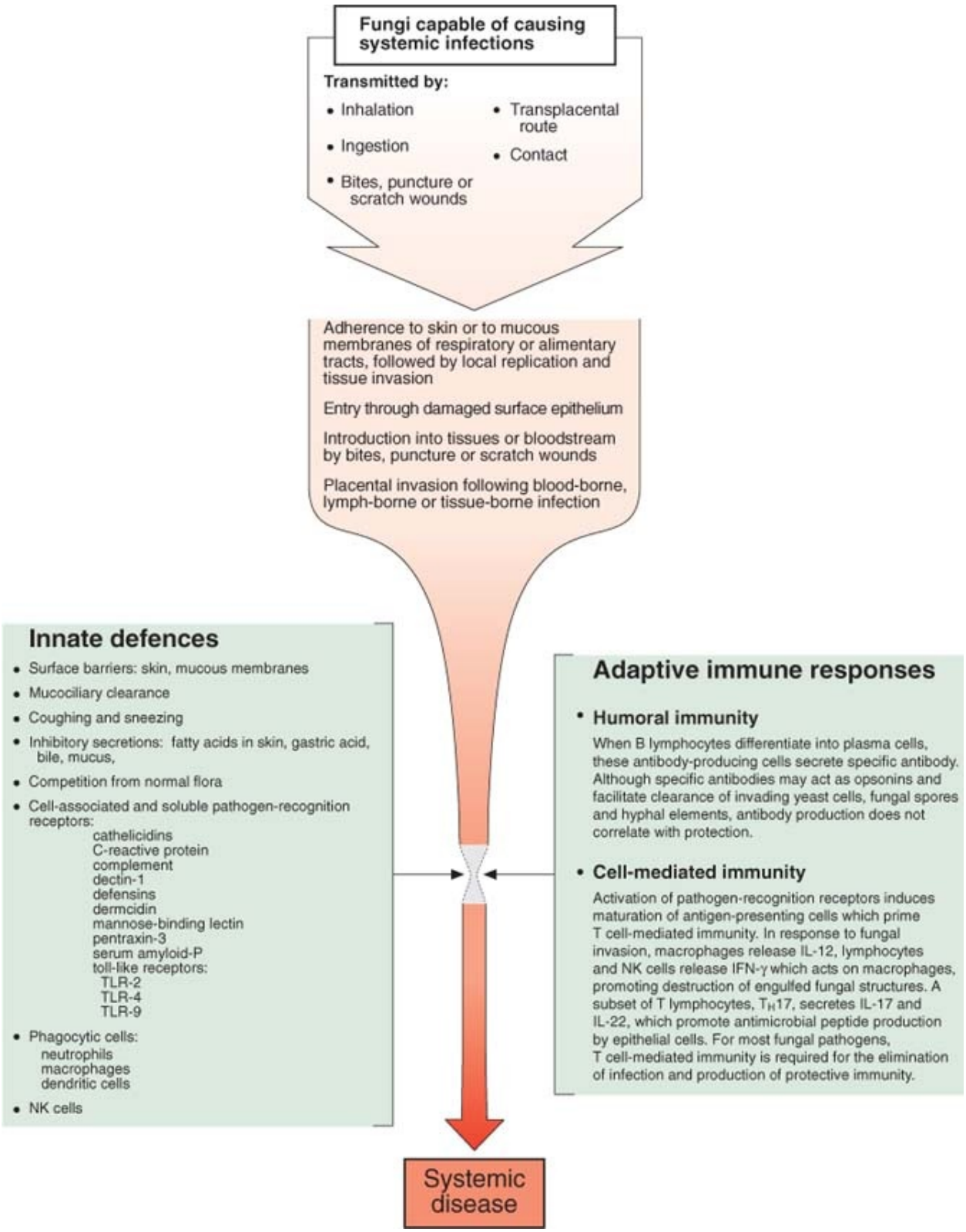


Table 3.15 Components of innate and adaptive immunity which contribute to protection against fungal pathogens.

Component	Comments
Innate immunity	
Cells	
Dendritic cells	As antigen-presenting cells, dendritic cells play a central role in the induction and activation of T cells; secrete IL-12 which promotes $CD4^{+}T_{H}1$ development; possess an array of pattern-recognition receptors including dectin-1 which binds to β-glucans
Epithelial cells	Anatomical barrier to fungal invasion, especially in the respiratory and gastrointestinal tracts of animals; pathogen-recognition receptors on these cells for fungal pathogens include dectin-1
Macrophages	Receptors for fungal pathogens on macrophages include TLR-2, TLR-4 and dectin-1; when activated by cytokines released from $CD4^{+}T_{H}1$ lymphocytes, especially IFN-γ, macrophage destruction of engulfed fungal structures is enhanced; NADPH oxidase in these phagocytes is essential for defence against aspergillosis
Neutrophils	These phagocytic cells have a central role in engulfment and destruction of fungal forms such as yeasts and spores; neutrophil granules contain cationic antimicrobial peptides active against fungi; NADPH oxidase forms part of their antifungal components; TLR-2 and other pathogen-recognition receptors on neutrophils aid the recognition and removal of fungal structures
NK cells	Although principally concerned with the destruction of virus-infected cells and certain tumour cells, NK cells release IFN-γ following the production of IL-12 by macrophages; activation of macrophages by IFN-γ promotes destruction of engulfed fungal structures by these phagocytic cells
Cell-associated and soluble pathogen-recognition receptors	
Cathelicidins	Cationic antimicrobial peptides present in the secondary granules of neutrophils; active against bacteria and fungi
C-reactive protein	Acute phase protein with a pentameric structure which is synthesized in the liver; promotes complement activation and opsonization of bacterial and fungal pathogens
Dectin-1	This receptor for β-glucans is a C-type lectin present on epithelial cells, phagocytes and dendritic cells
Defensins	Cationic antimicrobial peptides active against bacteria and some fungi
α Defensins	Present in the primary granules of neutrophils and intestinal Paneth cells
β Defensins	Produced by epithelial cells of the epidermis, respiratory and genitourinary tracts
Dermcidin	Antimicrobial peptide produced by sweat glands and active against bacteria and fungi
Mannose-binding lectin	Acute phase protein synthesized in the liver; binds mannose-containing bacterial and fungal polymers, promoting complement activation and phagocytosis
Pentraxin-3	Soluble factor which recognizes specific fungal motifs
Serum amyloid-P	Acute phase protein synthesized in the liver; binds to components of Gram-positive bacteria and fungi, promoting complement activation, opsonization and phagocytosis
Toll-like receptors (TLRs)	Conserved family of pathogen-recognition receptors; activation of TLRs usually induces expression of pro-inflammatory cytokines
TLR-2	Present on a wide range of cells including macrophages, dendritic cells, neutrophils and endothelial cells; binds zymosan, a component of fungal cells
TLR-4	Present on the membrane of phagocytes; following interaction with fungal components, contributes to production of cytokines and chemokines
TLR-9	Intracellular pathogen-recognition factor which interacts with fungal DNA
Receptor for β-glucans	Macrophage receptor which binds β-glucans and zymosan; promotes phagocytosis of fungal pathogens
Adaptive immunity	
Cells	
B lymphocytes	B cell activation follows cross-linking of B cell surface receptors by antigen, either directly or by dendritic cells, with suitable stimuli from T helper cells. Cytokine secretion by T cells, such as IL-2 and IFN-γ influences the antibody isotype when B cells differentiate into plasma cells and secrete specific antibody
	Different subsets of T lymphocytes participate in immune responses to infectious agents, generating cell-mediated immunity.

T lymphocytes	Involvement of $CD4^+$ T helper cells is probably the most effective host defence against systemic fungal disease. Dendritic cells not only present fungal antigen to $CD4^+$ T_H1 cells but also produce IL-12 which activates this subset. Activated $CD4^+$ T_H1 cells release IFN-γ which enhances intracellular killing of engulfed fungi by macrophages. Production of IL-17 and IL-22 by T_H17 cells promotes secretion of antimicrobial peptides by epithelial cells
Secretions	
Antibodies	Fungal pathogens often elicit specific antibody responses which are useful for the serological diagnosis of disease but raised antibody levels do not necessarily correlate with protection
Cytokines	A range of cytokines including IL-12 produced by macrophages and dendritic cells, and IFN-γ produced by NK cells and T lymphocytes, influence the functioning of cells that contribute to cell-mediated immune responses to fungal pathogens; IL-17 and IL-22 produced by T_H17 lymphocytes stimulate production of antimicrobial peptides by epithelial cells

The ability of the host to detect viral invasion and to respond rapidly to the pathogen is a requirement for the elimination of the invading infectious agent. The innate immune response has developed as a rapid and regulated defence system in which the recognition of an invading pathogen can occur by binding to a specific cytoplasmic receptor or toll-like receptor (TLR). These TLRs recognize conserved patterns of proteins, lipoproteins, double-stranded RNA or unmethylated DNA which are pathogen-associated molecular patterns. These interactions induce multiple signalling pathways which promote activation of cells and secretions that contribute initially to innate defences and later to adaptive immune responses.

Induction of innate immune responses to viral infections results in the production of type I interferons and activation of NK cells. The term interferon derives from the ability of these cytokines to inhibit viral replication. Production of type I interferons, the earliest immune response of the host to viral infection, is followed closely by the activation of NK cells. Type I interferons, a family of related polypeptides, include interferon-α (IFN-α), IFN-β, IFN-κ, IFN-λ, and a number of other cytokines with similar biological activity. The typical stimulus for type I interferon synthesis is viral infection, specifically double-stranded RNA produced during viral replication in an infected cell. Type II interferon, or IFN-γ, is produced following antigenic or mitogenic stimulation of lymphocyte subsets. Interferon-α is produced by leukocytes, especially macrophages, following viral infection. In response to a viral infection, fibroblasts and epithelial cells produce IFN-β. The induction, production and other characteristics of IFN-α, IFN-β and IFN-γ are summarized in Table 3.16.

Interferons

Type I interferons are produced by cells of the innate immune system and also by infected cells. There are two distinct ways whereby interferons can be produced: by infected cells which detect components of virus replication within

them, and by cells of the innate immune system which detect the presence of virus through toll-like receptors. Pathogen-derived nucleic acids can be recognized by a subset of toll-like receptors located intracellularly. These receptors can detect virions after engulfment by phagocytosis or pinocytosis. Pattern-recognition receptors involved in the detection of viral pathogens include TLR-3, TLR-7 and TLR-9. Immature dendritic cells, tissue macrophages and plasmacytoid dendritic cells express one or more of these receptors and produce type I interferons in response to signals from toll-like receptors. Plasmacytoid dendritic cells are especially important in the production of type I interferons.

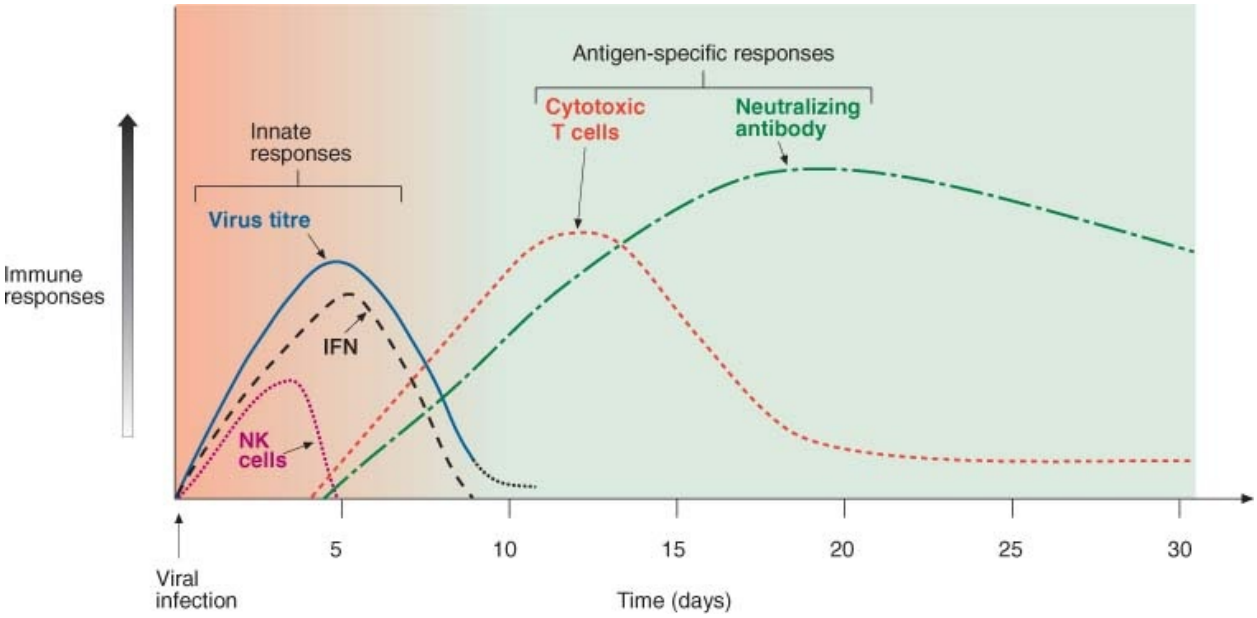
Within hours of viral infection, IFN-α and IFN-β are produced by infected host cells or by sentinel cells of the innate immune system. In acute systemic viral infection, interferon levels approximate virus titre as virus replication proceeds (Fig. 3.16). Interferons bind to the interferon receptor on adjacent host cells and induce production of antiviral protein, enabling them to resist infection. Interferons do not directly block viral replication but activate genes with direct antiviral activity. Type I interferons, bound to the interferon receptor on a cell, induce changes which include interference with protein synthesis and degradation of mRNA. Three important mechanisms relating to interference with viral replication involve synthesis of double-stranded RNA-dependent protein kinase, activation of latent cellular endonuclease and production of Mx proteins.

Protein kinase R (PKR) is expressed at low levels in many cell types but, after induction by interferon-receptor signalling, sufficiently high levels to interfere with virus replication are produced. The main target of PKR is the translation initiation factor eIF2α which it inactivates through phosphorylation. As PKR inhibits all protein synthesis, both cellular and viral, it prevents virus replication and may also result in cell death. Interferon receptor signalling induces expression of 2′,5′-oligoadenylate synthetases which, after recognition of double-stranded RNA, activate a latent cellular ribonuclease, RNase L, which degrades mRNA and also ribosomal RNA, thereby suppressing viral infection. The third antiviral mechanism induced by type I interferons involves Mx proteins which inhibit viral transcription of a range of RNA viruses and also interfere with virus assembly.

Table 3.16 Induction, production and biological activity of selected interferons.

Interferon	Induction	Production	Comments
Interferon-α	Viral invasion, double-stranded RNA	Leukocytes, especially macrophages	Multiple isoforms produced; induces an antiviral state in many cell types; activates NK cells
Interferon-β	Viral invasion, double-stranded RNA	Fibroblasts, epithelial cells	Single type produced; induces an antiviral state in many cell types; activates NK cells
Interferon-γ	Antigenic or mitogenic stimulation of cells	$CD4^+$ T_H1 cells $CD8^+$ T cells NK cells	Single type produced; acts as a macrophage-activating cytokine; stimulates activation of class I and class II MHC molecules on antigen-presenting cells; promotes differentiation of naïve $CD4^+$ T cells to the T_H1 subset

Figure 3.16 Outline of innate immune responses and antigen-specific immune responses following a typical acute systemic viral infection. IFN: interferon; NK: natural killer.



NK cells

Within days of a viral infection, activated NK cells are present in the tissues. By killing host cells expressing viral antigen on their surfaces, NK cells contribute to the elimination of cellular reservoirs of infection. The ability of NK cells to protect host cells against infection is enhanced by cytokines secreted by macrophages and dendritic cells. These include type I interferons, IL-12, IL-15 and IL-18. Binding of IFN-α and IFN-β to NK cells enhances the lytic activity of these cells and other cytokines produced by dendritic cells, and macrophages promote the antiviral activity of NK cells. Some viruses block MHC class I expression when they infect host cells, thereby preventing presentation of viral antigen in association with MHC class I molecules to cytotoxic T lymphocytes. In the absence of MHC class I molecules, NK cells recognize and destroy such

cells because the absence of MHC class I molecules releases NK cells from a normal state of inhibition.

By responding at an early stage to viral infection, NK cells contribute to host protection before adaptive immune responses have developed. Direct cytolysis through a perforin/granzyme mechanism, production of IFN-γ which protects cells from viral infection and activates macrophage antiviral activity, and destruction of infected cells through antibody-dependent cellmediated cytotoxicity are the major contributions of NK cells to antiviral immunity.

Macrophages contribute to antiviral immunity through phagocytosis of viruses and virus-infected cells, sometimes with the involvement of specific antibodies and complement. These phagocytic cells can destroy virus-infected cells and produce antiviral molecules such as IFN-α and tumour necrosis factor-α.

Adaptive immune responses to viral infections

Innate immune responses to viral invasion are succeeded by specific adaptive immune responses. Adaptive immunity is mediated by antibodies which block virus binding and entry into host cells and by cytotoxic T lymphocytes which can eliminate infection by killing virus-infected cells.

Humoral immunity

Antibody-mediated immunity is effective against viruses only at the extracellular stage of infection, before they enter host cells. In the case of cytopathic viruses which are released following destruction of host cells, antibodies play an important protective role also. In many instances, immunity against re-infection is mediated by circulating virus-neutralizing antibodies. Antiviral antibodies function mainly as neutralizing antibodies which prevent virus attachment and entry into host cells. These antibodies bind to the viral envelope, capsid antigens or other surface antigenic components. Secreted antibodies of the IgA isotype prevent attachment of viruses to host cells on mucosal surfaces. Antibodies produced by the dam and secreted in colostrum passively protect newborn animals against many viral pathogens. Specific antibodies can prevent attachment of viruses to host T cell receptors, thereby preventing entry into the cell. They can also promote clearance of virus particles from the circulation by clumping viruses and facilitating their removal by phagocytic cells. Lysis of some enveloped viruses by the membrane attack complex can occur when IgG or IgM antibodies bind to surface viral antigens and activate complement. When

viruses are coated by antibody, with C3b bound to the fixed antibody, they become opsonized virus particles, easily engulfed and destroyed by phagocytic cells. Infected host cells displaying viral antigen on their surfaces can be lysed by activation of the complement sequence through the involvement of bound antibody. Virus-infected cells, when coated with antibody and with C3b bound to their surfaces, may be engulfed and destroyed by phagocytes. Antibody bound to virus-infected host cells can initiate their destruction by NK cells in antibody-dependent cell-mediated cytotoxicity. Despite the diverse activities of antibodies in antiviral immunity, they are only effective against extracellular viral pathogens. Elimination of viruses within host cells requires the participation of NK cells or cytotoxic T lymphocytes which can kill such virus-infected cells.

Cell-mediated immunity

Although antibodies have an important role in the clearance of viruses from the circulation, preventing viraemia and spreading to target tissues at the acute phases of infection, they cannot eliminate intracellular infection. The principal T cell surveillance system which operates against viruses is selective and highly efficient and results in the destruction of virus-infected cells by cytotoxic T lymphocytes. In the course of a viral infection, $CD8^+$ T cells undergo rapid proliferation, reaching their peak at about 10 days before declining slowly (Fig. 3.16). Expansion of the T cell population is accompanied by differentiation into effector cytotoxic $CD8^+$ T cells which release cytokines, particularly IFN-γ and tumour necrosis factor, and kill infected cells directly through the release of perforin and granzymes or through the induction of apoptosis by Fas/Fas ligand interactions. Interferon-γ and tumour necrosis factor not only promote recruitment of inflammatory cells to the site of viral infection but also interfere with virus replication. A small proportion of the $CD8^+$ T cells which derived from the clonally expanded population of T cells, with distinct survival receptors, persist as memory cells. These memory cells, which received appropriate support from $CD4^+$ T cells during the initial activation, may survive for many years.

Most virus-specific cytotoxic T lymphocytes are $CD8^+$ T cells which recognize viral antigen displayed in association with MHC class I molecules on nucleated cells. Differentiation of $CD8^+$ cytotoxic T lymphocytes requires cytokines produced by $CD4^+$ T helper cells or co-stimulators from infected cells. The principal antiviral activity of cytotoxic T lymphocytes is associated with their

destruction of infected cells but other antiviral functions relate to activation of nucleases in infected cells which can degrade viral nucleic acid and production of IFN-γ which exerts some antiviral activity.

Destruction of virus-infected cells can occur through direct cellular interactions involving cytotoxic T lymphocytes and NK cells, through cytokine release and through granule exocytosis. Cytotoxic T lymphocytes can signal to target cells using Fas, a member of the TNF receptor family expressed on many cell types. When Fas ligand, expressed on activated T cells, binds to Fas, it initiates a signalling cascade which leads to apoptotic cell death.

Although NK cells and cytotoxic T lymphocytes recognize target cells by different means, they employ the same lytic mechanisms for the destruction of target cells. Specialized cytotoxic components released from cytotoxic T lymphocytes can kill target cells by damaging their cell membranes. These cytotoxic components, perforin and granzymes, are present in cytoplasmic granules termed lytic granules. When a target cell is recognized, the granules relocate to the site of contact between the cytotoxic T cell and the target cell and release the pore-forming protein, perforin, together with granule-associated enzymes referred to as granzymes.

Within the lytic cytoplasmic granules, perforin molecules are inactive monomers. When released into the extracellular space, where they encounter increased calcium levels, they undergo polymerization enabling them to form pores in the target cell membrane. Structurally and functionally, perforin shares common features with the complement component C9.

Granzymes, proteases which are contained in the lytic granules of cytotoxic T lymphocytes, are released on activation of these cells. These enzymes are serine proteases which apparently require the pore-forming action of perforin to enter the target cell in association with other small molecules and calcium ions. On entering the target cell, granzymes induce a series of changes in the cytoplasm which lead to the rapid initiation of apoptosis through activation of caspases, induction of DNA fragmentation and interference with cellular repair mechanisms. Granzyme A initiates apoptosis by a caspase-independent pathway which results in DNA fragmentation. Granzyme B cleaves a number of pro-caspases which results in apoptosis of the target cell. The mode of action of granzyme C is not clearly defined but this enzyme is reported to induce apoptosis by a caspase-independent pathway. Although many steps in the cytotoxic activity of perforin and granzymes remain to be elucidated, it is evident that the contents of lytic granules, when released, lead to rapid apoptosis

in target cells.

Neither NK cells nor cytotoxic T lymphocytes are damaged by the contents of their lytic granules. Both perforin and granzymes are synthesized as inactive molecules. Activation of perforin and granzymes occurs after their release from the lytic granules. High levels of calcium ions and other microenvironmental changes are required for their activation. As an additional safeguard, it is reported that cathepsin B, which lines the granule membranes, cleaves perforin on the cytotoxic T lymphocyte side of the contact point with the target cell.

Viruses which cause systemic infections in animals can be transmitted by many different routes. Common routes of transmission include the respiratory and alimentary tracts. Anatomical barriers, antiviral cytokines and phagocytic cells contribute to innate defences against viral invasion (Fig. 3.17). Adaptive immune responses, which develop more slowly than innate responses, are mediated by specific antibodies which neutralize circulating viruses and by cytotoxic T lymphocytes which destroy virus-infected cells. Components of innate and adaptive immunity that contribute to protection against viral infections are presented in Table 3.17.

Concluding comments

In an environment in which opportunistic infectious agents and pathogenic microorganisms are ubiquitous, protection against infection is a requirement for survival. The cooperative interactions of components of innate and adaptive immunity provide an effective defence against many pathogenic microorganisms. With its capacity to respond to a vast array of antigenic determinants on infectious agents, the immune system can respond to tissue invasion with effective humoral and cell-mediated responses. Characteristics of some infectious agents such as RNA viruses and retroviruses, with high mutation rates, can limit the effectiveness of immune responses, and long-lasting protection against some of these pathogens is difficult to achieve.

Table 3.17 Components of innate and adaptive immunity which contribute to protection against viral infections.

Components	Contribution to protection
Soluble factors	
Antibodies	Clear viruses from circulation, prevent viraemia and spread of viruses to target organs; neutralize extracellular viruses and prevent attachment of viruses to host T cell receptors; promote phagocytosis of virus particles; secretory IgA, produced locally, can protect mucosal surfaces

Antibodies bound to infected cells	Promote killing of virus-infected cells by NK cells and macrophages through ADCC
Antibodies + complement	Promote lysis of some enveloped viruses by assembly of the membrane attack complex; lysis of infected host cells; opsonization of coated viruses or infected cells by C3b facilitates destruction by phagocytes
Interferons	Interferon-α and interferon-β induce an antiviral state in many cell types; when bound to interferon receptors, these cytokines induce changes which include interference with viral replication, activation of latent cellular endonuclease and production of Mx proteins which inhibit viral transcription; interferon-γ activates macrophages and promotes differentiation of $CD4^+$ T cells
Cells	
NK cells and macrophages	Kill virus-infected cells by ADCC; NK cells also produce IFN-γ
$CD4^+$ T_h1 lymphocytes	Release of cytokines from these cells, especially IFN-γ, activates macrophages and enhances destruction of ingested viruses; promote maturation of cytotoxic T lymphocytes from precursor T cells
Cytotoxic T lymphocytes	These $CD8^+$ T lymphocytes, which produce IFN-γ and tumour necrosis factor, kill infected cells directly through release of perforin and granzymes or by induction of apoptosis

ADCC, antibody-dependent cell-mediated cytotoxicity.

Figure 3.17 Modes of transmission of viral pathogens which cause systemic disease. Innate defences and adaptive immune responses against these pathogens are outlined. IFN-γ: interferon-γ ; TNF-α: tumour necrosis factor-α; ADCC: antibody-dependent cell-mediated cytotoxicity.

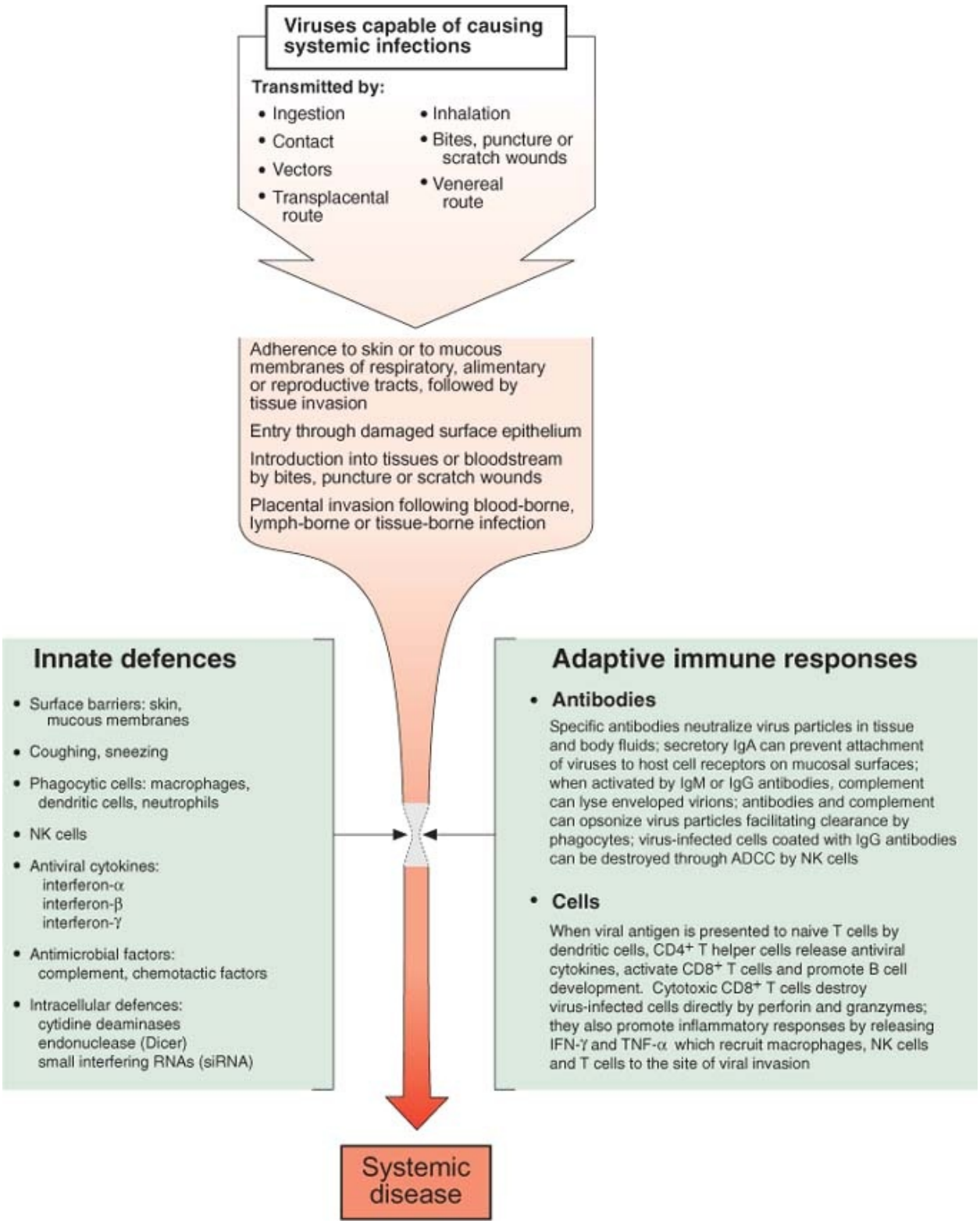
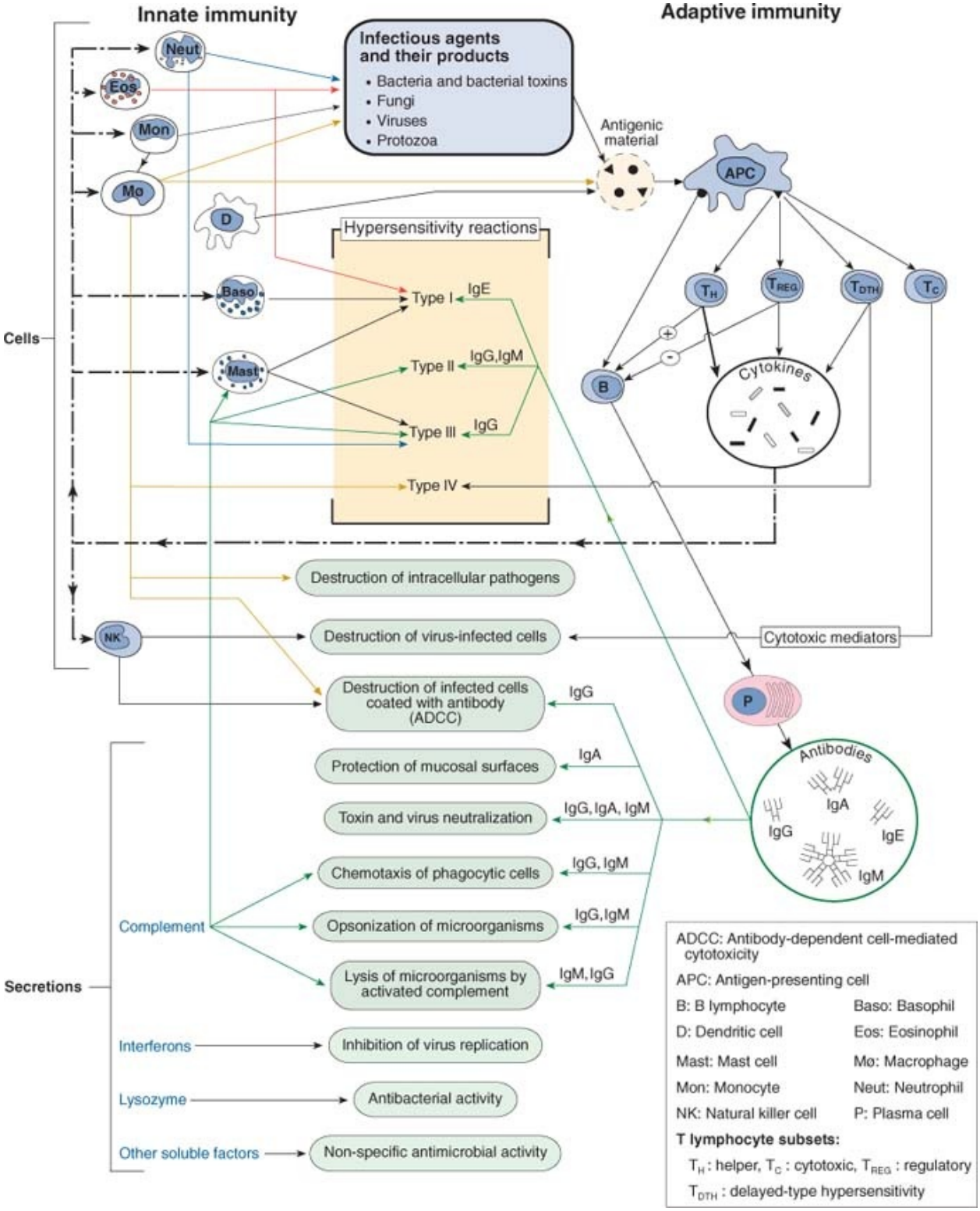


Figure 3.18 The principal cells and secretions which collectively contribute to protection against infectious agents. Cooperation between innate immunity and adaptive immunity enhances host defenses. Occasionally, immune reactions

generate deleterious responses such as hypersensitivity reactions.



Immune responses can also result in deleterious hypersensitivity reactions to innocuous substances such as foreign protein and some therapeutic substances.

In sensitized animals, some allergic responses can result in anaphylaxis with potentially fatal consequences.

The cells and secretions that collectively constitute the innate and adaptive branches of the immune system are outlined in Fig. 3.18. Cooperation between components of innate immunity and adaptive immunity is a requirement for effective protection against pathogenic microorganisms. The immune system itself is not exempt from defects, either developmental or acquired. Defects in one or more components of the immune system can result in increased susceptibility to opportunistic infection by microorganisms of environmental origin. A deficit affecting components of the immune system essential for the development of protective immunity invariably leads to overwhelming infection. Changes in the functioning of the immune system can be related to age, metabolic influences, neoplastic changes involving myeloid or lymphoid tissues and many other factors. At extremes of age, body defences tend to function suboptimally. In the absence of colostral protection, neonatal animals are susceptible to infections caused by environmental pathogens. A gradual decline in immunological competence occurs as animals approach the end of their normal life span.

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Chapter 4

Immunodeficiency diseases

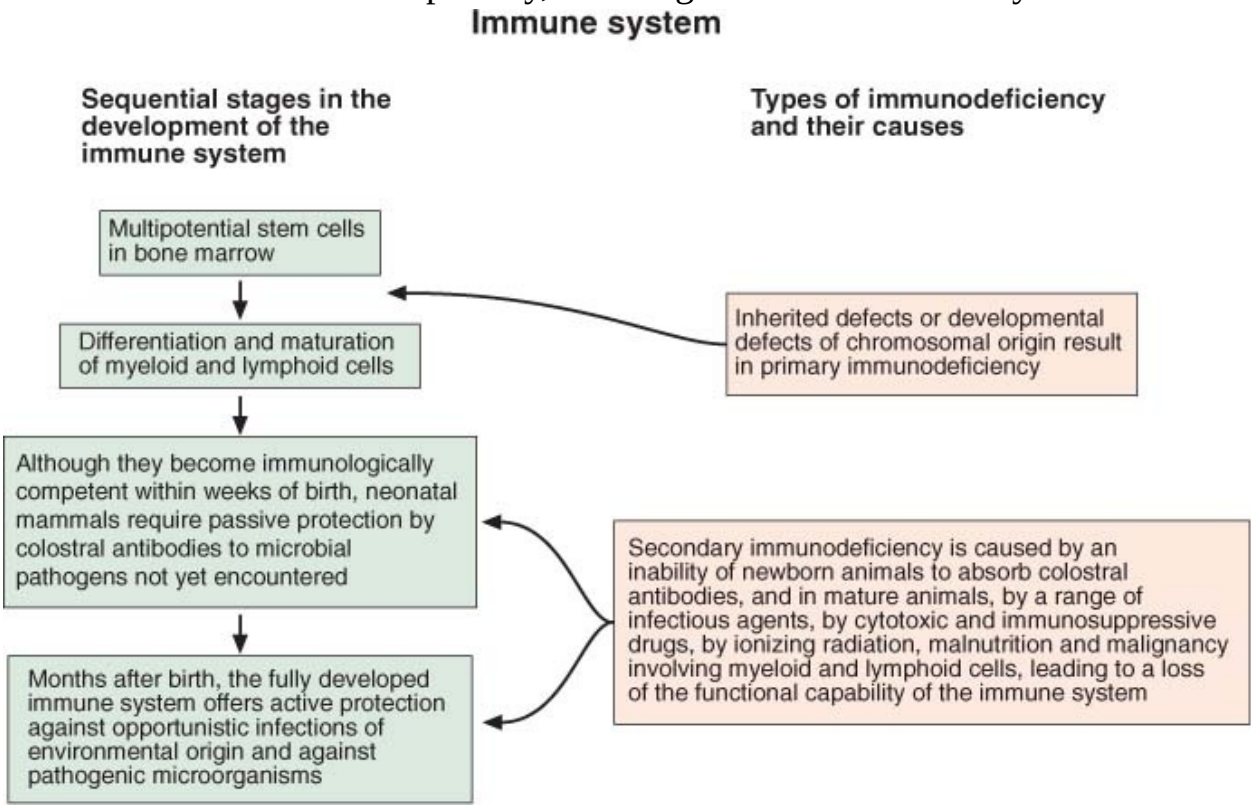
In mammals andalso in avian species, resistance to infection is a fundamental requirement for survival. Like other complex systems, the immune system is subject to failures in some or all ofits components and these failures can have serious consequences for the host. Defects in one or more components of the immune system can result in increased susceptibility to opportunistic infection by microorganisms of environmental origin. If the defect affects components of the immune system essential for the development of protective immunity, overwhelming infection is the inevitable outcome. Immunodeficiency diseases therefore result from the absence or failure of one or more elements of the immune system. Primary immunodeficiency diseases are due to genetic or developmental defects in the immune system. Although these defects are present at birth, they may not become evident until later in life. Secondary or acquired immunodeficiency is the loss ofimmune competence and develops as a consequence of exposure of cells of the immune system to infectious agents, treatment with immunosuppressive drugs, neoplasia or malnutrition. The most common secondary immunodeficiency disease in the human population is acquired immunodeficiency syndrome or AIDS. This syndrome results mainly from infection with human immunodeficiency virus 1 (HIV-1) which is a double-stranded RNA retrovirus which infects $CD4^+$ T lymphocytes. Severe depletion of $CD4^+$T cells results in drastic functional impairment of cell-mediated immunity and death from opportunistic infections. Since its discovery in 1981, AIDS has increased to epidemic proportions worldwide and it is estimated that more than 40 million people are infected with HIV-1 and that more than 25 million people have died from AIDS.

As a consequence ofimmunodeficiency diseases, there is a marked increase in susceptibility to bacterial, fungal, viral and protozoal infections. The nature of the infection in a particular human or animal depends largely on the component of the immune system that is defective. A defect in humoral immunity usually results in increased susceptibility to infections with pyogenic bacteria. When

cell-mediated immunity is defective, there is an increased susceptibility to viruses and other intracellular pathogens. Combined deficiencies of both humoral and cell-mediated immunity result in an increased susceptibility to all types of pathogenic microorganisms. In humans with immu-nodeficiencies involving T cells, an increased incidence of neoplastic diseases caused by oncogenic viruses is reported. In addition, certain immunodeficiency diseases are associated with an increased incidence of autoimmunity which may be related to a deficiency of regulatory T cells which maintain self tolerance. Because immunodeficiency may result from defects in lymphocyte maturation or activation, or from defects involving components ofinnate immunity, immunodeficiency diseases are clinically and pathologically heterogeneous. Sequential stages in the development of the immune system leading to immunological competence and deficiencies which interrupt its development leading to immunodeficiency are shown in Fig. 4.1. Causes of primary immunodeficiency and secondary immunodeficiency and the components of the immune system affectedare presented in Fig. 4.2. More than 200 primary immunodeficiency diseases have been reported in the human population and the genetic basis for more than half of these has been determined. Primary and secondary immunodeficiency diseases are being recognized with increasing frequency in domestic animals, especially in dogs and horses. The history, clinical signs and laboratory reports may suggest defects in the animal's immune system in some instances. Primary immunodeficiencies relating to T and B lymphocytes can be identified by combining clinical, laboratory and post-mortem data (Fig. 4.3). A history ofinfectious diseases during the first weeks oflife, increased susceptibility to opportunistic pathogens and recurring or continuous infections unresponsive to treatment is consistent with an immunodeficiency disease. The blood picture may reveal low numbers of white blood cells, either as lymphopenia or neutropenia. Changes in the concentrations or ratios ofimmunoglobulins andalteration in serum complement concentration are further evidence of defects in the functioning of the immune system. When compared with the human population, a relatively small number ofimmunodeficiency diseases are described in domestic animals. It is probable, however, that when recurring diseases in animals are subjected to closer scrutiny, many of these will be found to have an immunological basis related to defects in the development or functioning of the immune system.

Figure 4.1 Sequential stages in the development of the immune system leading to immunological competence and defects which interrupt its development or

lead to loss of functional capability, resulting in immunodeficiency.



Deficiencies in innate immunity in humans andanimals relating to problems in the development or functioning of phagocytic cells and in the complement system are summarized in Table 4.1 and illustrated in Fig. 4.4.

Severe combined immunodeficiency diseases

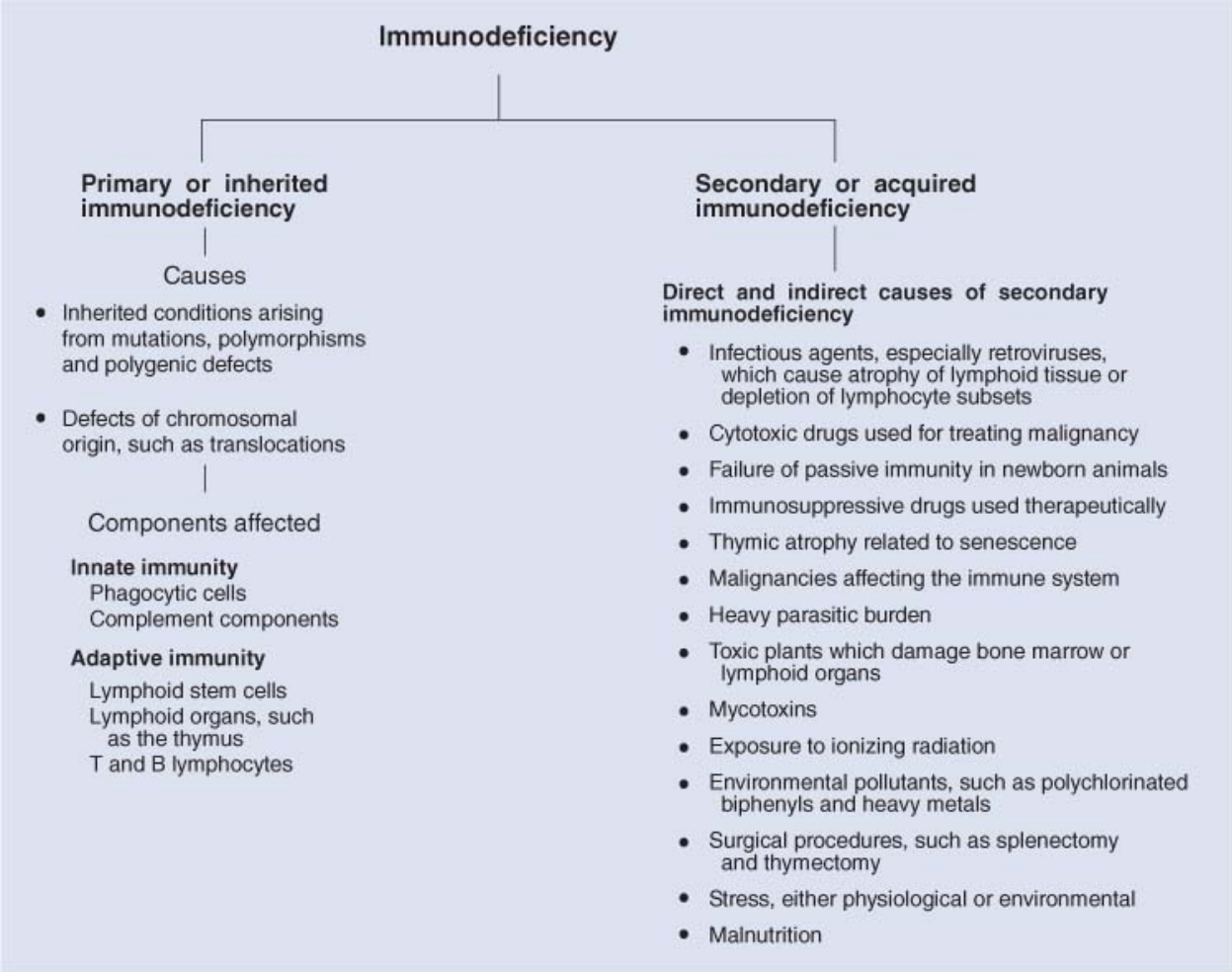
The heterogeneous group of diseases known as severe combined immunodeficiency diseases arises from defects in lymphoid development that affect T cells either alone or in combination with B cells and NK cells. Severe combined immunodeficiency disease (SCID) is often due to developmental defects at the level of the lymphoid stem cell in the bone marrow. Other causes of SCID include adenosine deaminase deficiency, purine nucleoside phosphorylase deficiency and defects in the recombinase activating genes, *RAG1* or *RAG2*.

Defects that occur early in embryological development can have a profound effect on the entire immune system. A severe form of SCID that occurs in humans, reticular dysgenesis, results from developmental problems at the level of the haematopoietic multipotent stem cell in the bone marrow. This condition,

which has an autosomal recessive mode ofinheritance, leads to developmental failure of B cells, T cells and granulocytes (Fig. 4.4). The resulting general failure ofimmunity leads to infection by a variety of microorganisms and early death of affected babies.

If lymphoid stem cells, which develop from multipotential stem cells in the bone marrow, do not populate the thymus by the sixth week of gestation in the human embryo, the thymus does not become a lymphoid organ. Up to 50% of SCID cases are X-linked due to mutations in the gene encoding the γ_c chain of the IL-2 receptor. This γ_c chain also forms part of the receptors for the cytokines IL-4, IL-7, IL-9 and IL-15. Thus, this mutation impairs responses to a range of cytokines, leading to defective T cell maturation. Of particular importance in the development of this disease is IL-7 whose receptor requires the γ_c chain for signalling. The inability of IL-7 to stimulate growth of thymocytes results in impaired maturation of T cells. Similarly, the receptor for IL-15, which also uses the $\gamma_c$chain, is defective and failure of IL-15 to exert its strong influence on NK cell development accounts for the deficiency of NK cells. Accordingly, in X-linked SCID, there is impaired maturation of T cells and of NK cells and greatly reduced numbers of mature T cells and NK cells; the number of B cells is usually normal or increased. A small number of patients with T cell lymphopenia and normal B cell numbers show an autosomal recessive rather than an X-linked pattern of inheritance and are similar phenotypically to patients with X-linked SCID. Cytokine transduction of signals, which elicit specific responses in target cells, involves enzymes called Janus kinases (JAKs), transcription factors called signal transducers andactivators of transcription. Patients with autosomal recessive SCID have mutations in the genes for the IL-7 and chain receptor. The α chain, which associates with the γ_c chain, is required for the intracellular signalling by the protein JAK3 kinase. An autosomal recessive form of SCID results from a mutation in the genes that encode for enzymes involved in the rearrangement of the immunoglobulin genes of pre - B cells and the T cell receptor genes of pre-T cells. These recombinase activating genes termed *RAG1* and *RAG2* are essential for rearrangement, and consequently mutations in either gene result in an absence of T cells and B cells; however, NK cells are not affected.

Figure 4.2 Causes of primary and secondary immunodeficiencies and components of the immune system affected.



Other autosomal recessive defects that disrupt T cell and B cell function include adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency. The enzyme adenosine deaminase catalyses conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. Deficiency of this purine-degrading enzyme leads to the accumulation of deoxyadenosine and deoxyadenosine triphosphate, metabolites which are toxic to lymphoid stem cells. By inhibiting the enzyme ribonucleotide reduct-ase, which is required for DNA synthesis and cell replication, adenosine deaminase deficiency also leads to reduced numbers of B and T lymphocytes. Although lymphocyte cell numbers are usually normal at birth, they decline rapidly after birth. A decline in NK cell numbers also occurs. Defects in the development or maturation of B lymphocytes and T lymphocytes are illustrated in Fig. 4.4 and summarized in Table 4.2.

Figure 4.3 Changes in the number of circulating lymphocytes, the composition of lymphoid organs and the responses of lymphocytes associated with primary

lymphoid immunodeficiency. Increased susceptibility to infection is a prominent feature ofimmunodeficiency diseases in animals.

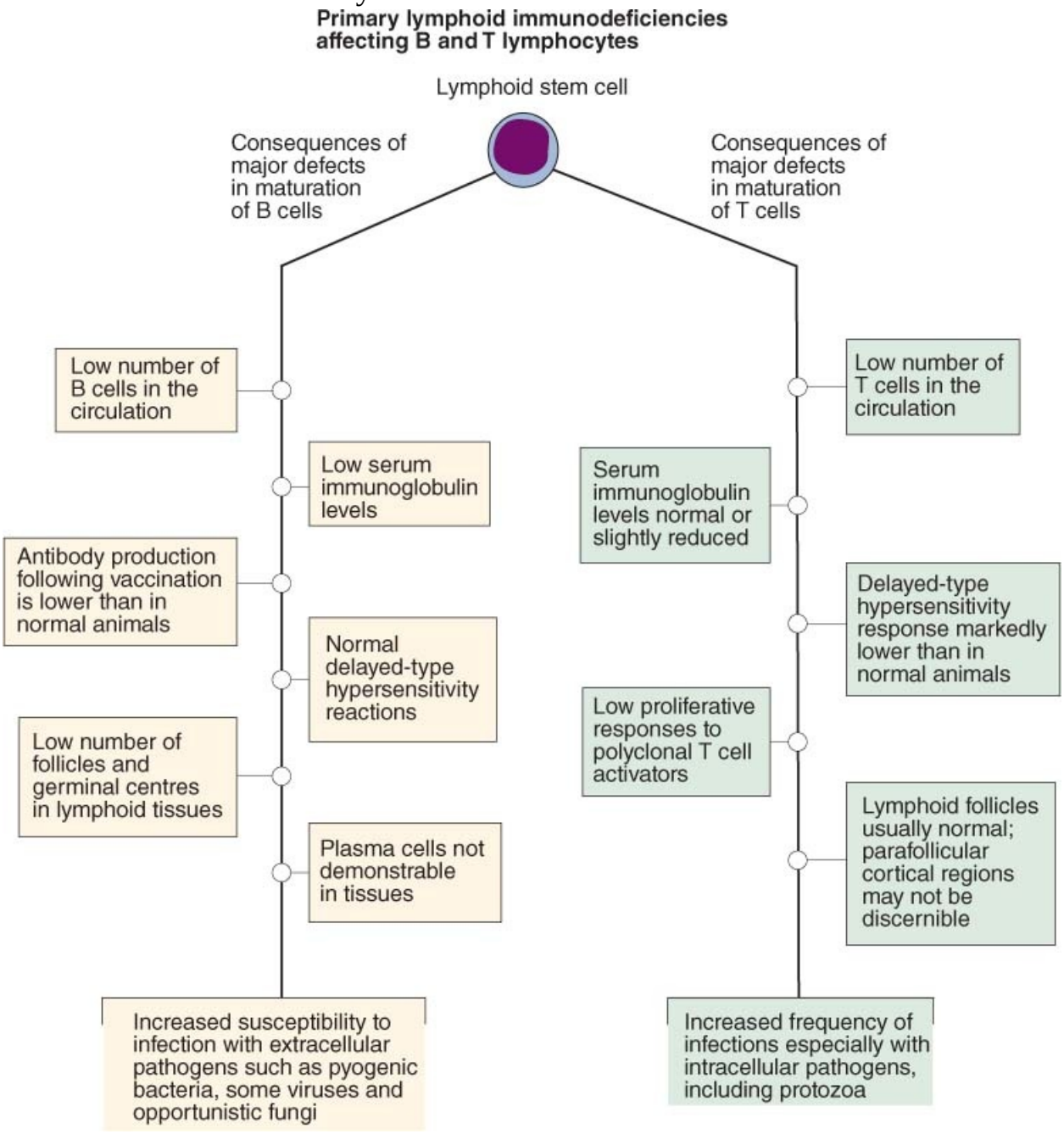


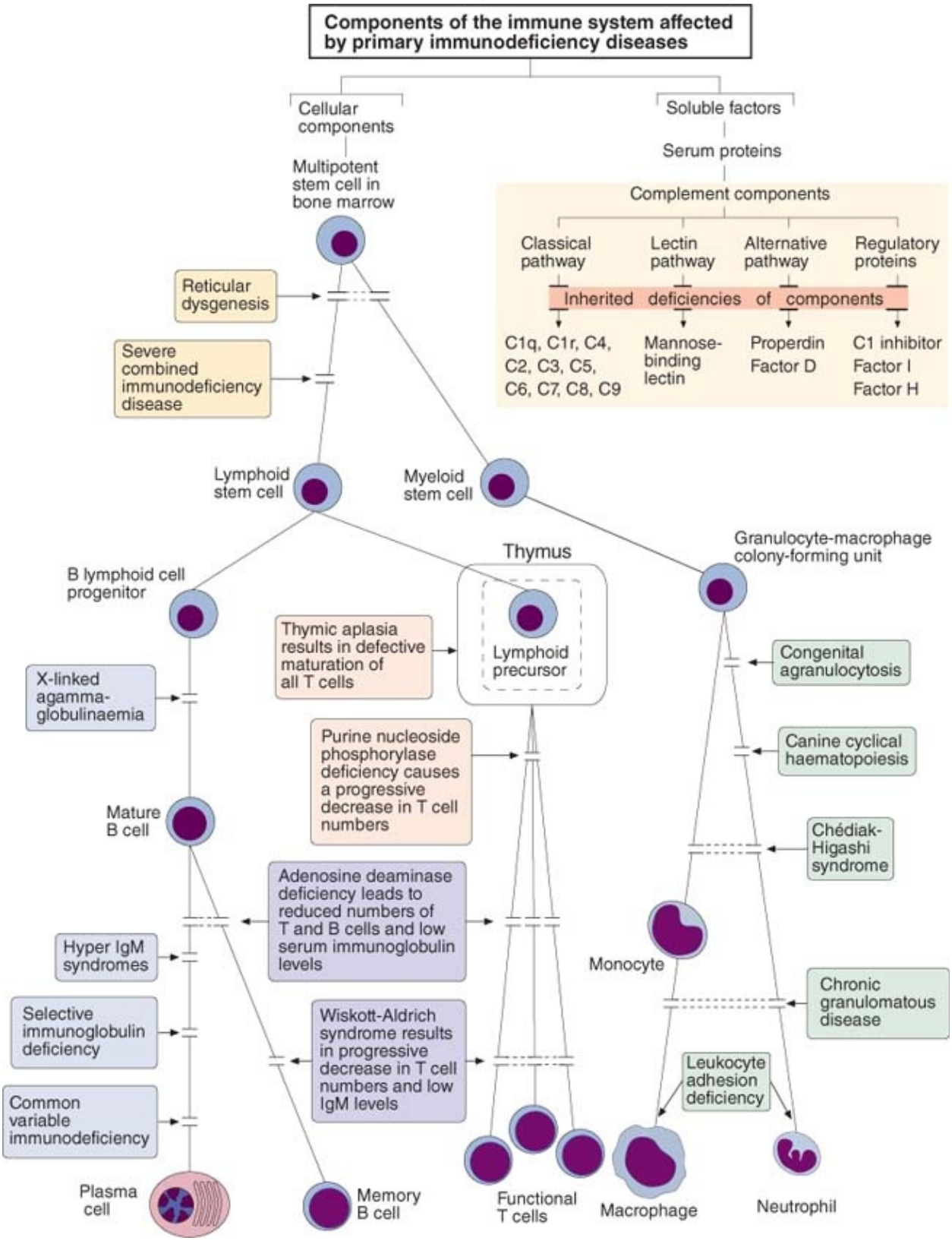
Table 4.1 The nature and consequences of primary immunodeficiencies relating to innate immunity in animals and humans.

Immune components in which deficiencies occur	Type of defect / associated clinical condition	Comments
	Neutropenia /	

Neutrophils	quantitative deficiencies can range from an almost complete absence of cells, agranulocytosis, to a marked reduction in peripheral blood neutrophils	In humans, congenital neutropenia, an autosomal recessive condition, is a consequence of a genetic defect involving the myeloid stem cells. Once neutrophil levels drop below 500 cells / mm^3, a dramatic increase in the incidence of bacterial infections occurs.
	Leukocyte adhesion deficiency / increased susceptibility to infection with both Gram-positive and Gram-negative bacteria	This immunodeficiency disease, which arises due to impairment of the adhesion ofleukocytes to vascular endothelium, interferes with recruitment of phagocytic cells to sites ofinfection. Two types ofleukocyte adhesion deficiency (LAD) have been described, type 1 and type 2. In LAD type 1, a defect in the β chain of the integrin molecule on the leukocyte surface due to mutations in the CD18 gene results in impaired neutrophil adherence to the vascular endothelium. In addition, chemotaxis is affectedand consequently neutrophils are unable to migrate out of blood vessels. Because the β subunit is common to three integrins found on polymorphonuclear leukocytes and on mononuclear cells, including NK cells and B and T lymphocytes, adhesion and migration of many white blood cell types is affected by this autosomal recessive disease. Prolonged recurring bacterial infections during infancy are a feature of this disease. Leukocyte adhesion deficiency has been reported in HolsteinFriesian cattle and in Irish Setters. Clinically, this deficiency in animals is characterized by recurring bacterial infections. At post-mortem examination there are large numbers ofintravascular neutrophils which have not migrated into the tissues. In LAD type 2, neutrophils lack sialyl Lewis X, the carbohydrate ligand required for binding to E-selectin and P-selectin on cytokine-activated endothelium. This autosomal recessive condition results in poor random mobility and weak chemotactic responses by neutrophils. Because neutrophils fail to adhere to vascular endothelium and lack rolling adhesion, pus formation is not a feature of pyogenic bacterial infections in affected children. The disease is characterized by recurring bacterial infections.
	Myeloperoxidase deficiency / delayed killing of phagocytosed microorganisms	Although reported to be the most common neutrophil dysfunction, the consequences of myeloperoxidase deficiency are not as serious as other neutrophil conditions. In the absence of myeloperoxidase, killing of engulfed microorganisms is delayed but other functions such as chemotaxis, phagocytosis and degranulation are normal.
Neutrophils and rarely macrophages	Chronic granulomatous disease / characterized by recurring intracellular bacterial and fungal infections	Chronic granulomatous disease is the title given to a number of defects of the phagocyte oxidative respiratory burst which can be inherited in an X-linked or autosomal recessive manner. Because of the defects in the oxidative pathway whereby phagocytes generate hydrogen peroxide and the resulting reactive products such as hypochlorous acid, which kill engulfed bacteria, patients with this disease are susceptible to bacterial and fungal infections. The inability of neutrophils and sometimes, of macrophages to generate reactive oxygen intermediates such as superoxide anions results in failure to kill engulfed microorganisms. The most common form of chronic granulomatous disease is due to mutation of a gene on the X chromosome coding for cytochrome b. As a consequence of the persistent infections, chronic cell-mediated immune responses develop involving T cell-mediated macrophage activation and the formation of granulomas, hence the name of this disease. This inherited disease is manifested by the development of serious infections in early childhood. A similar disease inherited in an X-linked manner has been reported in Irish Setters.
Neutrophils and occasionally macrophages	Chédiak-Higashi syndrome / defective lysosomal function in neutrophils andabnormal granules or organelles in other cells including macrophages, melanocytes and NK cells; platelet abnormalities also occur	The Chediak-Higashi syndrome is an autosomal recessive disease that occurs in humans, cattle, mink, Persian cats, white tigers, beige mice and killer whales. The neutrophils, monocytes, macrophages, melanocytes and NK cells contain abnormal giant cytoplasmic granules, and platelet functions are defective. The giant lysosomes found in neutrophils form as these cells mature from myeloid stem cells. A defect in a gene encoding a protein involved in intracellular vesicle formation results in a failure oflysosomes to fuse properly with phagosomes. In this syndrome, neutrophils have a deficiency of cathepsin G and elastase. Because phagocytes in affected humans andanimals have defective degranulation mechanisms, there is impaired fusion oflysosomes with phagosomes and these phagocytic cells exhibit diminishedability to kill ingested microorganisms. Decreased responsiveness to chemotactic stimuli is evident also in neutrophils and monocytes from affected humans andanimals. Recurring infections with pyogenic bacteria are a feature of this syndrome. Some affectedanimals have ocular abnormalities anda tendency to bleed excessively after surgical procedures.
Neutrophils and occasionally macrophages	Canine cyclical haematopoiesis / increased susceptibility to bacterial infections	This autosomal recessive disease of collies, called canine cyclical haematopoiesis (grey collie syndrome), results in regular fluctuations in leukocyte numbers. Affected dogs have decreased skin pigmentation, eye lesions and silver grey hair. A decrease in granulopoiesis, which occurs at intervals of approximately 12 days, lasts for about 3 days. During periods ofleukopenia, affectedanimals are susceptible to bacterial infections. A defect in neutrophil maturation in the bone marrow at the level of the pluripotent haematopoietic stem cell is the suspected cause of this disease. A cyclical pattern in the production of haematopoietic growth factors has been reported in affected dogs.

Monocytes, macrophages	Interferon-y deficiency / increased susceptibility to weakly pathogenic mycobacteria and some Gram-negative bacteria	A mutation in the gene encoding the interferon-γ receptor on monocytes and macrophages results in an inability of these cells to respond to the activation effect ofinterferon-γ. Children with this genetic defect are susceptible to infection with weakly pathogenic mycobacteria and some Gram-negative bacteria.
Complement	Genetic deficiencies of complement proteins / deficiencies of components involved early in the classical pathway predispose to immune complex diseases; deficiencies of C3 increase susceptibility to pyogenic infections; lectin pathway deficiencies are associated with increased susceptibility to bacterial infections in young children	Complement has a central role in inflammatory reactions and many cells express one or more membrane receptors for products of complement activation. Functions of complement include chemotaxis, opsonization, cell activation, lysis of target cells and priming of adaptive immune responses. Deficiencies of most complement components and regulatory proteins have been described. The clinical conditions arising from these deficiencies are not all of equal importance; clinical consequences range from increased susceptibility to infection to tissue damage caused by immune complexes. The early components of the classical pathway are particularly important for the elimination ofimmune complexes and deficiencies in any of these components result in a marked increase in immune complex diseases such as systemic lupus erythematosus and glomerulonephritis. As the three complement activation pathways, namely classical, mannose-binding lectin andalternative, require C3 activation, a deficiency of C3 results in increased susceptibility to bacterial infections. Deficiencies of terminal pathway components are associated with recurring bacterial infections often caused by Gram-negative bacteria. Activation pathways for complement are regulated by a number of factors, and deficiencies in these regulatory proteins are associated with abnormal complement activation anda number of related clinical abnormalities. The proteolytic activity of C1r and C1s is inhibited by a plasma protein called C1 inhibitor. In humans, an autosomal dominant inherited disease called hereditary angioneurotic oedema is due to a deficiency of C1 inhibitor. Patients with this deficiency have episodes of circumscribed oedema which may affect different parts of the body. If oedema affects the upper respiratory tract it may cause a potentially life-threatening airway obstruction. Other deficiencies of complement regulation reported in humans include factors I and H. Complement deficiencies have been observed in both domestic and laboratory animals, especially in inbred lines. Brittany spaniels with a congenital deficiency of C3 and rabbits with a C6 deficiency have been reported. Recurring infections with pyogenic bacteria are a feature of complement deficiencies in animals, especially with C3 deficiency, confirming the importance of C3 in the opsonization of pyogenic bacteria. A deficiency of factor H, which inactivates C3b in the alternative pathway, has been described in Yorkshire pigs. Affectedanimals fail to thrive and develop anaemia and glomerulonephritis.

Figure 4.4 Defects in the development or maturation of lymphoidand myeloid cells which result in primary immunodeficiencies in humans and animals. Inherited deficiencies in complement components are also shown.



Immunodeficiency diseases that affect horses include severe combined

immunodeficiency (SCID) in Arabian horses, agammaglobulinaemia in male foals, common variable immunodeficiency affecting adult horses, IgM deficiency in foals, and failure of foals to ingest or absorb colostral antibodies either due to failure of the mare to produce adequate amounts of colostrum or to a lack of appropriate management of the neonatal foal. Severe combined immunodeficiency is the most important congenital immunodeficiency recognized in Arabian or Arabian–crossbreed horses. The condition is inheritedas an autosomal recessive defect. Affected foals fail to produce functional B or T lymphocytes, and few circulating lymphocytes are present in their blood. Neutrophil and monocyte function is usually normal. Precolostral serum samples have no detectable IgM. Maternal antibodies, acquired through ingestion of colostrum, usually confer passive protection for up to 3 months. Once these passively transferred immunoglobulins are catabolized, affected foals become agammaglobulinaemic; most die before 6 months of age from recurring infections caused by opportunistic pathogens. Adenoviral pneumonia is reported to be one of the most common causes of death. Enteritis, hepatitis or infections in other organs sometimes occur in affected foals without pulmonary involvement. On post-mortem examination, apart from evidence of bacterial and viral infection, there is marked hypoplasia of both primary and secondary lymphoid tissue. The spleens of affected foals lack lymphoid follicles and germinal centres, lymph nodes lack germinal centres and cellular depletion is evident in their paracortical regions. Thymus development is so limited that thymic tissue may be difficult to find in affectedanimals. The disease is due to a mutation in the gene located on chromosome 9, coding for the catalytic subunit of a DNA-dependent protein kinase. Deficiency of the protein kinase results in failure of the variable region of the immunoglobulin heavy chain on B cells and of the variable region of the T cell receptor. As severe combined immunodeficiency disease is inherited in an autosomal recessive manner, its occurrence indicates that both parents carry the mutation and this can be confirmed by DNA analysis. PCR tests can be used to detect animals that are heterozygous or homozygous (affected foals) for the mutated gene. Genetic analysis has shown that more than 8% of Arabian horses carry the gene.

Severe combined immunodeficiency disease has been described in different breeds of dogs. An X - linked SCID in Basset hounds is characterized by lymphope-nia, with increased numbers of B lymphocytes and few T lymphocytes. Affected pups usually remain clinically normal until maternally-derivedantibody levels begin to decline between 8 and 12 weeks of age. As

passive immunity of maternal origin wanes, recurring bacterial and viral infections of the respiratory and gastrointestinal tracts occur. Few affected pups survive past 4 months of age. Death results from septicaemia and systemic viral infections. Apart from evidence of systemic infection at post-mortem examination, there is marked hypoplasia of primary and secondary lymphoid organs. Lymph nodes, tonsils, Peyer's patches and thymus are small and may be undetectable. The defect is due to mutations in the gene encoding the common gamma chain (γ_c) shared by the receptors for the interleukins IL-2, IL-4, IL-7, IL-9 and IL-15. Because of their inability to express functional IL-2 receptors, T lymphocytes do not mature and B lymphocytes respond to T-lymphocyte-independent antigens only, with no class switching from IgM to IgG. A SCID has been described in the Welsh Corgi breedand in Jack Russell terriers; sporadic cases of SCID have been reported in Rottweiler, Toy Poodle and some mixed-breed pups.

An autosomal recessive mutation which arose in the CB-17 inbred strain of mice resulted in severe combined immunodeficiency. In homozygous mice with this defect, referred to as SCID mice, mature B and T lymphocytes are absent. Affected mice have no serum immunoglobulins, are unable to produce cell-mediated immune responses andare highly susceptible to opportunistic infections. The defects in SCID mice result from an inability to express antigen-specific receptors on their lymphocytes due to defects in DNA recombinase activating genes required for rearrangement of T cell receptors and immunoglobulin gene rearrangement. In this condition, development of B cells ceases before expression of cytoplasmic or cell-membrane immunoglobulins. Likewise, T cell development is arrestedat an early stage before antigen-specific receptors are expressed. Blood cells other than lymphocytes develop normally in these mice; red blood cells, monocytes and neutrophils are present and function in a normal manner.

Thymic aplasia or hypoplasia

During early embryological development, cells derived from the bone marrow migrate to the epithelial thymus which develops from the third pharyngeal pouches with a contribution from the fourth pharyn-geal pouches. Responding to inductive factors from the thymic epithelial reticular cells, these pro-thymocytes become competent T lymphocytes. On leaving the thymus, mature T

lymphocytes seed other lymphoid organs with subsets which are responsible for cell-mediated immune responses. Accordingly thymic aplasia has a profound influence on T cell function: all populations of T cells including helper, cytotoxic and regulatory cells are affected. As a consequence of this deficiency, there is marked susceptibility to infections with viruses, intracellular bacteria, fungi and protozoa.

Table 4.2 Nature and consequences of primary immunodeficiencies in adaptive immunity resulting from defects in development, maturation or activation of lymphocytes.

Immune component involvedand the basis of the defect	**Type of defect**	**Comments**
Lymphoid stem cell in bone marrow	Severe combined immunodeficiency disease (SCID)	Early death of affected babies invariably occurs due to overwhelming infection by a variety of microorganisms.
Defective T cell maturation due to a defect in the gene encoding the common γ-chain cytokine receptor for IL-2 anda number of other interleukins	Severe combined immunodeficiency disease; up to 50% of these SCID cases are X-linked	Severe recurring infections occur at an early age in affected humans and dogs. Affected babies usually die before they reach 2 years of age; few affected pups survive past 4 months of age.
Mutation in genes encoding enzymes involved in the rearrangement in immunoglobulin genes and T cell receptor genes, termed *RAG1*and *RAG2*	Autosomal recessive forms of SCID. Affected humans andanimals are highly susceptible to opportunistic infections	Severe recurring infectious diseases in babies; in Arabian horses, this condition is of major importance and most affected foals die before 6 months of age. An autosomal recessive mutation in the CB-17 inbred strain of mice resulted in SCID; affected mice, referred to as SCID mice, lack mature B or T lymphocytes.
Accumulation of metabolites which are toxic to lymphoid stem cells due to a deficiency of purine-degrading enzymes	SCID due to deficiency of the purine-degrading enzymes adenosine deaminase and purine nucleoside phosphorylase	Progressive decline in B and T lymphocytes occurs after birth. Increased susceptibility to infectious diseases accompanies the decline in lymphocyte numbers.
Thymus	Thymic aplasia or hypoplasia. Congenital thymic aplasia in humans is referred to as the DiGeorge syndrome	Due to defective cell-mediated immunity, viral, fungal, bacterial and protozoal infections recur. Athymic mice, which are hairless and have rudimentary thymic glands, have defective cell-mediated immune responses.
Defective T cell function, low numbers of abnormal platelets and decreased IgM levels	Wiskott-Aldrich syndrome	Affected humans are prone to infections with encapsulated bacteria and with viruses. The close cooperation between T cells and B cells fails to develop; with increasing age, patients have reduced numbers of lymphocytes.
B cell defect with early failure of B lymphocyte development	X-linkedagammaglobulinaemia	The agammaglobulinaemia is accompanied by repeated pyogenic bacterial infections in humans; affected babies have no or few B cells in their circulation. The condition becomes clinically evident as maternally-derived immunoglobulin levels decline. An inbred mouse strain, called CBA / N, has an X-linked defect in B cell development.
B cell defects which occur as X-linkedandas autosomal recessive forms	Hyper IgM syndromes	Defective class switching with normal or elevated IgM levels and low IgG, IgA and IgE levels. Some affected patients are susceptible to opportunistic infections.
Immunodeficiency states with low or no production of particular immunoglobulins	Selective immunoglobulin deficiency	Described in humans andanimals; selective IgA deficiency is the most common form. Clinical features range from recurring bacterial infections to an absence of clinical problems.
Decreased concentrations of all immunoglobulins	Common variable immunodeficiency	Typically occurs later in life in humans andanimals, with impairedantibody responses to infectious agents and vaccines. A defect in the differentiation of B cells is suspected; an increased risk ofinfection is reported in this condition.

In humans, a mutation that arises from a deletion in chromosome 22 during

embryological development is referred to as the DiGeorge syndrome. Congenital thymic aplasia or hypoplasia, hypoparathyroidism, facial deformities and congenital heart disease are prominent features of this condition. In addition to T cell deficiency, calcium homeostasis is affected resulting in clinical evidence of tetany. Peripheral blood T lymphocytes are either absent or greatly reduced in number and do not respond to polyclonal T activators. Although B cells are usually present in normal numbers, response to immunization may not be optimal due to the absence of T helper cells which are required for some specific antigens.

An important animal model of T cell immunodeficiency resulting from abnormal thymic development is the nude (athymic) mouse. Mice homozygous for the genetic trait designated *nu*, which is controlled by a recessive gene on chromosome 11, are hairless and have rudimentary thymic glands. They have defective cell-mediated immune responses andare unable to make antibodies to many antigens. These mice, however, do have low numbers of circulating T lymphocytes and the majority of these cells carry T cell receptors of γδ type rather than the αβ type. In normal mice, the αβ T cell receptor is usual on circulating T lymphocytes. Thymic aplasia has been recorded in some domestic animals; inbred lines of dogs and cattle with absent or small thymic glands have been reported. Affectedanimals have defective cell-mediated immune responses.

Wiskott-Aldrich s yndrome

An uncommon X-linked immunodeficiency condition, characterized by thrombocytopenia, eczema and immunodeficiency, is referred to as the Wiskott-Aldrich syndrome (WAS). Affected patients have low numbers of abnormal platelets, decreased IgM levels and defective T cell function. They are prone to infections with encapsulated bacteria and with viruses, probably because of the gradual loss of T cells. The genetic basis of the disease is a mutation in the X - linked gene coding for the Wiskott-Aldrich syndrome protein (WASP) which is expressed by haematopoietic stem cells and is probably important in the regulation of lymphocyte and platelet development and function. In T lymphocytes, WASP is involved in the rearrangement of the actin cytoskeleton when these cells are activated following antigen recognition. When examined by scanning electron microscopy, T lymphocytes from patients with this syndrome have an abnormal appearance; they also have fewer microvilli on their cell surfaces than normal T cells. During antibody production, there is close

interaction between T lymphocytes and B lymphocytes as the cytoskeleton of the T cell reorientates itself towards the B cell. This close cooperation fails to occur in WAS and consequently T-lymphocyte support for B lymphocytes is lacking and B cells are unable to respond to polysaccharide antigens. Low IgM levels are another feature of this syndrome. With increasing age, affected patients have reduced numbers of lymphocytes and develop a more severe form ofimmunodeficiency. It is probable that WASP defects interfere with the complex processes involved in the activation and migration of lymphocytes andalso with lymphocyte survival.

Primary immunodeficiency diseases involving B lymphocytes

When a B cell defect occurs in humans with early failure in B lymphocyte development, it results in a complete absence of mature B cells and, accordingly, no immunoglobulin production. As maternal IgG levels decline, this autosomal recessive form of agam-maglobulinaemia results in widespread infections in affected babies. A number of autosomal recessive agammaglobulinaemic conditions relate to mutations in the μ heavy chain gene and to defects in the expression of the pre-B cell receptor which prevent normal B cell development.

Agammaglobulinaemia, which is inherited in an X-linked manner in humans, is characterized by an absence ofimmunoglobulins in the serum, and is called X-linkedagammaglobulinaemia or Bruton-type agammaglobulinaemia. The condition usually becomes evident clinically before 6 months of age with the decline in maternally-derived IgG acquired transplacentally. The agammaglobulinaemia is accompanied by serious and repeated pyogenic bacterial infections. The defect in X-linkedagammaglobulinae-mia is a failure of pre-B cells in the bone marrow to develop into mature B cells. This failure is caused by mutations or deletions of the Bruton tyrosine kinase gene. Bruton tyrosine kinase is involved in transducing signals from the pre-B cell receptors which are required for the continued maturation of these cells, and, because of this defect, B cell differentiation is blockedat the pre-B cell level. Patients with X-linkedagammaglobulinaemia usually have low or undetectableimmunoglobulin levels, few or no B cells in theirblood or lymphoid tissue, no germinal centres inlymph nodes and no plasma cells in tissues. The bone marrow of male patients with this deficiency disease usually contains

normal numbers of pre-B cells. In contrast to B cell development, T cell maturation, numbers and functions are usually normal. An inbred mouse strain called CBA/N has an X-linked defect in B cell development which is a consequence of a point mutation in the tyrosine kinase gene.

An unusual B cell immunodeficiency, characterized by defective immunoglobulin class switching, results in normal or elevated levels of serum IgM with low levels of IgG, IgA and IgE. This condition is termed the hyper-IgM syndrome. There are both X - linkedandautosomal recessive forms of this syndrome, and some affected patients are susceptible to opportunistic infections. The X-linked condition is caused by mutation of the gene encoding the CD40 ligand protein which is expressed on the surface of activated T cells. Through the interaction of the CD40 ligand with the CD40 molecule on the B cell surface, this effector CD40 molecule transmits essential signals for inducing immunoglobulin class switching. The mutant forms of the CD40 ligand produced in patients with the hyper-IgM syndrome do not bind to or transduce signals through the CD40 molecule and, therefore, do not stimulate B cells to undergo heavy chain isotype switching, a step which requires the involvement of T cells.

A number ofimmunodeficiency states characterized by low or no production of particular immunoglobulin isotypes have been reported in humans. Of these, selective IgA deficiency is the most common. The defect in IgA deficiency results from failure of the terminal differentiation of B cells. As a consequence of this defect, normal differentiation to the plasma cell stage does not occur. The clinical features ofindividuals with immunoglobulin isotype deficiency range from recurring bacterial infections to an absence of clinical problems. The numbers and functional responses of T cells in patients with selective immunoglobulin isotype deficiencies are usually normal.

An immunodeficiency condition in humans which is characterized by decreased concentrations of all immunoglobulin isotypes and occurs in the second or third decade oflife is referred to as common variable immunodeficiency. In addition to reduced levels of serum immunoglobulins, impairedantibody responses to infectious agents or vaccines and increased incidence ofinfection occur in patients with this condition. Male and female members of the human population are affected equally. The underlying cause of common variable immunodeficiency is not known but the fact that mature B lymphocytes are present in patients suggests a defect in the differentiation of B cells into plasma cells. Although the exact pattern ofinheritance is unknown,

common variable immunodeficiency has a genetic component and is considered to be a primary immunodeficiency disease.

In addition to SCID in Arabian horses and failure of foals to ingest or absorb colostral antibodies, there are a number ofimmunodeficiency states in foals and horses which affect immunoglobulin synthesis. These include common variable immunodeficiency, agammaglobulinaemia, IgM deficiency, Fell pony syndrome and transient hypogammaglobulinaemia. Common variable immunodeficiency, which occurs in adult horses, is attributed to an intrinsic inability of B lymphocytes to synthesize immunoglobulins. Serum levels of IgG, IgM and IgA are low or undetectable and few or no B cells are present in the circulation or in lymphoid tissues. Chronic or recurring infections, often caused by opportunistic pathogens and unresponsive to antimicrobial therapy, are commonly encountered in male or female horses with common variable immunodeficiency. Different forms of IgM deficiency have been described in horses. In foals, low or undetectable levels of IgM occur after maternal immunoglobulin levels decline, and respiratory or enteric infections may prove fatal before they reach 10 months of age. Occasionally, selective IgM deficiency occurs in adult horses. Agammaglobulinaemia is characterized by low or undetectable levels of all immunoglobulin classes in serum andan absence of B lymphocytes from the circulation. The disease, which has been described in male Thoroughbred foals and in other breeds of horses, is considered to be an X-linked inherited disease similar to X-linkedagam-maglobulinaemia in humans. Affected foals develop chronic bacterial infections as passive colostral protection declines. A condition in Fell ponies characterized by immunodeficiency, anaemia, lymphopenia and low IgM levels is presumed to be due to an inherited defect. Clinical findings in affected foals, which include opportunistic infections resulting in pneumonia and diarrhoea, are consistent with an immunodeficient state. Circulating T lymphocytes in affectedanimals are reported to be in the normal range but B lymphocyte numbers are low.

Deficiency in the synthesis of IgG has been described in some breeds of cattle but the clinical consequences are not well defined. An IgA deficiency has been reported in dogs belonging to the Beagle, German Shepherd, Irish Setter and Shar-Pei breeds. In some affected dogs an increased frequency of allergies has been reported. Transient hypogammaglobulinaemia has been reported in foals and pups. Clinical signs of the condition coincide with the waning of maternal antibodies acquired through colostrum, often presenting as recurring respiratory infections. A gradual improvement takes place over a period of months

associated with the production ofimmunoglobulins. A defect in lymphocyte maturation, recorded in European breeds of cattle, is inheritedas an autosomal recessive condition. Calves are normal at birth but signs of hair loss, parakeratosis and poor growth rate are evident by 2 months of age. A decrease in lymphocyte numbers and function is evident andantibody responses are reduced. In addition to skin lesions, post-mortem findings include atrophy of the thymus, lymph nodes and spleen. Affected calves show a positive response to oral treatment with zinc, and it has been suggested that in this hereditary condition there is an excessive requirement for zinc and that thymic hypoplasia results from insufficient dietary zinc.

Secondary immunodeficiency

Acquired or secondary immunodeficiency results from atrophy oflymphoid tissue or depletion oflymphocyte subsets caused by infectious agents, especially viruses, by treatment with cytotoxic drugs used for chemotherapeutic intervention in malignancy, and from a variety of negative influences including toxic plants, environmental pollutants and exposure to ionizing radiation. In the human population, infection with human immunodeficiency virus (HIV) has continued to spread with an estimated 45 million people infected worldwide. In addition to infection with HIV, human T cell lymphotropic virus 1 and measles virus induce marked suppression of cellular immune responses leading to secondary bacterial and fungal infections. Because HIV and human T cell lymphotropic virus 1 are retroviruses and become integrated via a provirus into the human genome, they can persist and potentially cause life-long damage to cells of the immune system. Associated with HIV infection, there is a progressive loss of $CD4^+$ T lymphocytes, coinciding with the failure of the infected person to control a variety ofinfections with opportunistic pathogens. Measles-related illness is a major cause of mortality in children in developing countries. However, although measles virus initially induces suppression of cellular immune responses, the host’s immunosuppression lasts for a limited period of time and there is a progressive clearance of virus from the tissues.

Atrophy oflymphoid tissue or diminished immune responsiveness is a feature ofinfection with particular viral agents in dogs, cats, cattle, horses, pigs, monkeys and poultry. In addition to infectious agents, many drugs, chemicals and other environmental factors can directly or indirectly contribute to the occurrence and severity of secondary immunodeficiency (Fig. 4.2). Suppression

of the functioning oflymphatic tissue or other components of the immune system has been related to equine herpesvirus infection of foals, bovine viral diarrhoea virus infection of cattle and classical and African swine fever infection of pigs. Canine distemper virus replication produces lymphocytolysis and leukopenia resulting in marked immunosuppression which predisposes infected dogs to the protozoan pathogen *Toxoplasma gondii* and to secondary bacterial infections. Parvoviral infection of dogs causes marked immunosuppression, a consequence of the destruction oflymphoid tissues along the alimentary tract. Feline leukaemia virus infection is associated with severe immunosuppression in some animals, resulting in opportunistic infections. As a consequence ofinfection with the parvovirus of cats which causes feline pan-leukopenia,there is extensive damage to lymphopoietic cells and to myeloid cells in the bone marrow and to lymphoid organs. Destruction of these target tissues results in panleukopenia and marked immunosuppression. The lentivirus which causes feline immunodeficiency replicates principally in $CD4^+$ T lymphocytes. There is a progressive deterioration in cell-mediated immunity associated with lymphocyte depletion. Cats with acquired immunodeficiency induced by this len-tivirus develop leukopenia, anaemia and chronic respiratory, enteric and skin infections. Infection of young chickens with virulent serotypes ofinfectious bursal disease virus causes marked immunosuppression. When infection spreads to the bursa of Fabricius, depletion of B lymphocytes occurs with impaired immune responses, lowered resistance to infectious agents and ineffective responses to vaccines.

A number of drugs administered to animals to suppress inflammatory responses or for the treatment of autoimmune diseases have immunomodulatory activity. Corticosteroids are potent immune modulators and some steroids induce lymphocytopenia with depletion of $CD4^+$ T cells. In contrast, steroid treatment can induce neutrophilia, a consequence of release of mature neutrophils from the bone marrow anda decrease in cells leaving the circulation. Steroids also have an inhibitory effect on cytokine synthesis. Cytotoxic drugs used for treating animals with malignancy induce immunosuppression. Because antineoplastic agents act primarily on rapidly dividing and growing cells, they affect many cells involved in immune responses, particularly lymphocytes and neutrophils. Methotrexate, a structural analogue of folic acid, blocks folic-acid-dependent synthetic pathways required for DNA synthesis. Treatment with this compound depresses immunoglobulin synthesis and inhibits polymorphonuclear cell involvement in inflammatory responses. By cross-linking DNA strands, cyclophosphamide exerts toxic effects on the bone marrow and induces leukopenia. The activity of

both T cells and B cells is adversely affectedand, consequently, cell-mediated immunity andantibody production are suppressed. In addition to the immunosuppressive effects of antineoplastic agents, immunosuppression is attributed to the activity of environmental pollutants such as polychlorinated biphenyls and heavy metals.

A relationship between nutrition and resistance to infection has been suggested by clinical observations and by epidemiological data. Nutritional deficiencies have been associated with impaired responses affecting cell-mediated immunity, phagocytic function and complement levels. The influence of nutritional deficiencies on immune processes has been attributed to the requirement for particular trace elements and vitamins for the optimal functioning oflymphoid organs, especially the thymus, for lymphocyte and neutrophil maturation, and for the synthesis ofimmunoglobulins. Nutrients that have been reported to enhance resistance to infectious agents include vitamins A, C and E, zinc, manganese, copper and selenium. Due to the complexity of host-pathogen interactions, however, a correlation between the inclusion of particular nutritional factors in the diet and resistance to infection is difficult to demonstrate conclusively. Nevertheless, it is reasonable to assume that an optimal diet enhances the functioning of cellular and humoral components of the immune system.

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Chapter 5

Vaccines and vaccination

Vaccination

Passive immunization can be used to confer temporary immunity against an infectious agent or its products. Although transferred antibodies give immediate protection, the duration of protection is limited since the transferred antibodies are catabolized in the recipient. In contrast, active immunization, which involves the administration of antigen to a susceptible animal, results in the recipient producing its own immune response which persists for a much longer time than passive immunity. In addition, subsequent administration of the same antigen results in a marked increase in the level of response obtained.

The process of stimulating protective immune responses in animals against pathogenic microorganisms by exposing them to non-pathogenic forms or components of the microorganisms is referred to as vaccination. A successful vaccine induces an effective adaptive immune response directed at appropriate target antigens on the pathogen without causing disease in the recipient. Among the types of vaccines currently in use or being developed are those composed of inactivated microorganisms, live attenuated microorganisms, microbial products, synthetic peptides and DNA of microbial origin (Fig. 5.1). The duration of protection following vaccination is influenced by many host factors including age, immune competence and the presence of maternal antibodies in the animal's circulation. Through their ability to reduce morbidity and mortality, vaccines can contribute to the alleviation of welfare problems in domestic animals, to improvement in productivity and to reduction in the need for chemotherapeutic intervention. Where feasible, vaccination is a highly effective method for controlling infectious diseases in animals. In common with many disease control measures, however, vaccination has defined limitations. Effective vaccines for controlling equine infectious anaemia and African swine fever are not available at present. Protective immunity against *Staphylococcus aureus* using vaccination cannot be induced in a predictable manner and prevention of fungal infections through vaccination has had limited success.

In a large population of animals of comparable age and immune and health status, the response to vaccination is not uniform. The immune response is influenced by many genetic and environmental factors and the outcome of vaccination tends to follow a normal distribution (Fig. 5.2). A small percentage of animals have a weak response to vaccination and if challenged might be susceptible to infection. The majority of the animal population respond adequately and a small percentage respond strongly to vaccination. The addition of appropriate adjuvants to vaccines can enhance and prolong the duration of the immune response, decrease the antigen concentration required for effective immunization and promote the development of cell-mediated immune responses (Heldens *et al*., 2008).

When feasible, effective and safe, vaccination is one of the most cost-effective measures for controlling infectious disease, not only in companion animals but also in food-producing animals. Some infectious diseases with wildlife reservoirs, such as rabies, can also be controlled in particular animal species through vaccination. The benefits of vaccination, therefore, are not confined to reduced morbidity and decreased mortality in vaccinated animals, as transmission of zoonotic diseases such as rabies in the human population can be substantially reduced by dog and cat vaccination Although many of the vaccines currently licensed for use in animals are produced by conven-tional methods, the advent of biotechnology has provided an opportunity for developing vaccines with improved efficacy and greater safety. Inactivated vaccines often contain many irrelevant antigenic substances, some with undesirable biological activity. Live attenuated vaccines can produce adverse reactions including immunosuppression. Despite these limitations, conventional vaccines will continue to be used until superseded by safer, more effective subunit or genetically engineered live vaccines.

Figure 5.1 Vaccines currently in use or being developed.

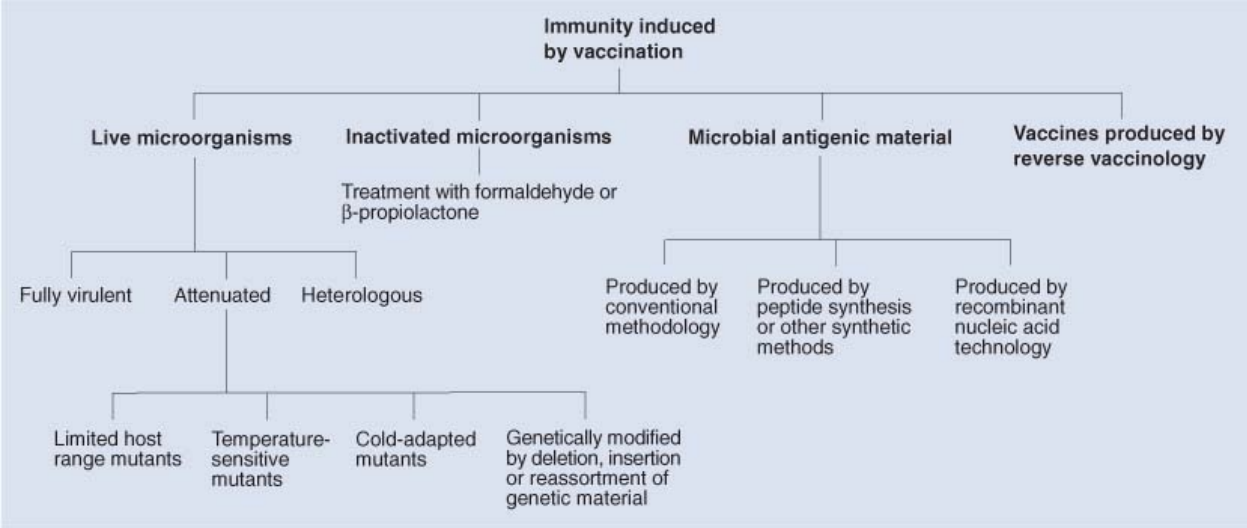
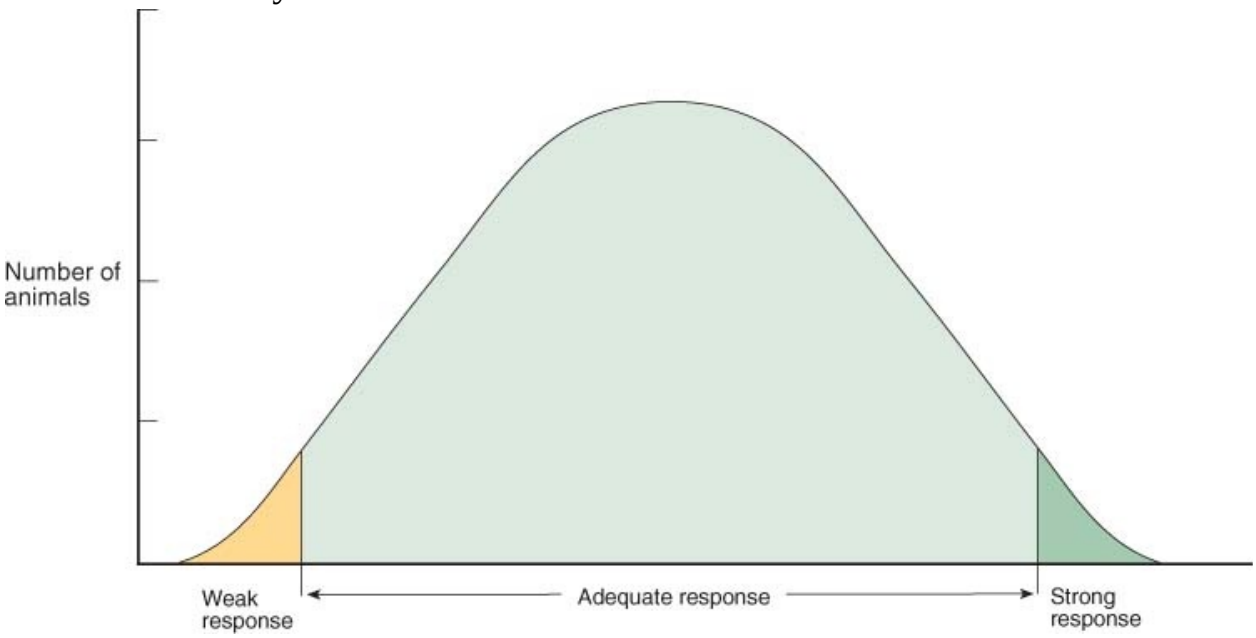


Figure 5.2 The antibody responses of a randomly selected population of vaccinated healthy animals follow a normal distribution.



Inactivated vaccines

Infectious agents can be killed without substantia lly altering the immunogenicity of their antigens which induce protective immunity. Although most inactivating chemicals do alter the immunogenicity of infectious agents, some, such as formaldehyde, cause limited antigenic change. When preparing inactivated vaccines, care has to be taken to ensure complete inactivation of the infectious agents as the chemicals used can cause aggregation of particles

thereby allowing survival of some microorganisms in the centre of aggregated material. Chemicals used for the preparation of inactivated bacterial and viral vaccines include formaldehyde, β-propiolactone and ethylenimine. Many bacterial vaccines used in animals are prepared by inactivating bacterial cultures (bacterins) or rendering their toxins inactive (toxoids) by chemical treatment.

A major limitation of inactivated vaccines is that some protective antigens are not produced readily *in vitro*. In addition, some components of killed vaccines can interfere with host immune responses. Inactivated viral or bacterial preparations can be partially purified and combined with adjuvants to enhance their immunogenicity. Because they are processed as exogenous antigens in the body, many inactivated vaccines can induce high levels of circulating antibody but are less effective at stimulating cell-mediated and mucosal immunity. As inactivated vaccines do not contain agents which can replicate, a greater antigenic mass and more frequent administration of vaccine (booster injections) are required to achieve results comparable to those obtained with live attenuated vaccines. Advantages of inactivated vaccines include stability at ambient temperatures, safety for recipients due to their inability to revert to a virulent state, and a long shelf life.

Live attenuated vaccines

Apart from the orf vaccine which is used in sheep, few virulent living organisms are used as vaccines in animals. The virulence of living organisms is reduced by attenuation, a process that involves adapting them to grow under conditions whereby they lose their affinity for their usual host and do not produce disease in susceptible animals. The bacillus of Calmette-Guerin, a strain of *Mycobacterium bovis*, was attenuated by culture in a bile-supplemented medium over many years. Bacteria can also be rendered avirulent using genetic manipulation.

Viruses can be attenuated by growing them in monolayers prepared from species to which they are not naturally adapted. Chick embryo attenuation has been employed successfully for rabies virus. Prolonged culture of canine distemper virus in canine kidney cells produced strains of reduced virulence suitable for immunization of dogs.

Even without attenuation, antigenically related viruses can be used to induce active immunity in certain species which they do not normally infect. Measles virus has been used to vaccinate dogs against distemper and, although these

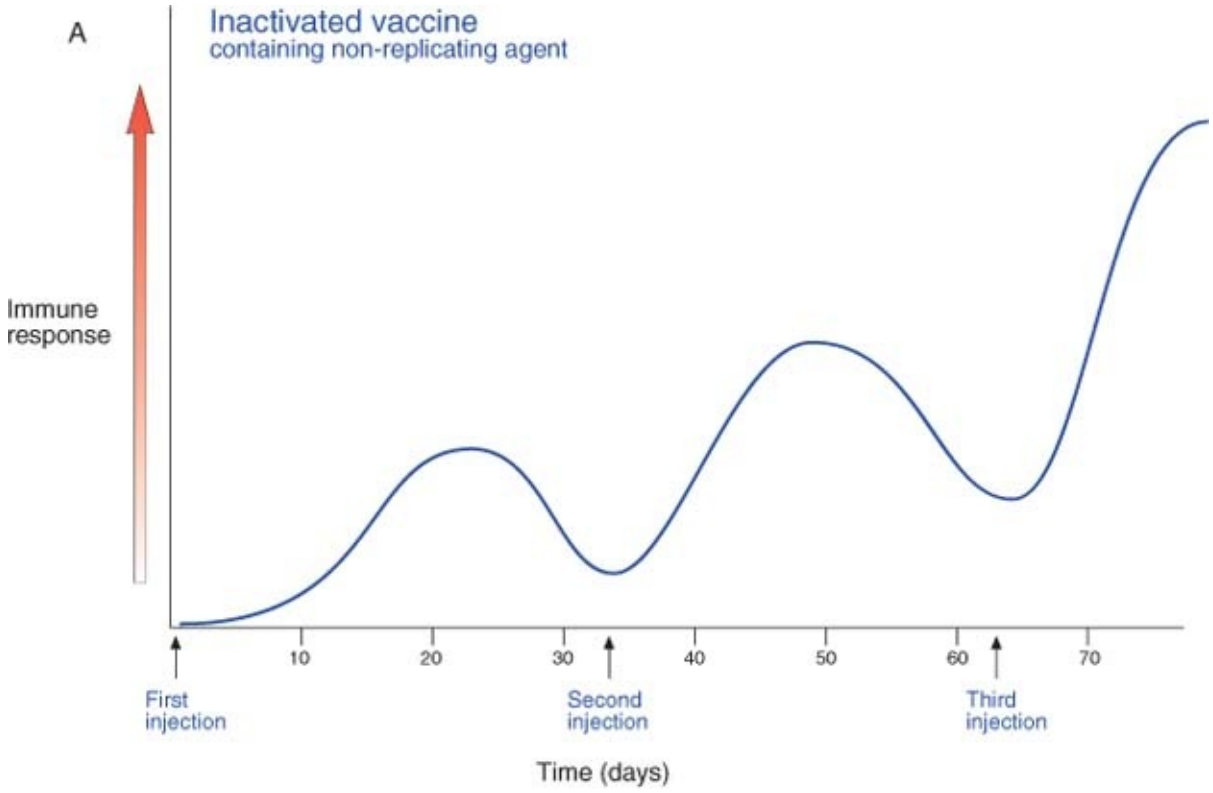
viruses cross-react, maternal antibodies to distemper virus in pups do not neutralize the live measles vaccine virus. The use of turkey herpesvirus to control Marek's disease in chickens is another example of protection induced by an antigenically related virus.

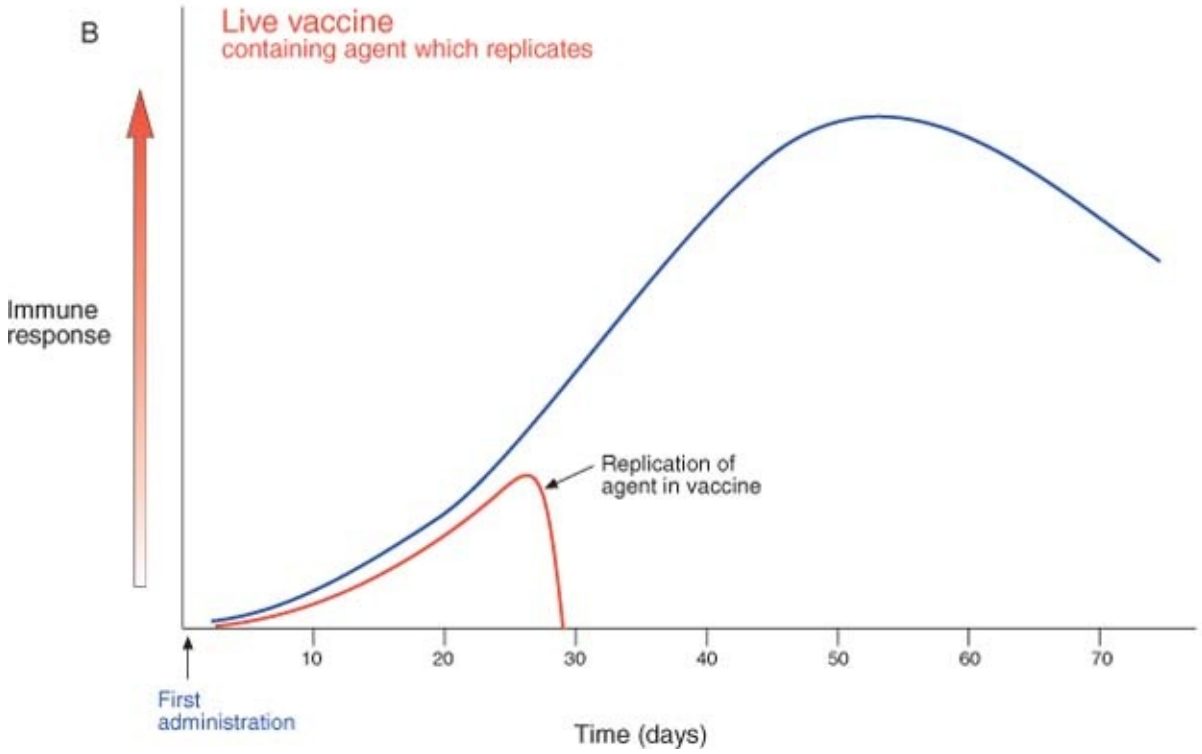
Live attenuated vaccines have many potential advantages over inactivated vaccines. They can be administered by a number of routes and present all the relevant antigens required for the induction of protective immunity since they multiply in the recipient. They usually induce a satisfactory level of cell-mediated and humoral immunity at sites where protection is required such as mucosal surfaces. Because they replicate in the body, adjuvants are not required. Booster doses, if required, can be given at widely spaced intervals as these vaccines induce a good immunological memory.

Disadvantages of these modified live vaccines include their possible immunosuppressive effects, especially in young animals or where an immunodeficient state exists. While live attenuated vaccines have been used for decades, the exact nature of the changes responsible for attenuation is often unknown. The circumstances in which reversion to virulence might occur cannot be predicted in a reliable manner. Live attenuated viral vaccines can be contaminated with infectious agents which can induce disease in recipients. Because maternal antibodies, acquired through ingestion of colostrum, can neutralize live viral vaccines, administration of such vaccines to young animals should be deferred until maternal antibodies have declined to low levels. The use of particular live viral vaccines in pregnant animals is contraindicated because of the risk of congenital defects in the developing foetus.

A live viral vaccine has a limited shelf-life and should be refrigerated during transportation and storage to ensure its viability. Comparisons of the immune responses to an inactivated vaccine and to a live vaccine are illustrated in Fig. 5.3.

Figure 5.3 Comparison of the immune responses to repeated administration of an inactivated vaccine (A) and to a single injection of a live vaccine (B).


A
Inactivated vaccine
containing non-replicating agent
Immune response
10
20
30
40
50
60
70
First injection
Second injection
Third injection
Time (days)


B
Live vaccine
containing agent which replicates
Immune response
Replication of agent in vaccine
10
20
30
40
50
60
70
First administration
Time (days)

Vaccines produced by recombinant nucleic acid technology

Recombinant vaccines are classified into three categories by the US Department of Agriculture (Mackowiak *et al*.,1999). Type I vaccines are composed of antigens produced by recombinant nucleic acid technology or genetic engineering. Type II vaccines consist of genetically attenuated microorganisms, and Type III vaccines are composed of modified live viruses or bacteria into which DNA encoding protective antigens are introduced by cloning.

Type I vaccines are composed of subunit proteins produced by recombinant bacteria or other microorganisms. DNA encoding the required antigen is cloned in a suitable bacterium or yeast strain in which the recombinant antigen is expressed (see Chapter 9). These vaccines usually contain adjuvants which are required to enhance the immunogenicity of the purified antigen derived from the recombinant microorganism.

Type I vaccines have been developed for a number of bacterial and viral pathogens. They have been used against the virus of foot - and - mouth disease, feline leukaemia virus, and *Borrelia burgdorferi*, the cause of Lyme disease.

Type II recombinant vaccines consist of virulent microorganisms that are rendered less virulent by gene deletion/knock-out or site-directed mutagenesis. The genome of large DNA viruses, such as herpesvi-ruses, contains many non-essential genes. Using genetic engineering techniques, an Aujeszky’s disease vaccine in which an attenuated virus lacking the gene encoding thymidine kinase (TK) was developed. As TK is normally required by wild-type herpesviruses to replicate in non-dividing cells such as neurons, TK-attenuated herpesviruses can infect neurons but are unable to replicate in these cells. Such deletion mutants induce a protective immune response in pigs. Deletion of the glycoprotein I (gI)-encoding gene in porcine herpesvirus 1 permits differentiation of naturally-infected pigs, which produce antibodies against gI, from vaccinated pigs which do not produce such antibodies. Thus, vaccination programmes can proceed in countries where the disease is being eradicated without interfering with serological recognition and removal of infected pigs. Similar strategies have also been used to provide vaccines targeting pathogenic bacteria of veterinary importance. In these bacteria, genes essential for key metabolic processes are often targeted for modification. As an example, a live recombinant bacterial vaccine prepared from *Streptococcus equi* TW928 was designed, in which a 932-

bp deletion in the *aroA*-encoding gene was developed (Jacobs *et al*., 2000). Submucosal administration of this knock-out vaccine confers protection in horses. Attenuated vaccine strains can be detected by PCR methods that confirm the genetic alteration in the chromosome.

The failure of some vaccines used in veterinary medicine to induce a protective immune response can result from problems related to delivery. Development of delivery systems that are effective, safe and convenient for administration and suit animal producers' needs is therefore challenging for those engaged in vaccine production. The use of live viruses for the delivery of veterinary vaccines is a possible solution to current difficulties. Type III vaccines are composed of modified live organisms called vectors into which a gene encoding an antigenic determinant is introduced. This engineered organism then serves as a delivery system in the recipient. In order to produce safe viral vaccine vectors it is necessary to ensure that the vector itself does not pose a threat to vaccinated animals or humans. This can be achieved by attenuating the viral vector or by generating live attenuated viruses with precise genetic alterations that ensure their suitability as vectors.

Recombinant nucleic acid technology provides a more complete understanding of the genetic organization of viruses, permitting the identification of suitable regions for insertion of foreign DNA. Several types of potentially useful viral vectors from a variety of viruses such as pox viruses (including vaccinia virus and fowlpox virus), adenoviruses, herpesviruses and retro-viruses, have been developed (Sheppard, 1999; Meeusen *et al*.,2007). Potential advantages of the use of viral vectors for vaccine delivery include possible administration to large groups of animals by aerosols or in water rather than by injection of individual animals. Such mass administration procedures would be particularly relevant to poultry and pig producers. If properly designed, the vector should express those antigens from the pathogen that are required to induce a protective immune response thereby reducing or eliminating the chance of disease in animals exposed to the infectious agent in a modified live form. A distinct advantage of vectored vaccines is that they can induce both humoral and cell-mediated immune responses, including strong cytotoxic T cell immunity. In addition, some vectored vaccines may be capable of inducing local immune responses on mucosal surfaces.

To ensure vector stability and the appropriate expression of foreign DNA, only a limited amount of that genetic material can be incorporated into the vector genome. Consequently, each vectored vaccine can produce only one or a

relatively small number of foreign antigens in the host animal for the induction of a protective immune response. A possible complication of vectored vaccines is that they may express altered tissue tropism as a result of the acquisition of foreign DNA. Prior exposure of animal populations to the virus used to construct the vector could substantially limit the effectiveness of a vector-based vaccine.

Currently a small number of viral vectored vaccines have been approved for use in animals. A vaccinia vaccine vector carrying the rabies G glycoprotein has been used successfully as an oral vaccine administered to wild carnivores in bait. The G glycoprotein induces virus-neutralizing antibodies in vaccinated animals which protect against rabies. Other examples include a canarypox virus-vectored vaccine against canine distemper virus in dogs, West Nile virus in horses and a fowlpox virus-vectored vaccine designed to protect against avian influenza virus in poultry. Selected microbial diseases of animals and vaccines produced by biotechnology which are available for their control are presented in Table 5.1.

Table 5.1 Selected microbial diseases of animals and vaccines produced by biotechnology that are available for their control.

Microbial pathogen	Species affected	Associated disease	Vaccine characteristics
Brucella abortus	Cattle	Brucellosis	Spontaneous rifampicin-resistant rough mutant
Canine distemper virus	Dogs	Distemper	Canary pox virus-vectored vaccine
Porcine herpesvirus 1	Pigs	Aujeszky's disease	Thymidine kinase-deleted marker vaccine
Porcine circovirus 2	Pigs	Post-weaning multisystemic wasting syndrome	Inactivated baculovirus expressing porcine circovirus 2 ORF2 protein, with adjuvant
Streptococcus equi	Horses	Strangles	Live vaccine administered submucosally: Δ*aroA*
West Nile virus	Horses	West Nile virus infection	DNA vaccine

Synthetic peptide vaccines

If the structure of epitopes that can induce a protective immune response is known, it is possible to chemically synthesize peptides corresponding to these anti-genic determinants. Only a small portion of antigenic molecules interact with specific receptors on B cells and T cells. For B cells, an antibody interacts with up to five amino acids in its antigen-binding site. Epitopes for T cell receptors can be composed of 12 to 15 amino acids.

The general approach with synthetic peptide vaccines is to identify potential epitopes in the protein antigen and to synthesize a series of peptides corresponding to that amino acid sequence. The immuno-logical activity of these molecules is then evaluated *in vivo*.This approach is appropriate only for epitopes consisting of contiguous amino acids referred to as linear epitopes. The majority of natural epitopes are non-linear and are, therefore, dependent on the conserved three-dimensional structure of the molecule. Antibodies induced by peptide vaccines may not react with the native molecule and, in addition, peptides are usually poor immunogens due to their small size. Immunogenicity can be enhanced with appropriate carrier molecules or adjuvants. Limited progress has been made with synthetic peptides for the induction of protective immune responses against infectious agents.

DNA vaccines

One of the most significant developments in vaccine production in recent years involves the use of DNA, encoding microbial antigens cloned in a bacterial plasmid, for immunization. The procedure involves injection of a plasmid containing the DNA sequence for a protective antigen whose expression is controlled by a strong mammalian promoter. For an infectious agent expressing that antigen, injection of this recom-binant plasmid into the skin or muscle of animals may result in the production of the protein inducing immunity against that infectious agent. This leads to the expression in host cells of the encoded genes with the development of a significant immune response to the gene product in the recipient. Unlike viral vectors, the recombinant plasmid cannot replicate in the mammalian cells but transfected host cells express the vaccine antigen. Methods of delivery include direct intramuscular injection and the use of liposomes or coated gold particles fired by a gene gun. Although transfection rates appear low, antigen production has been detected in animals vaccinated

with DNA intramuscularly for up to 6 months after injection. Because DNA vaccination induces intracellular processing of antigen, it seems to mimic a natural infection and is, therefore, an effective method of inducing T cell responses. Even small amounts of DNA can stimulate strong cell-mediated responses. Humoral responses, however, may not be as high as those obtained by injection of a purified antigen. A strategy in which priming with DNA vaccines is followed by boosting with attenuated viral vectors such as fowlpoxvirus and modified vaccinia virus has produced exceptionally strong immune responses (Ramshaw and Ramsay, 2000). The success of consecutive administration of DNA vaccines and attenuated viral vectors was attributed to the ability of the DNA vaccines to generate T cells of high affinity which were further stimulated by boosting with non-replicating viral vectors. Although immune responses may be delayed following DNA vaccination, a persistent response may occur. In contrast to modified live viral vaccines, maternal antibody does not appear to affect the immune response in young animals. An advantage of immunizing with purified DNA is the possibility of antigen presentation in its native form as it would occur during replication of an infectious agent in the body. By this method of vaccination, it is also possible to select genes for the antigen of interest without the need for a complex bacterial or viral vector.

The safety of DNA vaccines remains unresolved. The possibility that foreign DNA could integrate into the host chromosome and induce neoplastic changes or other cellular alterations has been suggested. It has also been suggested that DNA introduced into the body by this method of vaccination might induce anti-DNA antibodies to the recipient’s own DNA.

Reverse vaccinology

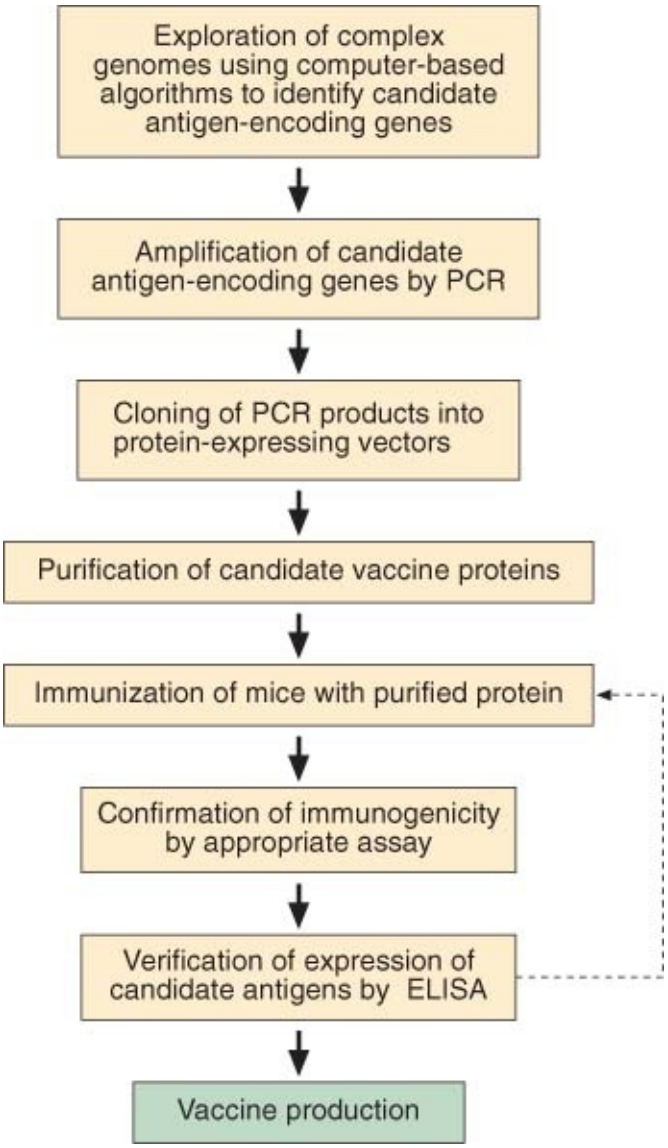
Availability of genome sequences for many infectious agents offers the possibility of exploring the complete proteome, which provides the potential for rational selection of vaccine candidates. This novel approach is termed reverse vaccinology and it can be combined with functional immunology to optimize epitope prediction, leading to development of DNA vaccines. Depending on the nature of the infectious agent, approaches used may differ.

Potential candidate vaccines have been developed using reverse vaccinology for bacterial pathogens, namely *Bacillus anthracis* (Ariel *et al*., 2002) and *Leptospira* (Koizumi and Watanabe, 2005). The completed genome of *Neisseria meningitidis* group B (NMB) was searched using suitable unbiased computer

algorithms to identify open reading frames (ORF) encoding surface-exposed protein antigens, which could later be evaluated as potential vaccines (Fig. 5.4) based on their ability to elicit a bactericidal antibody response (Pizza *et al*., 2000; Tan *et al*., 2010). The candidate ORFs were amplified by PCR and cloned into suitable protein-expressing vectors. Protein produced from each individual clone was identified by ELISA, then purified and assessed for immunogenicity in an experimental animal model. Immunogenicity was confirmed using a standard serum bactericidal antibody (SBA) assay, designed to measure the bactericidal activity of the serum in the presence of specific antibodies which bind to the pathogen and activate complement. Five candidate antigens produced for a vaccine against NMB were developed.

A recognized limitation of reverse vaccinology when applied to the development of vaccines for pathogenic bacteria or other pathogenic agents with complex genomes is that the strain under investigation may not represent the genetic diversity of the species. Comparisons of genomes from several different strains of bacteria such as *Streptococcus agalactiae* underscores this issue (Tettelin *et al*., 2005). In this case, a core genome of 80% exists between all strains, with each new strain containing approximately 18% of the corresponding genome consisting of unique DNA sequences. These sequences may be important and may not be included in a generic vaccine. Therefore, it may be important to evaluate genome sequences from multiple strains of a microbial pathogen to identify efficient targets for vaccine production.

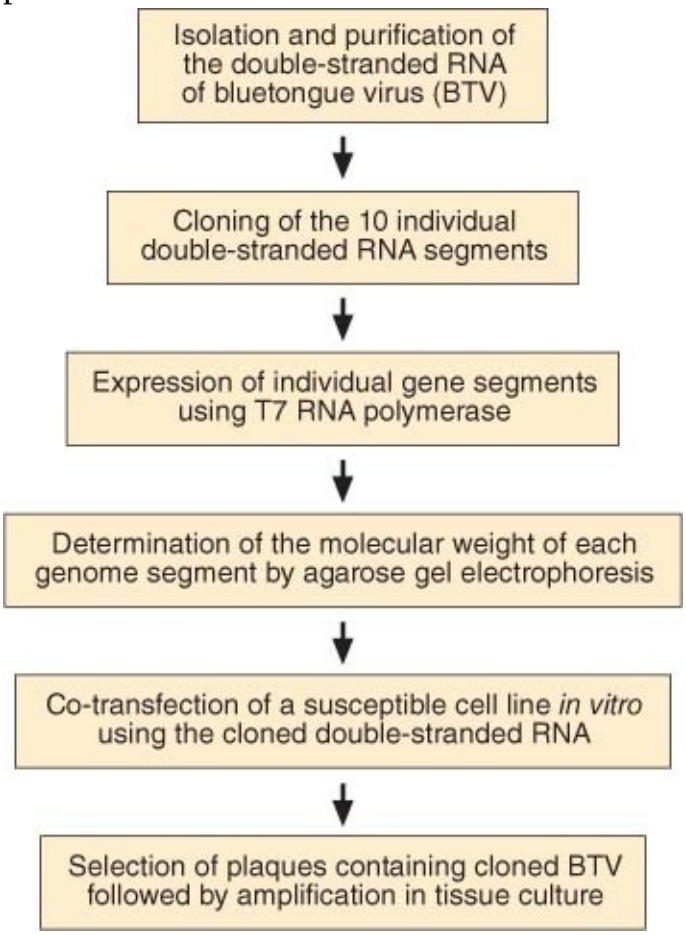
Figure 5.4 Steps involved in the exploration of complex genomes for identification of candidate surface-expressed proteins.



In contrast to the application of reverse vaccinology applied to pathogenic bacteria, a different strategy was applied to the development of viral vaccines. Bluetongue has been recognized as a viral disease of livestock for over 100 years. Current vaccines for this virus are not without their problems and new approaches have been developed to improve safety and protection. Reverse vaccinology has been applied in the development of attenuated viral strains that can confer protection (Roy *et al*., 2009). Traditional bluetongue vaccines have relied on the provision of attenuated virus following passage through chick embryos or sheep. The nature of the attenuating mutations is unknown and recent developments in reverse genetics may provide a solution to this issue.

Figure 5.5 *In vitro* cloning of a population of bluetongue virus particles using

reverse genetics for subsequent *in vivo* assessment of their suitability for vaccine production.



Bluetongue virus (BTV) is a member of the *Reoviridae* family with a genome consisting of 10 double-stranded RNA segments, each consisting of a unique ORF. A complementary DNA (cDNA) copy of each ORF is cloned into a T7 plasmid, between a flanking upstream strong T7 promoter and a downstream restriction site (Fig. 5.5). These ten individual clones represent the complete genome of BTV. T7 phage RNA polymerase produces individual RNA transcripts *in vitro* for each genome segment. Individual transcripts are initially assessed by denaturing agarose gel electrophoresis, following which they are co-transfected into a susceptible mammalian cell line and overlaid with agarose. BTV plaques that develop can be recovered and amplified by growth in tissue culture. In this way, attenuated virus can be produced *in vitro* following the introduction of directed mutations. These engineered viruses are later tested in ruminant animal models to identify the pathogenicity determinants of BTV. The data generated can be used later to design a vaccine strain containing several attenuated mutations.

Adjuvants

Substances that have the ability to increase or modulate the intrinsic immunogenicity of an antigen are referred to as adjuvants. Typically, if adjuvants are mixed with antigens before administration, they boost the immune response of antigens of low immunogenicity. They also enhance the immune response to small amounts of antigenic material. Although the modes of action of many adjuvants are not well understood, some such polyribonucleotides and lipopoly-saccharides are recognized as ligands for toll-like receptors on dendritic cells and macrophages.

For almost 80 years, aluminium salts have been used as adjuvants. These salts promote antibody production in primary immune responses through their stimulation of T_H2 cell responses. Formerly, it was suggested that the adjuvant activity of aluminium salts was due to the depot effect of these salts at the site of injection. Currently, the effect of aluminium salts is attributed to local concentration of antigen and improved uptake by antigen-presenting cells (Guy, 2007). Macrophage activation, IL-4 production by an unknown population of cells and expansion of antigenspecific B lymphocytes are among the effects associated with the use of aluminium salts in vaccine preparations. At present, some of the best understood adjuvants are those capable of acting as ligands for toll-like receptors which recognize conserved components of bacteria and viruses. These adjuvants induce immune responses by activating macrophages and dendritic cells. Adjuvants may possess a number of different features relevant to the enhancement of adaptive immune responses. These include depot formation, ability to act as carriers and immunostimulating activity (Box 5.1). Promoting antibody production was a common outcome of adjuvant use with many vaccines. The inability of many adjuvants to induce a strong cell-mediated response, however, was a serious limitation to the development of effective vaccines for particular microbial pathogens. Induction of a protective immune response against intracellular bacterial pathogens, many viruses and protozoal parasites requires an effective cell-mediated immune response. Availability of adjuvants capable of inducing strong cell-mediated immune responses to a range of antigenic components in vaccine preparations is required for the successful development of effective disease control programmes for food - producing animals and companion animals. The merits of individual adjuvants relate to

their formulation and their ability to enhance antigen uptake by antigen-presenting cells, to activate antigen-presenting cells and to stimulate T lymphocyte subsets, B lymphocytes or other cells which participate in antigen recognition, processing and presentation to lymphocytes. The place of adjuvants in the development and clinical use of vaccines has been intensively investigated in recent years (Alving and Matyas, 2005). As many of the potential vaccines being produced by recombinant technology or other developments in molecular biology are protein subunits or even short peptides which, on their own, are weakly antigenic, a new generation of adjuvants is required to enhance their immunogenicity. Categories of adjuvants in current use or being evaluated are listed in Table 5.2.

BOX 5.1 Modes of action of adjuvants.

- Retention and slow release of antigenic material from the site of injection
- Increased immunogenicity of small or antigenically weak synthetic or recombinant peptides
- Some adjuvants, such as derivatives of lipopolysaccharide which act as ligands for toll-like receptors, promote the development of adaptive immune responses
- Improved speed of response and persistence of response to effective antigens
- Increased immune response to vaccines in immunologically immature, immunosuppressed or ageing animals
- Stimulation of dendritic cell and macrophage activity and the enhanced processing of antigen by antigen-presenting cells
- Modulation of humoral or cell-mediated immune responses by the subset of T lymphocytes which is activated
- Stimulation of T and B lymphocyte responses

Bacterial derivatives

Because complex bacterial derivatives are related to pathogen-associated molecular patterns, they can be detected by toll-like receptors and promote the development of adaptive immune responses. Evidence of direct binding of bacterial derivatives to toll-like receptors is lacking, however, and it is established that lipopolysaccharide does not bind directly to TLR-4 (Guy, 2007). Although epidermal Langerhans cells express mRNA encoding a number of toll-like receptors, these antigen-presenting cells do not directly respond to toll-like receptor agonists. Detection of natural or synthetic agonists by toll-like receptors can orientate the immune response towards a T_H1 cell or T_H2 cell response, and

a number of immunostimula-tory compounds of bacterial origin can induce T_H1 cell responses following detection by toll-like receptors.

The adjuvant effect of mycobacterial cell wall components such as muramyl dipeptides and trehalose dimycolate is attributed to their ability to stimulate macrophages and dendritic cells, interferon-γ production and T helper cell activity. The activity of monophosphoryl lipid A, a derivative of lipopolysaccharide, is thought to be mediated by interaction with TLR-2 (Del Giudice and Rapuoli, 2005). Enhanced migration of dendritic cells, generation of T_H1 responses, increased expression of co-stimulatory molecules and production of IL-2, interferon-γ and IL-12 are associated with the use of this derivative of lipopolysaccharide.

Cytokines and related substances

A number of cytokines, including IL-1, IL-2, IL-12 and interferon -γ**3**, have been shown experimentally to be effective adjuvants, especially if combined with the antigenic material. Alternatively, cytokines can be administered as a single dose close to the time of vaccination. Using cytokines to direct the immune response in a given direction is a potential advantage of using these substances as adjuvants.

Emulsions

An emulsion is a mixture of two immiscible liquids with one dispersed throughout the other in small droplets. Emulsions used as adjuvants can be water-in-oil emulsions or oil-in-water emulsions. Oils used in the preparation of water-in-oil emulsions include mineral oils, vegetable oil, squalene and Montanide ISA 720. Freund's complete adjuvant contains heat-killed mycobacteria in mineral oil to enhance further the immune response to antigenic material. Because mycobacteria contain immunostimulating substances such as muramyl dipeptide, Freund's complete adjuvant is a potent stimulator of antigen-presenting cells, T cells and B cells. It induces strong cell-mediated responses and stimulates antibody production.

Oil-based adjuvants have a number of undesirable attributes. They may induce local and systemic inflam-matory reactions. Granuloma and abscess formation may occur at the inoculation site. The use of Freund's complete adjuvant is not permitted in food-producing animals, as oil remains at the injection site and the

killed mycobacteria in the adjuvant induce a positive reaction in the tuberculin test. Injection of mineral oils into tissues is a questionable procedure as some may have carcinogenic activity. Alternatives to Freund's adjuvants have been proposed and evaluated. These include vegetable oil emulsions composed of peanut oil, olive oil or sesame oil with emulsifying agents.

Table 5.2 Categories of adjuvants in current use or currently being evaluated.

Category/examples	Comments
Bacterial derivatives	
Muramyl dipeptides	Adjuvant activity attributed to stimulation of macrophages, dendritic cells, interferon-γ production and T_H cell activation
Monophosphoryl lipid A	Enhanced migration of dendritic cells, generation of T_H1 responses, production of IL-2, IL-12 and interferon-γ
Lipid A derivatives	
Heat-labile enterotoxins of *E. coli*	
Trehalose dimycolate	
Cytokines and related substances	
IL-1, IL-2, IL-12 Interferon-γ	Effective adjuvants if combined with antigenic material; can be used to direct the immune response in a given direction
C3d	
Emulsions	
Water-in-oil emulsions	These oil-based preparations are considered depot adjuvants; inclusion of heat-killed mycobacteria enhances immune responses; they stimulate antigen-presenting cells, T and B lymphocytes
Mineral oil	
Vegetable oil	
Montanide ISA	
Squalene	
Oil-in-water emulsion MF 59 (squalene with Tween 80 and Span 85)	Seems to act through localization of injected material in antigen-presenting cells; promotes T_H2 cell development and production of IgG antibody
Biodegradable particles	
Liposomes	Taken up by antigen-presenting cells and processed by MHC class II-dependent pathways
Virosomes	Fuse with cell membranes, enclosed antigen presented via MHC class I-dependent pathways, effective cytotoxic T lymphocyte responses reported
Proteosomes	Taken up by antigen-presenting cells; promote T_H1 responses
Virus-like particles	Immunogenicity strong without additional adjuvant
Mineral salts	
Aluminium hydroxide, aluminium phosphate, calcium phosphate, aluminium potassium sulphate	Adjuvant activity related to macrophage activation and increased uptake of antigen by antigen-presenting cells; promote T_H2 cell responses and enhance antibody production
Saponins	
Immunostimulating complexes (ISCOMs)	Saponin-based adjuvants augment T_H1 and T_H2 cell responses; ISCOM-based vaccines promote both humoral and cell-mediated immune responses; activity of ISCOMs attributed to interactions with macrophages and dendritic cells and activation of $CD4^+$T cells

An oil-in-water emulsion, MF 59, contains squalene stabilized by the addition of two emulsifiers, Tween 80 and Span 85. Unlike water-in-oil emulsions which

are considered as depot adjuvants, MF 59 seems to act through localization of injected material in antigen-presenting cells. This adjuvant, which is stable and highly versatile, has undergone extensive trials. Current information indicates that MF 59 promotes T_H2 cell development and preferential production of IgG antibody (Del Giudice and Rapuoli, 2005). The strong adjuvant activity of MF 59 has been demonstrated with viral and bacterial vaccines in laboratory animals and in primates. A subunit influenza vaccine incorporating MF 59 adjuvant has been licensed in a number of European countries.

Biodegradable particles

Antigenic material can be either encapsulated into biodegradable particles or carried on their surfaces through adsorption or covalent linkage. It is presumed that the particulate nature of the carrier particles targets antigen-presenting cells. Liposomes, membranous vesicles of naturally occurring phospholipids, can act as effective adjuvants for protein antigens which are incorporated into the vesicle membrane. These biodegradable particles are taken up by antigen-presenting cells, and their contents are processed largely via MHC class II-dependent pathways (Nataro and Levine, 2005). Uptake of liposomes can be facilitated by coating them with antibodies to promote interaction with receptors on antigen-presenting cells. Liposomes produced by reconstitution of viral glyco-proteins and lipids, referred to as virosomes, have many of the properties of the viruses from which they are derived. Virosomes have been used as delivery systems for antigens, drugs and other compounds. By binding to and fusing with cell membranes, viro-somes discharge their contents into the cytoplasm. Accordingly, antigens are presented via MHC class I-dependent pathways, and effective $CD8^+$ cytotoxic T cell responses to virosome-encapsulated antigens have been reported. It has been reported also that mucosal application of virosomes can elicit secretory IgA responses.

Structures derived from the outer membrane proteins of Gram-negative bacteria have been employed as adjuvants. These structures, termed proteosomes, can be produced by detergent extraction of the bacteria. Outer membrane proteins are highly hydrophobic and when isolated by detergent extraction undergo protein–protein interactions which result in self-assembly of structures resembling vesicles. During manufacture, suitable antigenic material can be incorporated into these structures with the exclusion of detergent (Nataro and Levine, 2005). Another approach involves the combination of antigen with

preformed lipopolysaccharide-solubilized proteosome particles. The surface hydrophobicity of proteosomes may improve their uptake by antigen-presenting cells and their ability to promote T_H1-type responses. The interaction of outer membrane proteins of Gram-negative bacteria and lipopolysaccharide with toll-like receptors on antigen-presenting cells may contribute to the enhanced immunogenicity of antigens complexed with proteosomes.

Virus-like particles are non-replicating recombinant viral capsids with a structure similar to the virus particles from which they are derived. These particles are formed by self-assembly of viral structural proteins synthesized in suitable eukaryotic or prokaryotic systems and accordingly have the native conformation of viral antigens but without the presence of viral nucleic acid. The immunogenicity of virus-like particles is reported to be strong without the requirement for an additional adjuvant. Extensive clinical trials with these particles have been carried out with favourable results recorded for a number of human viral diseases.

Mineral salts

Although aluminium salts have been used extensively as adjuvants for almost 80 years, their modes of action are not well characterized. Another mineral salt, calcium phosphate, has also been used for its adjuvant activity. Formerly, the activities of mineral salts were attributed to their depot effect and to the site of injection. Current information on the activities of mineral salts suggests that their adjuvant activity relates to activation of macrophages and increased uptake of antigen by antigen-presenting cells (Guy, 2007).

When used as adjuvants, mineral salts stimulate an earlier, higher and longer-lasting antibody response after primary immunization than occurs with soluble vaccines. Their stimulatory effect relates only to primary immune responses, as secondary immune responses are not affected. These adjuvants promote antibody production through the stimulation of T_H2 cell responses. Cell-mediated responses are stimulated minimally, a serious limitation of mineral salt-based adjuvants for vaccines aimed at intracellular pathogens. Granulomatous reactions, which can persist for many months at the site of injection, are an undesirable response to the inclusion of mineral salts in vaccines.

Saponins and immunostimulating complexes

Triterpene glycosides, isolated from the bark of the South American tree *Quillaja saponaria* Molina, referred to as saponins, have both toxic and adjuvant properties. Saponin-based adjuvants augment both T_H1-and T_H2-type responses. Fractionation has been used to select less toxic saponins, and semi-synthetic saponin derivatives have been developed (Guy, 2007).

Immunostimulating complexes (ISCOMs) containing the saponin Quil A, cholesterol and phospholipids, are cage-like structures into which antigen can be incorporated. ISCOM-based vaccines promote both humoral immunity and cell-mediated immune responses. Iscomatrix, which is similar in formulation and structure to ISCOMs, can be used without the incorporation of antigen into the structure. Induction of immunity following mucosal administration has been reported with ISCOM-based vaccines. The effectiveness of ISCOMs as adjuvants is attributed to their ability to interact with macrophages and dendritic cells and to activate $CD4^+$ T cells.

Administration of vaccines

Factors that influence the success of vaccination programmes in animals include the nature, composition and efficacy of the vaccine, the ages of the animal population at time of vaccine administration, routes of transmission of the infectious agents involved and the methods used to administer the vaccine. In mammals, most vaccines are administered by injection. For diseases that affect particular systems, vaccines may be given by a route that stimulates local immune responses at the usual site of invasion. Accordingly, the intranasal route may be used for vaccination against infectious bovine rhinotracheitis or feline viral rhinotracheitis. When large numbers of animals are involved, methods other than injection may be used. In farmed fish, vaccination is an important method for preventing infectious diseases. Depending on the nature of the vaccine, injection into the abdominal cavity or immersion of fish in a diluted solution of antigenic material may be used.

Irrespective of the species of animals being vaccinated, there should be strict adherence to the manufacturers' instructions, with clean equipment mandatory and vaccines used before reaching their expiry date. Animals selected for vaccination should not be receiving immunosuppressive therapy, and animals incubating disease will not be protected by vaccination. An interval of up to 10 days may elapse before vaccinated animals are protected by their own active

immune response.

When dealing with pregnant animals, timing of vaccination should take account of the requirement for colostral protection for newborn animals. Ewes should be vaccinated 6 weeks before lambing to ensure high colostral antibody levels, and vaccination of mares with tetanus toxoid should take place at least 4 weeks in advance of foaling. The administration of live viral vaccines to pregnant animals is usually contraindi-cated because of the risk of congenital defects in the developing foetus.

Poultry

A large number of vaccines are available for use in poultry and careful planning should form part of the vaccination programme. The flock history, type of production system and local diseases prevalent in the region influence vaccine selection (Cserep, 2008).

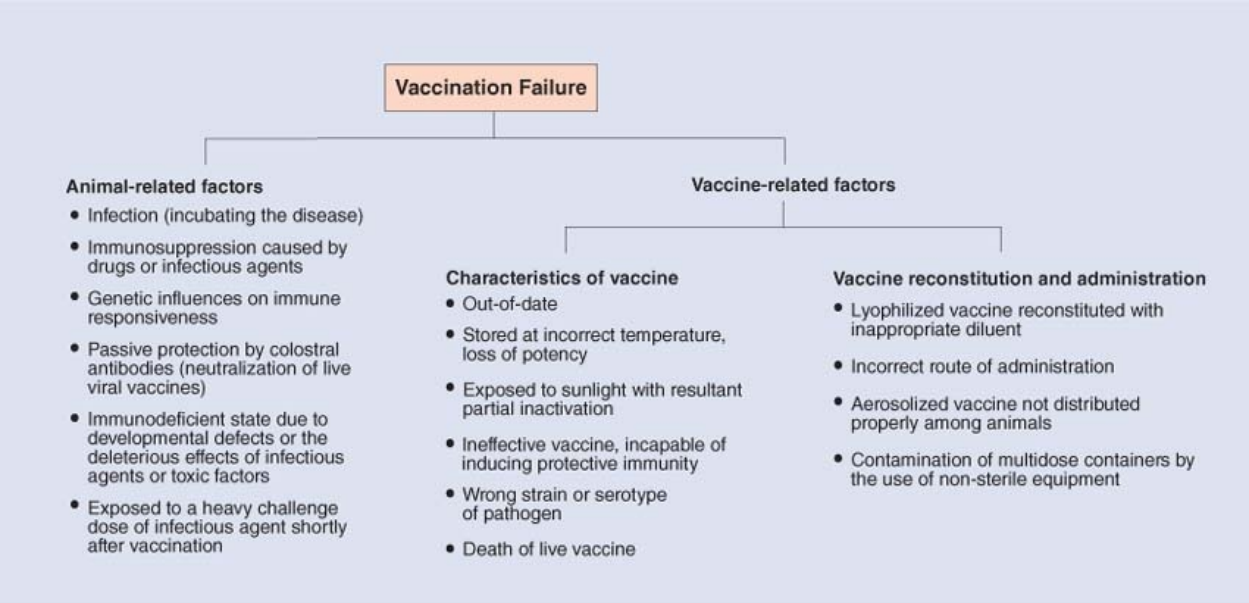
Most live vaccines are given in drinking water or by aerosol. Because tap water usually contains chlorine, another source of clean water should be used for reconstitution of live viral vaccines. Some poultry vaccines are administered by eye drops, by injection, in feed or by wing web inoculation. In recent years, *in ovo* vaccination has become an established procedure in many countries. A number of live viral vaccines including Marek's disease vaccine are administered by this route. The procedure, using an automated egg injection system, is carried out at 18 days when transfer from incubator to hatcher takes place.

Adverse reactions following vaccination

Even in clinically normal animals, vaccination is not a risk-free procedure and adverse reactions can emerge soon after vaccination or at a later stage. Undesirable responses to vaccination may be a consequence of contamination of the vaccine during manufacture, reconstitution or administration. Allergic reactions to vaccine components can occur, especially following repeated use of preparations containing protein from tissue culture fluid or chick embryo sources. Reactions at the injection site may be due to the introduction of pyogenic bacteria during injection, leading to abscess formation. Granulomas at the site of vaccine administration sometimes occur if water-in-oil adjuvants are incorporated into the vaccine. Neoplastic changes have been recorded at

injection sites in some species of animals. Adverse reactions following vaccination may be related to contamination, survival of infectious agents in the preparation, vaccine-induced immuno- suppression, allergic reactions to vaccine components or neoplastic tissue changes at the site of injection (BOX 5.2).

Figure 5.6 Factors which may contribute to vaccination failure.



BOX 5.2 Potential adverse reactions following vaccination.

- Local or systemic infection caused by contamination of live vaccine with extraneous agents
- Disease produced by the survival of infectious agents in a supposedly killed vaccine
- Disease produced by resistant infectious agents such as prions surviving in inactivated vaccines
- Disease production by live vaccine in immunosuppressed animals
- There may be a risk of congenital defects if particular live vaccines are administered to pregnant animals
- Vaccine-induced immunosuppression
- Development of hypersensitivity reactions to vaccine components (immediate or delayed responses)
- Adjuvants containing mineral oil may induce a granulomatous reaction at the injection site
- Induction of neoplastic changes due to the presence of oncogenic infectious agents or from the action of particular adjuvants
- Disease produced by the presence of infectious agents in live vaccines, undetectable by current conventional methods

Vaccination failure

The efficacy of vaccines is determined by many factors. Problems with vaccines produced by reputable companies are relatively uncommon if strict quality-control procedures are in place. Because of the large number of factors that can influence or, in some instances, determine the efficacy of vaccination, a small percentage of vaccinated animals may not be protected following vaccination (Fig. 5.2). The outcome of vaccination is determined not only by vaccine quality but also by animal-related factors that may strongly influence the results obtained. Vaccine-related factors that can contribute to vaccination failure include inherent characteristics of the vaccine and problems with reconstitution and administration. Animal-related factors include the possibility of animals incubating the disease at the time of vaccination, neutralization of live viral vaccines by colostral antibodies, and immunosuppression caused by drugs or infectious agents. Accordingly, vaccination failure may arise from errors in reconstitution or administration of a vaccine, characteristics of the vaccine and animal-related factors (Fig. 5.6).

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Chapter 6

Molecular diagnostic methods

The characteristics of living bacteria are associated with the genes present on their bacterial chromosome. This structure consists of a double-stranded deoxyribonucleic acid (DNA) helix with the properties required to control replication of the bacterium, store its genetic information and express some characteristics unique to the organism. Despite its relatively simple chemical composition, DNA has an incredible capacity for directing ordered and complex functions within a bacterial cell.

All of the above properties are carefully controlled by specific enzymes. For example, replication is controlled by the enzyme DNA polymerase, which is required for the formation of accurate copies of the original DNA molecule. Synthesis of messenger RNA (mRNA) is controlled by RNA polymerase. This message is subsequently decoded in a process involving other enzymes leading to the synthesis of a bacterial protein (see Chapter 9 for revision of the structure of DNA and an outline of replication and transcription).

Careful consideration of the chemical nature of the DNA structure shows that it possesses some useful properties that can be used for analytical purposes. This has led to the development of diagnostic molecular techniques designed to detect features associated with the DNA of bacteria and other microorganisms.

Analytical properties of nucleic acid

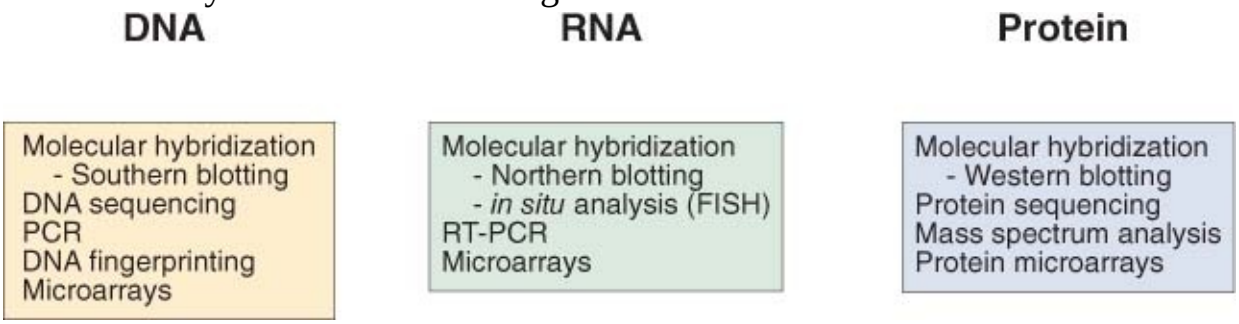
The analytical properties of DNA derive from its chemical structure. These have been used to develop many of the modern protocols for detection/diagnosis often reported in the scientific literature. A DNA molecule has three important analytical features which facilitate its use as a diagnostic target:

- Recognition properties: the base pairing rules in DNA can be used to develop experimental methods, and to detect unique nucleic acid sequences. This approach has resulted in the development of molecular techniques including DNA probe hybridization, DNA sequencing, the polymerase

chain reaction (PCR) and more recently, microarrays. All of these seemingly complex techniques have one simple feature in common: a given DNA sequence (termed a probe or a primer) binds to its complement through base pairing recognition. This binding event is detected through a signal molecule associated with one or other of the binding partners.

- Stability and robust flexibility: the DNA molecule is capable of existing for centuries, even in a desiccated state. For example, DNA has been detected in archeological material taken from Egyptian mummies, and using appropriate isolation methods it can be recovered from many types of preserved specimens. Forensic investigations now make use of DNA-based approaches to provide rapid results.
- Sequence features: when the DNA sequence of any cell is closely examined, one of the key features noted is the existence of repetitive strings of bases in DNA. This is particularly noticeable in the human genome. An example of this is the *Alu* sequences in human DNA. Repeat sequences also occur in animals and microbes. Precisely what roles these repeat sequences play remains to be determined. Nevertheless, repetitive sequences have facilitated the development of analytical approaches to investigate DNA.

Figure 6.1 A summary of modern diagnostic approaches using nucleic acids and proteins as target analytical molecules. The appropriate analytical procedures are determined by the nature of the diagnostic biomarker.



Using these three analytical features, powerful diagnostic protocols have been developed, based on our knowledge of DNA structure. Investigation of humans and animals for disease traits using molecular methods has become a reality. Based on our understanding of the chemical properties of nucleic acid, strategies often encountered include detection methods to identify the presence of an infectious agent, including a bacterium or virus, exploring tissue sections for the presence of cancer markers, forensic analysis for identification purposes and other applications. Collectively, these methods are referred to as molecular

diagnostics.

Several useful molecular methods have been developed, many of which have common features. In Fig. 6.1, the application point for various molecular diagnostic techniques is shown. All of these methods are based on concepts involving replication and transcription (Chapter 9).

Molecular hybridization

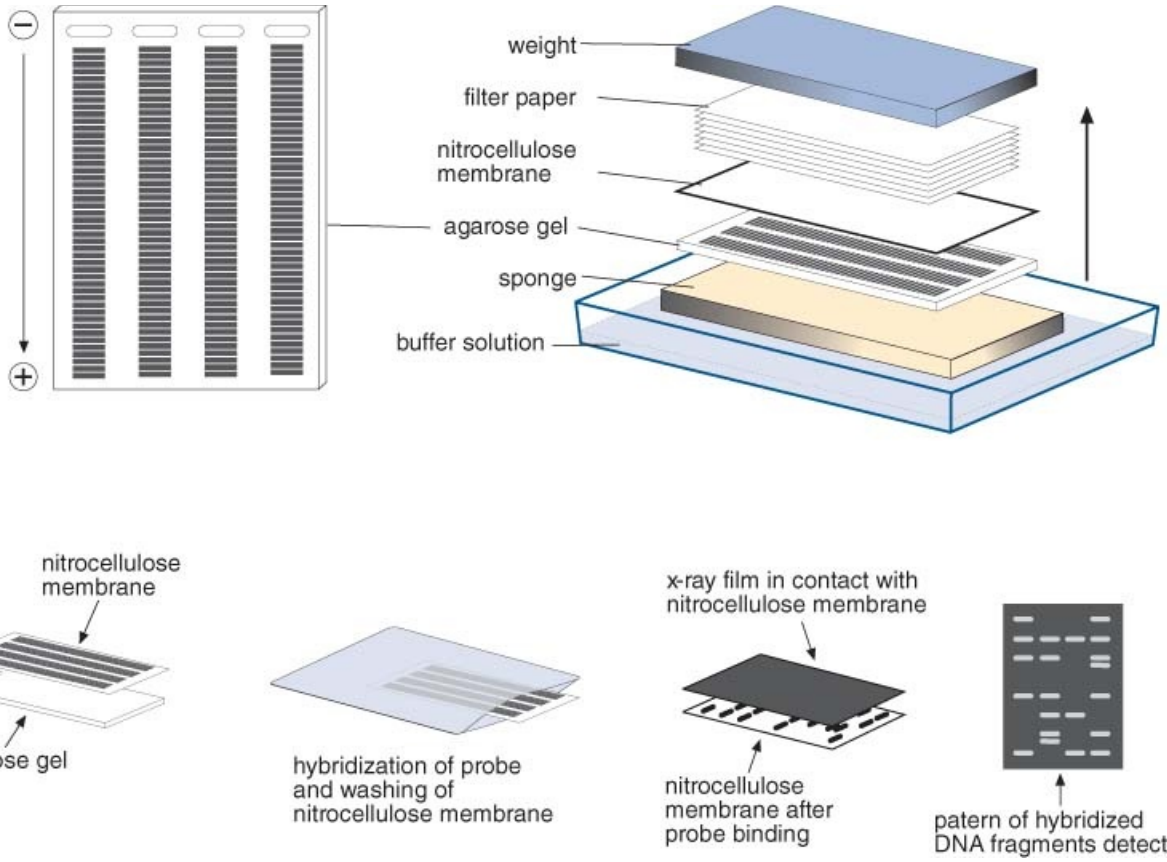
If a DNA (or RNA) molecule is labelled with a marker (such as a radioactive or non-radioactive signal molecule), it constitutes a DNA (or RNA) probe that can be detected. This labelled probe contains a sequence of bases that can find its complement, bind to it through hydrogen bonding and be detected. These are the steps that form the basis for all molecular hybridization methods. Any DNA probe, under suitable experimental conditions, would be capable of binding or hybridizing to its complementary strand in solution (based on established DNA base- pairing rules). It is this binding event that is subsequently detected, depending on the nature of the signal marker employed. Examples of molecular hybridization techniques include Southern and northern blotting (Fig. 6.1).

Southern blotting (Southern, 1975) involves hybridization between a labelled DNA probe (Fig. 6.2) and a previously fragmented DNA template produced following the action of a restriction endonuclease. These enzymes locate and cut at specific sequences in double- stranded DNA. The DNA digest pattern produced is then separated using conventional agarose gel electrophoresis. After the electrophoresis step, the DNA fragment pattern is blotted on to a nitrocellulose membrane, which is later hybridized with the DNA probe. Under stringent hybridization buffer conditions, involving extensive washing of the filter with salt solutions, a high degree of specificity is achieved, allowing the probe to locate and bind to its complementary sequence. Low stringency conditions can be achieved by reducing the salt concentration in the washing solutions. Radioactive isotopes such as ^{32}P or ^{35}S were originally used as labels for DNA probes in earlier versions of this technique, but have now been replaced by non- radioactive affinity labels including biotin and digoxigenin (DIG). Non-radioactive labels can be detected using streptavidin and anti-DIG antibodies conjugated to either horseradish peroxidase or alkaline phosphatase.

Northern blotting is a technique wherein a labelled probe hybridizes to RNA separated by gel electrophoresis similar to that described earlier.

DNA probes can also be chemically synthesized and simultaneously labelled during this process. Use of this approach permits the application of several probes simultaneously, as might occur when the target sequence is not precisely known. An example of a more recent development is the application of fluorescent *in situ* hybridization (FISH analysis). The latter is particularly useful for locating genes on specific chromosomes in a karyotypic array. An older and a technically less challenging strategy is *in situ* hybridization on tissue sections, similar to those used in pathology laboratories. Finally, western blotting is a technique that does not involve nucleic acid, but rather a marked protein (e.g. a monoclonal antibody directed against a denatured protein and labelled with a reporter) binding to a complex mixture of proteins previously separated after electrophoresis through a polyacrylamide gel matrix.

Figure 6.2 The key steps involved in Southern blotting. DNA fragments, previously separated by conventional agarose gel electrophoresis, are blotted on to a nitrocellulose membrane by capillary action. The agarose gel is placed in contact with the nitrocellulose membrane. Individual DNA fragments are drawn through the gel on to the nitrocellulose membrane by a gradient established using dry filter papers and a weight. Once DNA contacts the nitrocellulose membrane, it becomes irreversibly bound. In this process an exact image of the DNA fragmentation pattern in the original agarose gel is transferred. The DNA fragment of interest is identified by applying a radiolabelled probe consisting of a complementary DNA sequence. X-ray film in contact with the nitrocellulose membrane enables detection of the radiolabelled DNA probe.



DNA sequencing

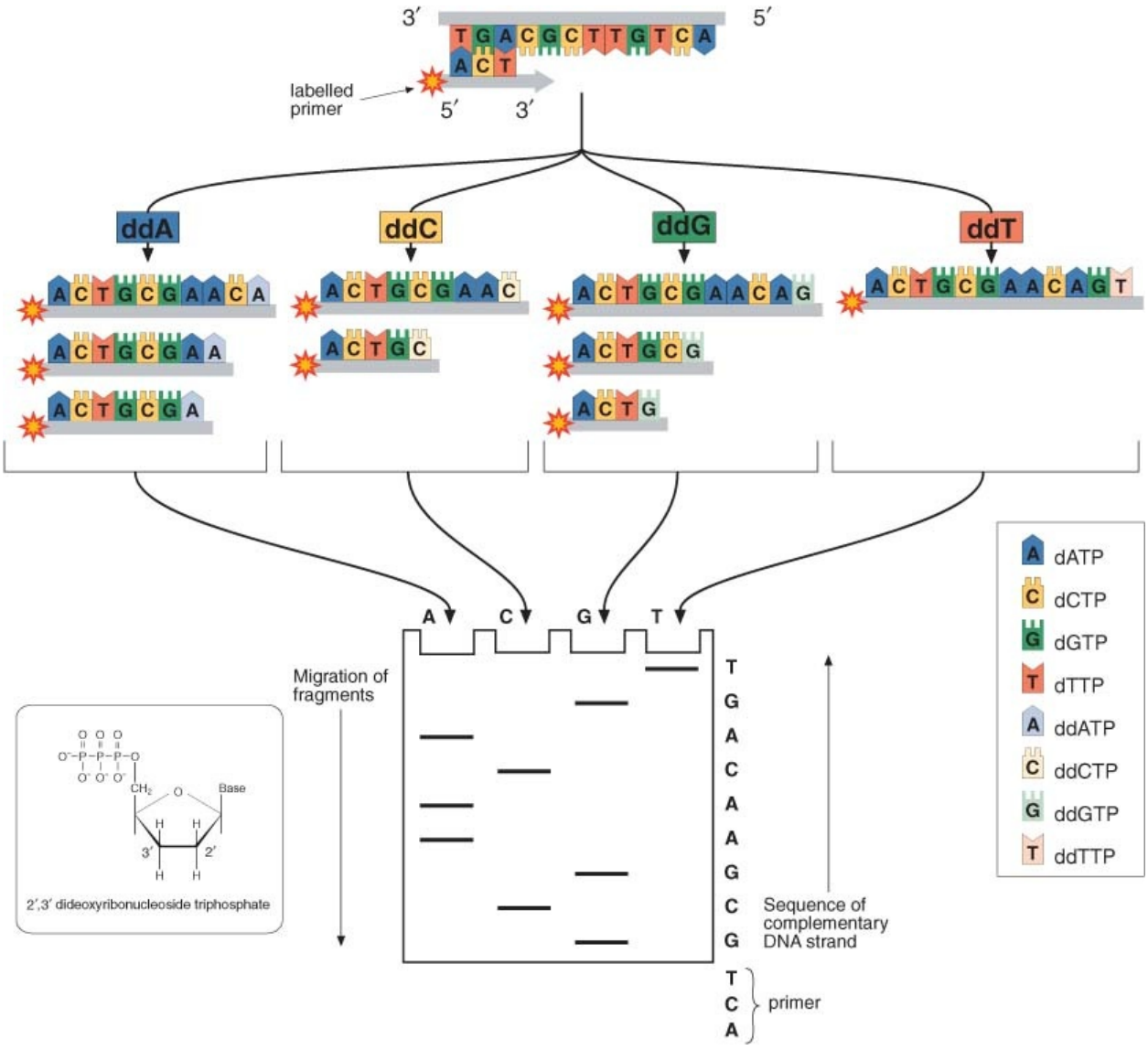
DNA sequencing undoubtedly represents the most powerful analytical/diagnostic approach that exists in the molecular armoury. Insight into the understanding of any DNA molecule derives from its nucleotide sequence. The nucleotide sequence can be used to deduce the primary protein structure of the corresponding protein which can later be compared with similar sequences from other organisms. DNA binding sites and other regulatory features of genes can also be identified.

The DNA sequence of a gene can be determined using either a chemical-based approach, developed originally by Maxam and Gilbert, and/or an enzymatic-based method developed by Sanger. The latter protocol, referred to as dideoxynucleotide sequencing, is the most commonly applied method. Its application on a routine basis has yet to enter mainstream diagnostic laboratories due to the level of technical expertise required. Nonetheless, many research and diagnostic laboratories use DNA sequencing, and increasingly avail themselves of the sequencing services provided by commercial companies.

The technical principles on which modern dideoxy DNA sequencing protocols are based involves the partial replication of a short DNA sequence using all four deoxyribonucleoside triphosphates (dNTP) and a chemically modified dideoxyribonucleoside triphosphate (ddNTP) lacking a hydroxyl group at the 2′ carbon on the ribose sugar ring (inset in Fig. 6.3). Like hybridization, this method is based on sequence recognition according to the base-pairing rules and accurate enzymatic synthesis, which are all features of the naturally occurring replication event. When applied to DNA sequencing (Fig. 6.3), they can be described as follows:

- primer hybridization,
- sequence reaction,
- detection,
- data analysis.

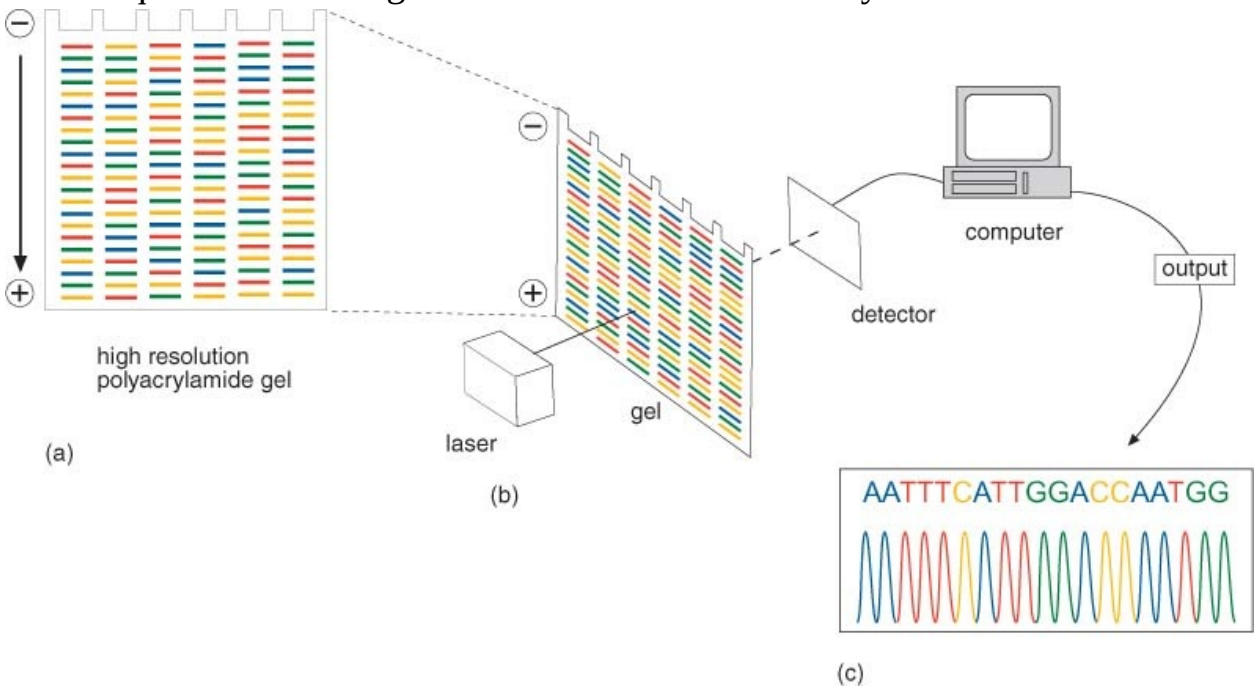
Figure 6.3 The Sanger dideoxyribonucleotide sequencing method. A DNA fragment of interest is hybridized with a short labelled primer sequence to provide a free 3′-OH end on to which the next deoxyribonucleoside triphosphate (dNTP) can be attached. Four independent reactions are set up, one for each of the four dideoxyribonucleoside triphosphate (ddNTP) bases. Using a carefully determined mixture containing dNTP and ddNTP nucleotides (shown in inset), a premature chain termination occurs at each individual nucleotide in the sequence following the incorporation of a dideoxyribonucleoside monophosphate (ddNMP). A set of DNA fragments is produced in each of the four reactions, as shown. These are subsequently separated by polyacrylamide gel electrophoresis. Each reaction mixture is loaded into a separate lane in the gel and DNA fragments are then resolved. The DNA sequence can be read directly from the gel.



The first step involves the annealing of a short primer sequence to the gene or DNA fragment to be sequenced. A short primer [17-mer] provides a free 3′-OH group on to which a given dNTP or ddNTP, specified by the base pairing rules can be added. If the former is incorporated, the chain continues to be extended. Annealing and extension continue on this DNA strand until a ddNTP is incorporated, at which point further chain elongation is inhibited. For each DNA fragment being sequenced, four separate reactions are set up, each of which contains all four dNTPs(dATP, dCTP, dGTP and dTTP) and one of the four ddNTPs (ddATP, ddCTP, ddGTP or ddTTP). The primer is labelled with either a ^{32}P or ^{35}S radiolabel or a fluorescently-labelled reporter group, as shown in Fig. 6.3. Ratios of dNTP to ddNTP are carefully adjusted to ensure that pre-mature chain termination occurs at every single base in the DNA fragment being

sequenced. Enzymatic synthesis of the complementary strand is catalysed by DNA polymerase, and further enzyme activity is arrested at the end of the reaction by the addition of formamide. Each of the four reactions now contains a mixture of nested radiolabelled/non-radiolabelled DNA fragments of differing lengths, all derived from the sequence of interest. These labelled fragments are then separated by high-resolution gel electrophoresis through a poly-acrylamide matrix. Finally, the DNA sequence is directly recorded from these bands after detection/ development of a suitable photosensitive film (e.g. an X-ray film can be used). Using this approach 200–250 bases can be determined.

Figure 6.4 High-throughput DNA sequencing using fluorescent dyes. DNA sequence reactions are carried out as described in Fig. 6.3, using four different fluorophores, each with a unique emission wavelength. Following the completion of the four sequencing reactions, the contents are combined and loaded into a single lane (a) and electrophoresed. The identity of every fluorophore is detected using a laser focused on a fixed position on the gel (b). Each excitation signal is recorded and can be viewed as a series of different excitation peaks with the unique colour identifying one of the four bases (c). The DNA sequence of the fragment is recorded automatically.



In a later development of this technology, fluorescent- based automated DNA sequencing was designed to reduce the manual manipulations involved whilst increasing the sample throughput (Fig. 6.4). This method has revolutionized

whole genome sequencing for both prokaryotic cells and eukaryotes. In automated DNA sequencing, either the bases (dye-terminator chemistry) or the primers (dye-primer chemistry) can be labelled with a fluorescent dye. For the dye-terminator format, the dNTPs are labelled with four different fluorescent dyes, each emitting a narrow spectrum of light when excited by an argon laser in the DNA sequencing instrument. The Stokes shift produced after excitation/emission is subsequently detected and recorded. In a 'four-colour one-lane' format (Fig. 6.4), samples are pooled following the completion of the sequencing reactions as described above, and loaded into a single lane of a separating gel slab or a capillary matrix. An alternative approach using one fluorescent dye is the 'one-colour four-lane' format. In this case, the protocol steps are similar to those described in Fig. 6.3. Data derived from the emissions produced by the dye labels are captured and converted to nucleotide sequence information. Automated DNA sequencing can facilitate the determination of approximately 500 bases per run.

These data are subsequently analysed using one of a number of algorithms, available over the worldwide web. One of the first steps in this case is to attempt to determine the biological function of the sequenced region. This process is termed sequence annotation and is used to ascribe a biological function to the sequence determined. Later, some of the interesting sequence features including DNA binding regions and others can be highlighted.

RNA can also act as a template for sequence, although it is rarely used due to the technical superiority of the DNA-based strategies.

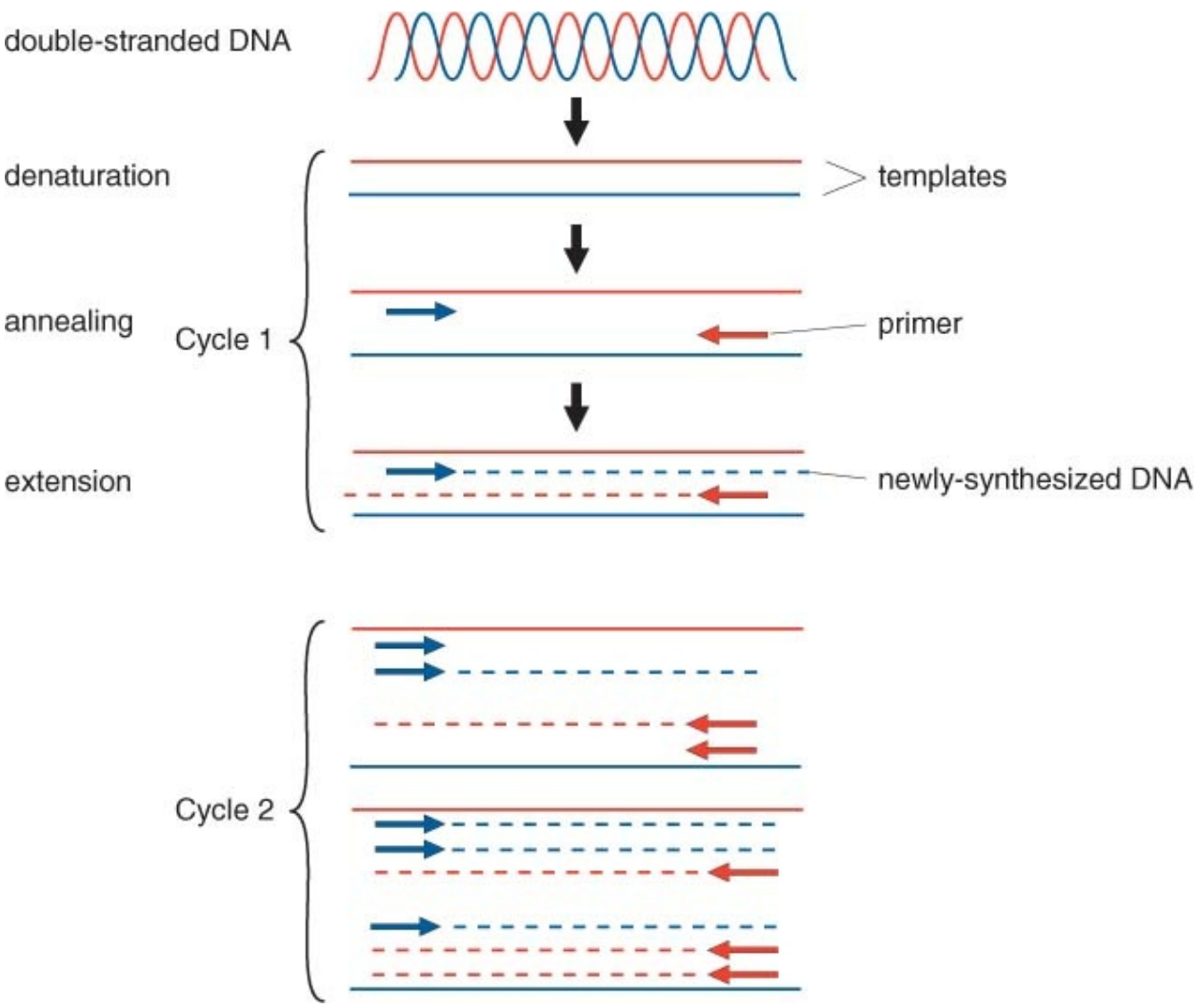
More recent advances in DNA sequencing technology have produced instrumentation capable of sequencing a bacterial genome within hours.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was developed out of the strategies used for DNA sequencing. Most of the steps in a PCR protocol are very similar to those for DNA sequencing. PCR was developed in the mid-1980s and is probably the single most important molecular technique, being applied almost on a routine basis. The typical PCR protocol consists of three repeated steps, resulting in the amplification of a discrete segment of DNA (or RNA, after the addition of a reverse-transcription step, RT–PCR, see below). In the first of these, the template DNA (derived, for example, from a crude preparation of

genomic DNA isolated from a microbial pathogen of veterinary interest or from blood or other tissue samples) is denatured, separating the two DNA strands (Fig. 6.5). This is followed by an annealing step wherein the reaction temperature is lowered allowing two synthetic DNA primers (Fig. 6.5) to bind (hybridize) to the template. These primers are located on opposite DNA strands. Finally, the temperature is increased again (typically to 74°C) and a thermostable DNA polymerase enzyme begins a round of synthesis. Collectively, all of these steps constitute one cycle, and in a conventional PCR reaction up to 30 cycles are carried out. This repetitive switching between temperatures allows amplification of a specific DNA target by up to one million times.

Figure 6.5 Conventional polymerase chain reaction (PCR) consists of three repeated steps, denaturation, annealing and extension. The DNA template purified from a microbial pathogen of veterinary interest is first denatured at a high temperature to separate the two DNA strands in cycle 1. Each DNA strand acts as a template. After lowering the temperature of the reaction, a specific primer (14-base single-stranded DNA fragment) is annealed at a complementary sequence of bases on the template. Using a mixture of deoxyribonucleoside triphosphates (dNTPs), a thermostable DNA polymerase enzyme (usually purified from *Thermus aquaticus*) extends the primer sequence, according to the base pairing rules, to synthesize the complementary strand. The product of the PCR reaction or amplicon is detected using a conventional agarose gel.



A programmable thermal cycler controls the rate of temperature change, the length of incubation at each temperature and the number of times each cycle is repeated. Multiple cycles produce an exponential increase in the amount of amplified DNA, based on the original gene target. The amplified PCR product, or amplicon, can then be detected by conventional agarose gel electrophoresis, stained with ethidium bromide and visualized using UV light.

Conventional PCR-based assays have been developed to detect a broad range of pathogenic agents associated with animals. These include the food-borne zoonotic pathogen *Salmonella*. PCR-based detection methods can also be used to identify the methicillin-encoding gene *mecA* in methicillin-resistant *Staphylococcus aureus* (MRSA), a bacterium increasingly recognized in companion animals. Molecular-based methods using primers directed against the genes encoding the O-antigens located on the surface of bacterial cells can be used to identify the important pathogenic serotypes of *Escherichia coli* including

enterohaemorrhagic *E. coli* (EHEC), such as *E. coli* O157, O111 and other isolates (Murphy *et al*., 2007). Commercial kits are also available for identification of these and other pathogenic organisms.

A potential limitation of DNA-based diagnostic methods is that they detect both viable and non-viable cells. This limitation can be overcome either by using an enrichment step before nucleic acid extraction or by performing an RNA-based detection method using reverse transcriptase (RT) mediated PCR in a protocol known as RT–PCR. As RNA is produced only in living cells, only viable cells are detected. RT–PCR differs from DNA-based PCR by the addition of a preliminary early step involving the enzymatic conversion of RNA into a DNA copy of the original template (cDNA) in a reaction catalysed by RNA-dependent DNA polymerase (reverse transcriptase). RT–PCR assays can be used to detect RNA viruses such as rotavirus, coronavirus and norovirus.

Real-time PCR

In contrast to conventional PCR, detection and simultaneous quantification of amplicons in real time is an important enabling technology in molecular diagnostics. The method facilitates the determination of the absolute numbers of a DNA target relative to a normalized gene within a living cell. Quantitative realtime PCR (qPCR) can be used to quantify bacteria, other microorganisms and individual genes.

Real-time PCR has gained wide acceptance due to its improved speed, sensitivity and reproducibility and the reduced risk of carry-over contamination. The real-time PCR instrument can be regarded as both a thermal cycler and a real-lime fluorescence detector, thereby facilitating the performance of automated procedures in a closed system. Real-time PCR uses fluorescence to detect the presence or absence of a particular DNA or RNA target. It is this detection process that differentiates real-time from conventional PCR.

A number of dye-labelling methods are currently used for real-time PCR and these can be broadly categorized as non-specific or specific. SYBR green I is an example of a non-specific intercalating dye that binds to double-stranded DNA. As the amplification reaction proceeds and PCR products accumulate over time, more dye is bound and the fluorescent signal increases. Specific dye labelling includes the use of *Taq* Man probes, molecular beacons and sunrise probes (Table 6.1). A *Taq* Man DNA probe is labelled on both ends with a fluorophore

reporter that emits a fluorescent signal, and a quencher dye that absorbs the emitted radiation. In the unhybridized form the emitted fluorescence is quenched due to the proximity of the two dye labels. When hybridized to the amplification product, the probe is hydrolyzed by the 5′-3′-exonuclease activity of *Taq* DNA polymerase, resulting in the separation of the two fluorescent labels and an increase in the fluorescent signal (Fig. 6.6).

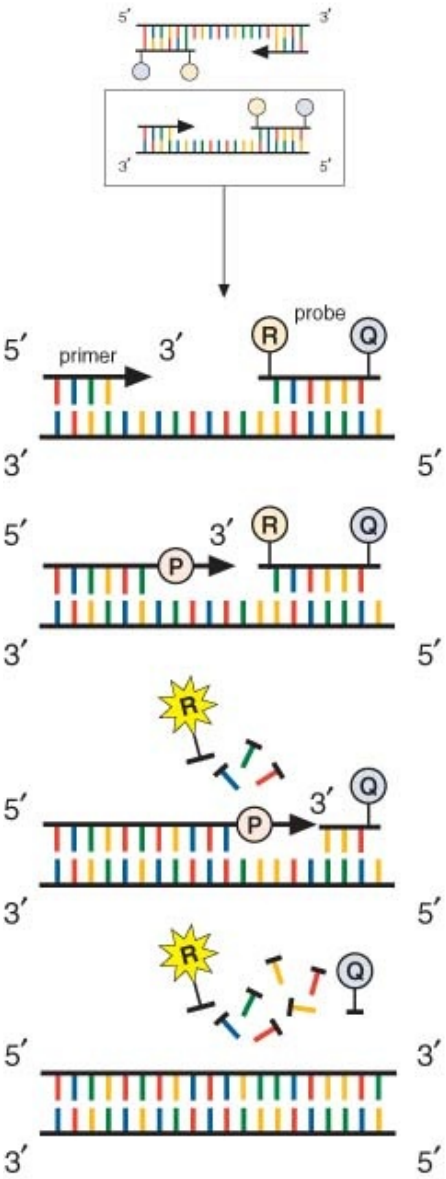
As the numbers of amplicons increase during a realtime PCR reaction, the fluorescent signal produced (using either of the dye labelling systems outlined above) also increases. By plotting the fluorescent signal (in arbitrary units) against the cycle number (Fig. 6.6), the relative DNA concentrations can be determined. A threshold value denoted as C_T is used to differentiate fluorescent signals produced from biological samples from that emitted by the background. Plotting $\log_{10}$ values of C_T against concentration produces a straight line (Fig. 6.6) and this facilitates the relative quantification of gene expression. This method can also be used to determine numbers of bacterial cells in a biological sample.

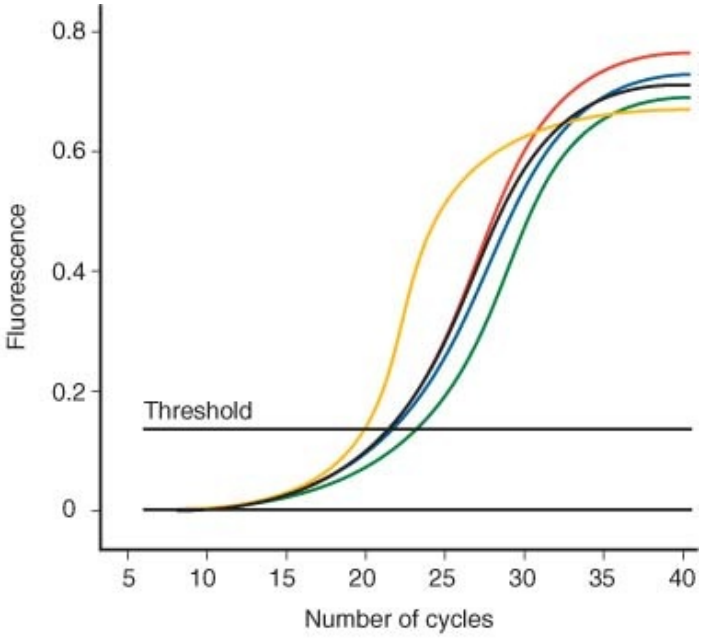
Table 6.1 Types of fluorescent dye labelling methods and hybridization probes used in quantitative real-time PCR.

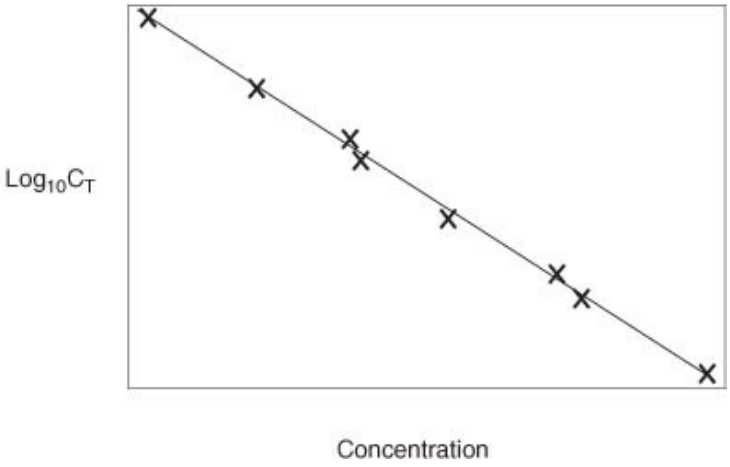
Detection chemistry	Type	Multiplex capability
DNA binding dye	SYBR green I (two primers)	no
Hybridization probe	*Taq*Man (two primers and one probe)	yes
Molecular beacons	Quenched probe (two primers and one probe)	yes
Sunrise primers	Quenched primer (two primers)	yes

Figure 6.6 Real-time PCR uses fluorescence for detection of PCR products. This method does not require an agarose gel for analysis of the reaction. Detection is accomplished in the real-time PCR instrument using fluorescence spectrophotometry. Two primers are shown together with a specific DNA probe labelled with a fluorescent reporter (R) and a quenching (Q) dye. When the two dyes are in close proximity, fluorescence is undetectable. A range of different probe designs are used (Table 6.1). As the amplification reaction proceeds, the 5′-3′-exonuclease activity of *Taq* DNA polymerase (P) degrades the probe, resulting in the separation of the two dyes, which allows the reporter dye to fluoresce. As the number of cycles increases, recorded fluorescence also increases as shown. A threshold limit is set, referred to as the crossing threshold (C_T), above which a sample is recorded as positive. By plotting $\log_{10}C_T$ values against concentration, a linear plot is obtained. Real-time PCR is quantitative

and can be used to determine the number of infectious agents in a sample or the DNA concentration.







Expression of any gene in a microbe or other cell can be determined by measuring the mRNA transcription using RT–PCR. To determine the level at which a gene of interest is expressed, mRNA transcribed under defined conditions, such as at the mid-logarithmic phase of growth, is measured and compared with a second gene, such as 16S rRNA, the expression of which is independent of the growth phase. The relative amount of mRNA produced from the gene of interest is then compared with that transcribed from 16S rRNA in the

same sample. The latter gene is regarded as a housekeeping gene and, by normalizing expression of the gene of interest to 16S rRNA, quantitative measurements of gene expression can be made. This technique is referred to as quantitative RT–PCR (qRT–PCR).

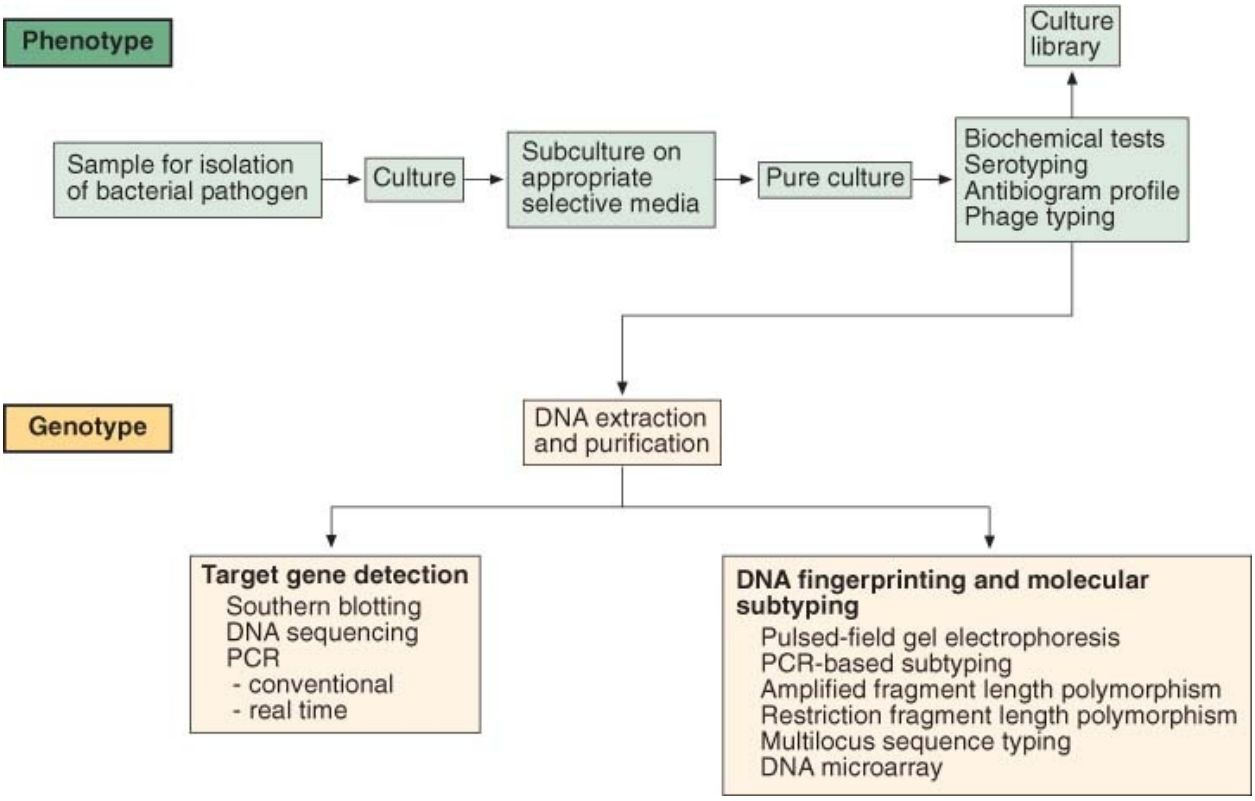
The most impressive feature of PCR technology is its ability to generate discrete DNA fragments from complex genomic backgrounds, including humans and animals. This feature makes PCR an attractive alternative to more traditional diagnostic methods. Taken together with additional technological innovations, amplification protocols based on PCR are becoming more common (O'Regan *et al*., 2008). Commercial kits are now available based on this protocol to detect and quantify a range of pathogenic organisms relevant to veterinary medicine.

Diagnostic approaches to surveillance and subtyping of bacteria, a comparison of phenotyping and genotyping methods

Characterization of a veterinary pathogen is essential for the support of epidemiological investigations of a disease outbreak. Laboratory methods used must be capable of identifying those organisms linked to the outbreak while excluding isolates unrelated to the outbreak. Conventional laboratory approaches are summarized in the top portion of Fig. 6.7. These methods include biochemical identification, a method that discriminates between isolates based on specific biochemical reactions and is usually capable of identifying strains only to the species level; serotyping applies specific antisera to detect surface O-antigens associated with lipopolysaccharide or H-antigens among members of the *Enterobacteriaceae*. This approach has been very useful for providing information on certain bacterial serotypes linked with clinical syndromes, such as the association between *E. coli* O157:H7 and haemolytic uraemic syndrome (HUS). Antibiogram profiling is used for determining the susceptibility profile of a bacterial isolate against a panel of antimicrobial compounds. Phage typing is also used in some disease outbreaks, in particular with the serovars of *Salmonella* Enteritidis and Typhimurium, to further identify isolates. This diagnostic method suffers from poor reproducibility and the necessity to maintain stocks of bacteriophages that require constant quality control. Furthermore, some isolates lack the necessary phage surface receptors rendering

them untypable by this method.

Figure 6.7 Steps involved in the characterization of a bacterial isolate prior to molecular investigation. Although this procedure relates to a bacterial pathogen, the methods used can be suitably modified for other infectious agents.



Whilst some phenotype-based methods, such as serotyping, have been used successfully, many are not universally applicable and their use is limited to the species for which they were originally developed. Moreover, variable gene expression, resulting from the loss or gain of genes, can alter the phenotype leading to misidentification. These factors limit the reliability of phenotype-based identification for surveillance, and furthermore these methods suffer from poor discriminatory ability.

The rapid development in molecular-based approaches has led to the design of new diagnostic protocols that are independent of the inherent limitations of traditional methods (Fig. 6.7). Molecular subtyping methods target variation within the genomes of bacteria whilst reducing the limitations encountered with more conventional phenotyping approaches.

Molecular subtyping for definitive

identification of bacteria

Phenotype-based methods facilitate the identification of bacteria such as *Pseudomonas aeruginosa* or *Listeria monocytogenes* to the genus and/or species level. For bacteria such as *Salmonella*, these methods are important for identification of a specific serovar such as Enteritidis, Typhimurium or other members of the 2,500 known serovars. In the case of pathogenic *E. coli*, O-serotyping defines the organism specifically as O157, O111 among over 200 other O-serotypes. Bacterial subtyping permits the identification of a bacterium below the species level and provides the means of tracking an organism, describing its molecular epidemiology and defining its transmission routes. This modern analytical approach provides a more refined identification of a bacterium based on its DNA fingerprint, and facilitates the recognition of over 600 different *E. coli* O157:H7 subtypes and more than 1,800 *Salmonella* Typhimurium subtypes as recognized by the Centers for Disease Control and Prevention (CDC) PulseNet network (Swaminathan *et al*., 2001).

Bacterial subtyping is important as it can be utilized to support quality control in food manufacture where beneficial microorganisms (probiotics) are deliberately added to food and drinks for health-promoting purposes. It also has applications in tracking the source of bacterial contamination in food manufacturing. Using bacterial subtyping methods (Table 6.2), food-borne cases of illness and outbreaks of infectious disease may be tracked through the application of DNA fingerprinting to definitively identify the aetiological agent involved, thereby linking veterinary medicine with public health. Transmission routes of MRSA between humans and animals have also been identified by subtyping isolates and comparing their DNA fingerprint patterns (O'Mahony *et al*., 2005; Strommenger *et al*., 2006). In general, there are three approaches to subtyping including restriction fragment length polymorphism (RFLP) analysis, PCR-based amplification of conserved repetitive sequences in bacterial genomes and DNA sequencing. No single subtyping method is routinely used by all laboratories and each method has its own advantages and limitations. The choice of method is influenced by the subtyping information required and the equipment available.

Table 6.2 Molecular-based subtyping methods used for tracing bacterial pathogens associated with disease outbreaks in animal and human populations.

Sequential development of analytical methods	Molecular basis of subtyping methods

First-generation methods	Plasmid DNA profiling Restriction digests of purified plasmids
Second-generation methods	Restriction endonuclease digestion of total DNA (including chromosomal and plasmid) Ribotyping
Third-generation methods	Pulsed-field gel electrophoresis (PFGE)
	PCR-based amplification:
	– *Rapid amplification of polymorphic DNA (RAPD)*
	– *PCR–RFLP analysis of conserved genes (flaA, recN* and others)
	– *PCR ribotyping*
	– *REP–PCR*
	– *ERIC–PCR*
	– *BOX–PCR*
	– *AFLP*
Fourth-generation methods	Multilocus variable-number tandem repeat analysis (MLVA)
	Multilocus sequence typing (MLST)
	DNA sequencing

Plasmid profiling

This original method involves the purification of all plasmids from a bacterium followed by their separation on an agarose gel where the plasmid profile can be compared in a collection of bacterial isolates. It is a relatively easy technique to perform and was used successfully to identify different serovars of *Salmonella*. Application of this method may be limited, however, as only a small percentage of some bacteria such as *Campylobacter* may contain plasmids. Nevertheless, plasmid profiling is important when characterizing genetic markers associated with antibiotic resistance.

Restriction endonuclease analysis (REA)

Total genomic DNA, including the chromosomes and plasmids, is purified and subjected to enzymatic digestion with a restriction endonuclease. The latter enzyme cleaves at specific recognition sites in DNA, producing a multi-band pattern or restriction fragment length polymorphic pattern detected after electrophoresis through an agarose gel. The RFLP pattern produced is often too complex to serve as a fingerprint and is difficult to analyse, limiting the utility of this subtyping method. Moreover, plasmids initially present in a strain may later be lost, altering the banding profile and complicating comparison of strains.

Ribotyping

Ribosomal-encoding DNA genes (rDNA) are naturally amplified in bacteria and have been used successfully as a target for identification. Large portions of these genes (the *rrs* genes code for 16S rRNA and the *rrl* genes code for 23S rRNA) have been conserved throughout evolution. In this procedure chromosomal DNA is purified and digested with a suitable restriction enzyme, then Southern blotted as outlined previously (Fig. 6.2), before hybridization with a species-specific rRNA probe. The pattern of fragments detected is referred to as the ribotype. Since these genes are highly conserved, pathogens can be identified using appropriately labeled 16 and 23SrRNA probes. These can be designed for any organism(s) whose sequence is known. Ribotyping has been used extensively for identifying a range of pathogens including food-borne pathogens such as *Campylobacter jejuni*, *Salmonella* and other pathogenic bacteria.

This analytical method is labour-intensive and time- consuming. Its power of discrimination is poor compared with other subtyping approaches. An automated ribotyping system was developed that provides excellent reproducibility and is capable of typing all organisms, making standardized ribotyping more accessible. However, the cost per sample is high and this laboratory protocol may need to be used in conjunction with another subtyping method.

Pulsed-field gel electrophoresis (PFGE)

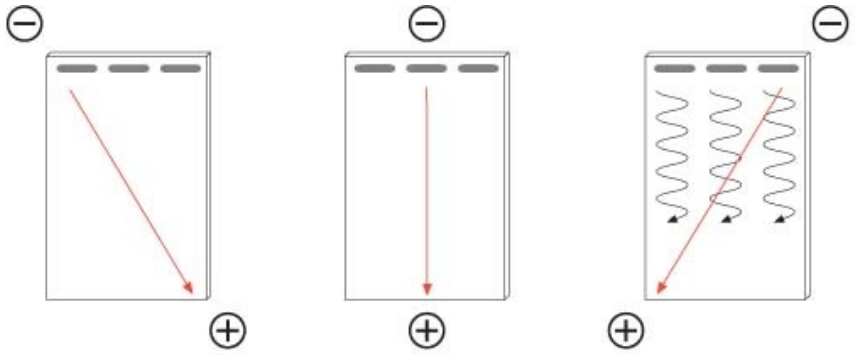
A major limitation with REA-based methods is the complexity of the fragmentation patterns generated, making them difficult to analyse. Macrorestriction analysis can overcome this limitation via the digestion of the bacterial chromosome using rare cutting restriction endonucleases. These enzymes cut chromosomal DNA into a small number of very large DNA fragments, which can be resolved using specialized electrophoresis equipment. Pulsed-field gel electrophoresis (PFGE) is regarded as the gold standard in molecular subtyping (Fig. 6.8). Bacterial cells to be subtyped are prepared by embedding in agarose plugs to prevent shearing of the DNA. Chromosomal DNA is then purified in these plugs following lysis of the bacterial cell wall and digestion of cellular proteins with a protease enzyme. Cell debris is then removed by washing, and the plugs containing the chromosomal DNA are now ready to be enzymatically digested. A thin slice is cut from the agarose plug and incubated at an optimal temperature in a suitable buffer containing a rare cutting

restriction enzyme (e.g. Xba1 for *Salmonella*). Following enzyme digestion of the chromosomal DNA, the digested DNA fragments are resolved according to size in an electrical field in which the orientation of the field is changed in a pulsed manner (Fig. 6.8). This protocol causes the DNA fragments to change their orientation in response to the direction of the electrical field such that larger DNA fragments reorient themselves more slowly compared with smaller digested fragments. The resulting macrorestriction PFGE profile produced consists of a small number of digested DNA fragments ranging in size from 10 to 900 kbp depending on the bacterium involved and the restriction enzyme used. Some examples of rare cutting enzymes and the bacteria subtyped using them are shown in Table 6.3.

PFGE is a highly discriminating subtyping protocol. Since its development in the mid-1980s it has been successfully applied to subtype several bacteria and used to determine the genetic relationships between case-related and unrelated isolates. As the method is relatively simple to perform, standardizing the technical elements would facilitate the comparison of PFGE profiles between laboratories nationally and internationally. PulseNet (www.cdc.gov/pulsenet) is an example of a globally standardized and operated PFGE-based subtyping network used to track food- borne pathogens across countries and continents. Following the development of the PFGE profiles as outlined above, the gel images of the DNA fragmentation patterns are digitized and converted to a TIFF file format. (All of these steps can be completed within 24 hours.) The TIFF file is then exported to a dedicated software program which analyses the PFGE profiles, comparing them against other similar profiles in a constantly expanding DNA fingerprint database. As the system is operated under strictly standardized conditions, these files can be exchanged between laboratories in different regions of the world. This approach is often used to track the dissemination of food-borne bacteria linked to an outbreak of disease.

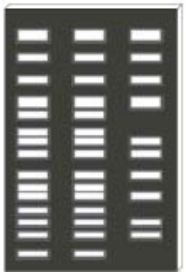
Figure 6.8 Analysis of the genome of *Salmonella* Dublin is illustrated. The presence of all Xba1 sites is determined using a computer program available over the worldwide web [http://insilico.ehs.es], followed by analysis using pulsed-field gel electrophoresis (PFGE). Total DNA of *Salmonella* Dublin is isolated and embedded in agarose plugs to prevent shearing of high molecular weight molecules. All preparatory steps are performed in these plugs. Xba1 is added to the purified DNA and the fragmentation pattern produced is resolved using a pulsed electrical field. The pulsed electrical field alters the direction of

the current, thereby causing DNA fragments to realign. Larger fragments realign more slowly than smaller DNA fragments, thereby facilitating the separation of very large (greater than 20 kbp) DNA fragments. On the basis of the pulsed-gel results, one of the three isolates has a profile distinct from the other two. These profiles are sometimes called pulsotypes. In the illustration shown, three different *Salmonella* Dublin isolates are digested with Xba1, one of which is CT_02021853.



Computational analysis of *Salmonella* Dublin CT_02021853 genome sequence to locate the Xba1[T'CTAG'A] restriction sites

Cleavage position	Length of sequence	Length of sequence (sorted)	Pulsed-Field Gel Electrophoresis
5097	5099	726639	
105107	100010	652995	
592107	487000	487000	727.5 kB
1001919	409812	409812	
1243266	241347	326283	
1535455	292189	296410	485.5 kB
1650799	115344	292189	
1977082	326283	246771	388.0 kB
2223853	246771	241347	
2301855	78002	191993	291.0 kB
2303058	1203	179839	
2956053	652995	167143	
2962569	6516	144688	194.0 kB
2983561	20992	115344	
3150704	167143	110443	
3295392	144688	100010	
3475231	179839	78002	97.0 kB
3771641	296410	42190	
3963634	191993	20992	
4074077	110443	6516	48.5 kB
4116267	42190	5099	
4842906	726639	1203	



Although relatively simple to implement, the PFGE subtyping method does

suffer from a number of limitations. These include the need for expensive specialist equipment. The method is also labour-intensive and cannot be automated.

PCR-based subtyping methods

Several PCR-based subtyping methods have been developed. In general these methods are simple to carry out and can be applied to any genome. Some of

Table 6.3 PFGE-based subtyping bacteria and commonly used rare cutting restriction endonucleases.

Bacterium	Rare cutting restriction endonuclease
Campylobacter jejuni	Sma1; Kpn1
Clostridium botulinum	Sac2
Escherichia coli O157:H7	Xba1; Bln1
Listeria monocytogenes	Asc1; Apa1
Vibrio species	Not1; Sfi1
Salmonella species	Xba1; Bln1
Staphylococcus aureus	Sma1

the approaches are shown in Table 6.2. A brief outline of four methods is given below.

Random amplification of polymorphic DNA (RAPD)

RAPD, also known as Arbitrarily Primed PCR (AP- PCR), was one of the first examples of PCR-based subtyping described. This method does not require any prior knowledge of the organism's DNA sequence. It uses a single short (10-mer) random primer and sub-optimal low stringency annealing temperatures in a PCR reaction to generate a DNA fingerprint. It is a relatively simple and rapid protocol, producing highly discriminating DNA fingerprints. Nevertheless, the method suffers from a lack of reproducibility, making the development and standardization of inter-laboratory protocols difficult. For this reason, RAPD has not gained widespread acceptance as a reliable subtyping protocol.

PCR-RFLP analysis

This approach can be applied to a gene target that exhibits a high degree of polymorphism and therefore can be used to discriminate between bacterial isolates. An example of this is *flaA* subtyping used to subtype *Campylobacter*

jejuni isolates. In this example, the gene of interest, the flagellin A subunit encoding *flaA*, is amplified by PCR (Corcoran *et al*., 2006). The amplified PCR product is then subjected to digestion using a suitable restriction endonuclease, in this case Hinf1, producing an RFLP profile. The RFLP profile is then used to compare different isolates of the same bacterium. Other targets used include 16S, 23S rRNA and the interspatial region 16-23S rRNA, *fliC* for *Escherichia coli* O157 and the *coa*-encoding coagulase gene in *Staphylococcus aureus*.

As the method focuses on the polymorphism associated with one gene to the exclusion of the remainder of the bacterial genome, its power of discrimination is limited. Furthermore, this approach is only suitable to describe epidemiological links in a specific temporal setting such as time and place and would therefore not be a suitable tool to assess the molecular epidemiology in a global setting or in a longitudinal study.

REP–PCR

Bacterial genomes contain several examples of repetitive sequences throughout their genomes. Examples of common repeats include the 38-bp repetitive extragenic palindromic sequence (REP), the 126-bp enterobacterial repetitive intergenic consensus (ERIC) and the 158-bp BOX repeat sequences (Table 6.2). REP– PCR makes use of the nucleotide sequence conserved within the repeat sequence to facilitate the design of primers located towards the extremities of these repeats and which amplify the DNA regions located between the repeats (Fig. 6.9). REP, ERIC and BOX repeats differ in their location and copy numbers between bacteria, making them attractive targets with which to discriminate between different bacterial species.

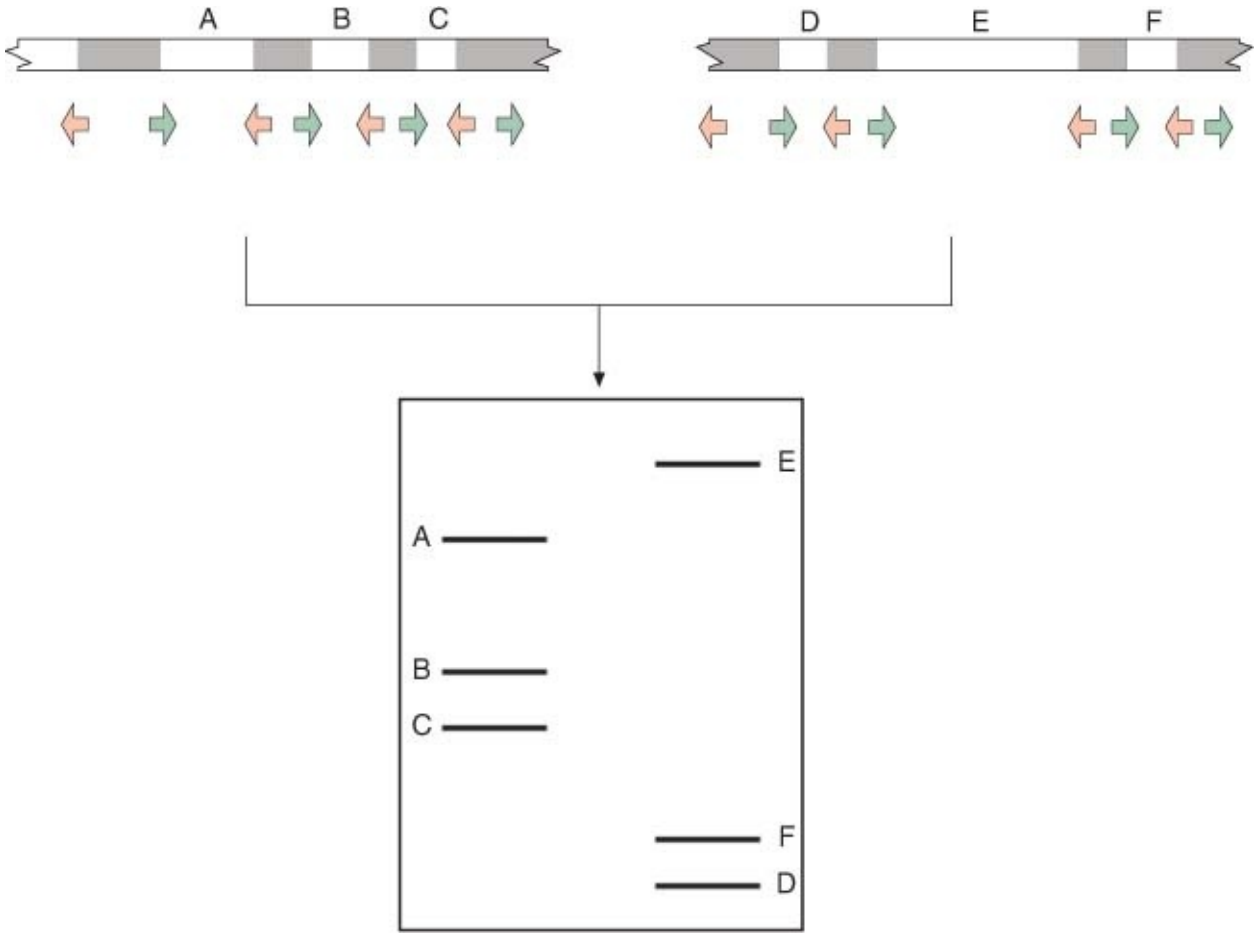
Unlike RAPD, REP–PCR is performed under high- stringency annealing conditions. This feature increases the possibility of standardizing this protocol. An automated gel-free REP–PCR assay has been developed for *Salmonella*. In this example, a chip is used in place of an agarose gel to resolve amplicons after REP–PCR. Associated software then converts the DNA profiles into digitized formats as before and enters them into a strain database which can be used to evaluate existing and new patterns generated using standardized reaction conditions.

Amplification fragment length polymorphism (AFLP)

Originally developed for and applied to plant genetics, AFLP has been adapted

for use in bacteria. The method does not require any prior knowledge of the target bacterial genome sequence. Bacterial genomic DNA is first digested with one or more restriction endonucleases to which synthetic short oligonucleotide adaptors of known sequence are attached using the cohesive ends generated by the restriction enzyme. This forms the sites to which adaptor- specific DNA primers are annealed and used to amplify the adaptor-ligated DNA fragment. A complex pattern of DNA fragments is produced following PCR, ranging in size from 50 to 100 bp with between 40 and 200 bands that are resolved by conventional agarose gel electrophoresis. In a more recent development a fluorescent label can be attached to the adapter, being subsequently incorporated into the amplicon and facilitating its resolution and detection using an automated DNA sequencer. The latter method is referred to as fluorescent (F)-AFLP. DNA fragment profiles can be managed as described earlier and used to compare with other bacterial isolates.

Figure 6.9 Segments of chromosomal DNA from two different bacterial isolates showing the distribution of repetitive extragenic palindromic (REP) elements (shaded boxes). Purified DNA is amplified with two REP-specific DNA primers (indicated by arrows), which bind and amplify the regions located between the repeats as shown. The resulting amplification profile for each isolate is illustrated.



Multilocus sequence typing (MLST)

Following technical advances in automated DNA sequencing allied to the associated falling costs, DNA- sequencing-based subtyping has emerged as a new approach to distinguish isolates of the same bacterial species. Multilocus sequence typing (MLST) is an example of such an approach wherein chromosomal DNA is purified and short segments of seven housekeeping genes are amplified prior to sequencing (Fig. 6.10). The genes used for MLST analysis all encode protein products essential to bacterial viability, and are therefore subject to selective pressure. These DNA sequences are then compared. Based on the sequence differences or polymorphisms detected, each unique sequence is termed an allele and is identified by a unique sequence type (ST) number. ST numbers associated with these loci are then used to compare isolates and infer genetic relationships. Bacterial isolates can be identified by a string of ST numbers, and for two individual bacterial isolates where the same ST number strings occur these isolates are defined as being indistinguishable by this method.

MLST profiling is a very sensitive method that can be used to distinguish between species within a genus including *Campylobacter jejuni*. The method may also be used to distinguish commensal *E. coli* isolates from pathogenic variants of the same genus such as serotype O157. Recently, this approach highlighted a human-to-poultry adaptation event involving *Staphylococcus aureus* (Lowder *et al*., 2009). The latter study illustrated the importance of subtyping to describe the impact of globalization on the emergence of a pathogen of veterinary and public health importance.

MLST protocols have been described for a variety of important veterinary pathogens. The method is easily standardized, and detailed protocols have been described and can be found at www.mlst.net. For example, seven primer pairs are used to amplify housekeeping genes in *Streptococcus zooepidemicus* and the sequences of these together with a complete list of each set of alleles are contained in this database. Data entries are regularly updated to include new isolates.

Figure 6.10 Illustration showing the steps involved in the determination of a multilocus sequence type (MLST) profile for a bacterial isolate. Clustering of different sequence types is shown in colour.

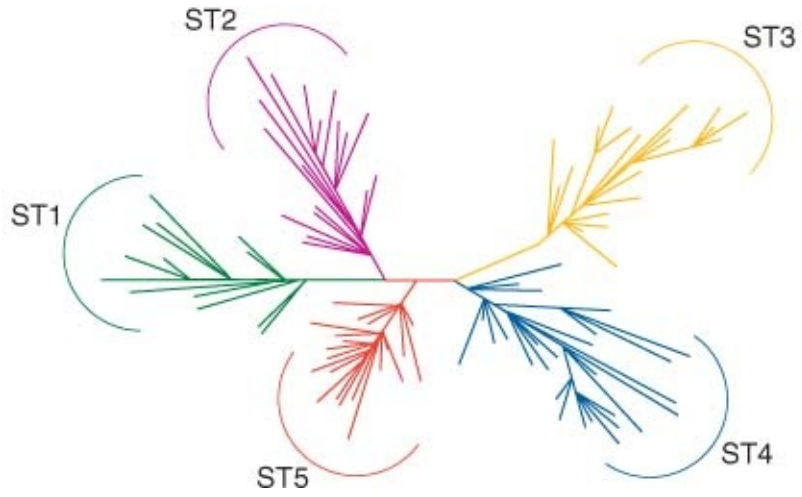
Seven genes, either housekeeping or essential, are selected for sequence analysis

⬇

Following PCR, the DNA sequence of a limited portion of each gene (300-400 bp) is determined

⬇

Sequences are aligned and compared with known DNA sequences from other bacteria of the same genus and species and allele numbers and sequence types (ST) are assigned



DNA microarray technology

Unravelling the complex details arising from the interaction of a veterinary pathogen and its host is a key step towards extending our understanding of the basis of infectious disease. In this way, important pathogen-associated virulence markers can be identified along with the delineation of the manner in which the host cell responds. In the future this approach could lead to the development of novel drugs.

Early examples of various hybridization techniques have been outlined previously (Figs 6.1 and 6.2) and these can be used to facilitate the identification of a specific DNA fragment (Southern blotting) or an mRNA molecule (northern blotting) containing a gene of interest. Modified hybridization protocols along with DNA sequencing and RT–PCR can be used to identify and measure levels

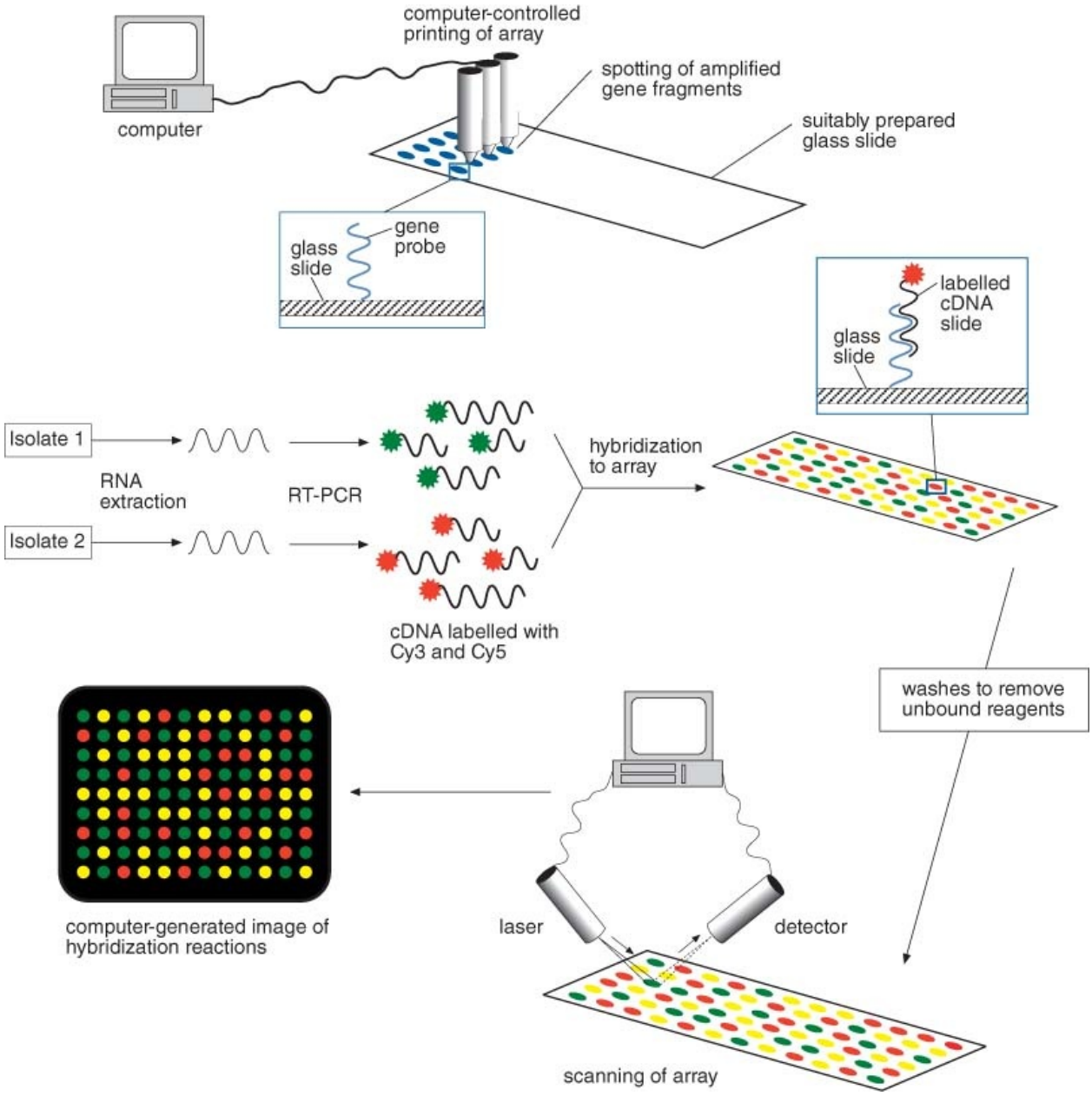
of gene expression under carefully defined growth conditions. In each of these examples, only limited information can be obtained. In contrast, by measuring the total response of a bacterial cell in which genes are up- and down-regulated, an assessment of the global changes occurring in gene expression across the genome can be obtained. In this way, transcriptional signals following the growth of a bacterium under different conditions can be determined. Similarly, genes involved in infection can be identified following the isolation of mRNA and hybridization to a DNA microarray containing the known genes from a particular bacterial pathogen.

The development of DNA microarrays is based on the use of a solid support to which a series of genes or chemically synthesized segments of those genes from a bacterium or any other organism of interest is attached. A technique called photolithography was adapted to produce silica-based microarrays which, depending on the density of the array, may contain thousands of DNA probes. As an example the *Salmonella* DNA microarray in use contains 4,500 individual DNA fragments.

The genes on the microarray may represent either complete amplicons obtained by PCR (Fig. 6.5) or synthesized oligonucleotides containing part of a gene's open reading frame (ORF). In order to analyse the transcriptome of any organism, the array must contain each gene identified following the determination of the complete genome sequence. Usually each gene is spotted more than once on to the array and at different locations as a control measure. Once attached to the solid-phase support, mRNA purified from a bacterial cell being studied can be hybridized and DNA fragments or probes on the microarray that bind to these mRNA molecules can be identified. Hybridizing signals represent the set of genes that are expressed in the bacterial cell, cultured under defined conditions (Fig. 6.11).

Figure 6.11 Illustration showing the key steps involved in DNA microarray analysis. Preparation of the DNA microarray involves selection of genes for inclusion on the array. Selected genes are amplified by PCR or synthesized chemically and are fixed in position at specific locations on an array slide. RNA is extracted from two bacterial isolates. cDNA is synthesized using an RT–PCR reaction. The cDNA from each isolate is labelled with a unique fluorescent dye as shown. The labelled cDNAs are combined and then hybridized to the array. Fragments of the labelled cDNA with complementary sequences bind to specific probes on the array. The pattern of binding is subsequently detected by scanning

the array slide to detect fluorescence. Where only one of the two labelled cDNA binds, the corresponding fluorescent signal is detected. At positions where both probes bind, a signal based on a combination of both fluorescent dyes is detected.



In bacteria, mRNA has a short half-life and is usually produced in only the required amounts under specific growth conditions. To use mRNA to probe a DNA microarray, the mRNA must be amplified in a RT–PCR reaction and labelled with a fluorescent dye (Fig. 6.11). Once the hybridization step is completed, any mRNA- derived labelled amplicon is subsequently detected by

scanning the DNA microarray and analysing the pattern of fluorescent spots with dedicated computer software. The intensity of the fluorescent signal obtained gives a quantitative measure of the expression of each gene. Genes that are up- and down- regulated are identified and clustered into functional groups. Expression of the genes identified on the microarray can later be verified by qPCR under the defined conditions used.

DNA microarrays can be used in several ways. The arrays can provide useful information for identifying those genes controlling growth of an organism under defined growth conditions including aerobic *versus* anaerobic metabolism. In environmental microbiology, DNA microarrays containing 16S rRNA sequences can be used to identify bacterial and other microorganisms present in a particular environment. This DNA microarray is termed a phylochip. Comparative genome analysis makes use of DNA microarrays to compare the gene index of different serovars of *Salmonella* (Reen *et al*., 2005). DNA chips have been developed to aid in the simultaneous identification of a number of important pathogens including bacteria and viruses that may share similar niches.

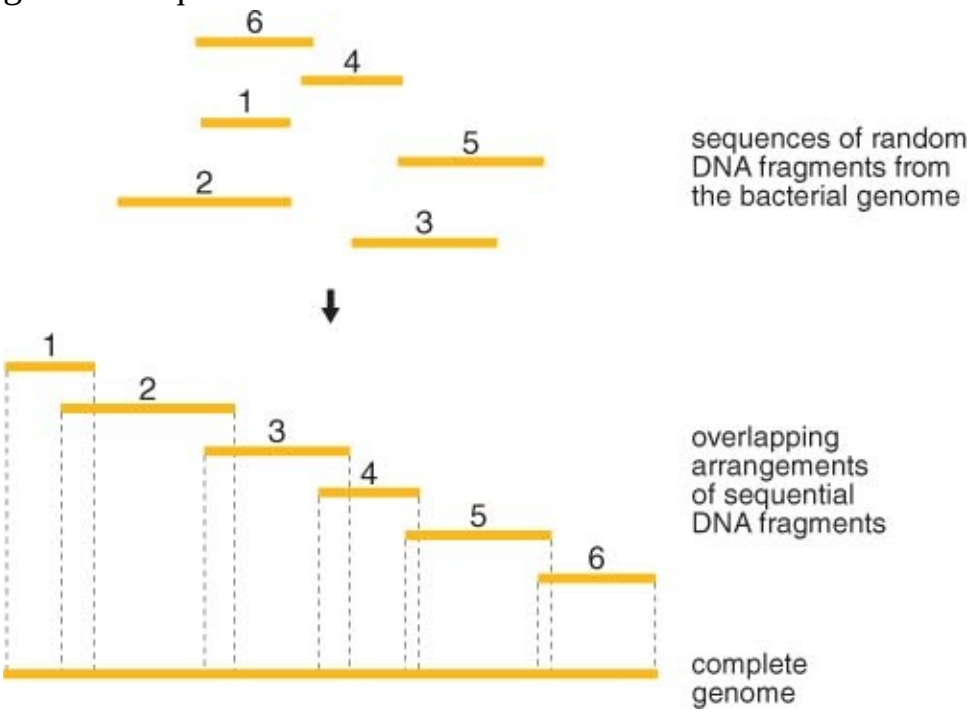
Whole bacterial genome sequencing

The first bacterial genome to have its DNA sequence completely determined was *Haemophilus influenzae* (1.8 Mbp) in 1995. Following advances in DNA sequencing technology along with improved computing protocols for managing enormous datasets, the numbers of whole bacterial sequences being completed has risen quickly. Currently there are 1,014 completed bacterial genomes deposited in online databases, with more than 3,500 at various stages of completion. Information obtained from genome sequencing projects can aid our understanding of infectious disease and microbial evolution.

The first technical approach to determine the DNA sequence for a bacterium necessitated the construction of an extensive library of randomly generated DNA fragments (Fig. 6.12). These short DNA fragments, from the organism's genome are cloned into a suitable vector. The DNA sequence from each randomly generated library insert is then determined using Sanger DNA sequencing (as outlined previously). Powerful bioinformatic computing tools are used to search the DNA sequences from the library of cloned DNA fragments to identify overlapping sequences. Using this approach, overlapping library clones can be identified and spliced together until the complete DNA sequence of the bacterium is determined.

Advances in sequencing technologies and computational analysis over the past few years have led to the development of faster analytical approaches, so

Figure 6.12 An illustration of the assembly and determination of a complete genome sequence for a bacterial chromosome.



called next-generation sequencing technologies. These new methods significantly increase the volume of sequence data that can be determined, taking only days to complete a bacterial genome. This makes whole bacterial genome sequencing available to all suitably resourced laboratories.

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Section II
Introductory Bacteriology

Chapter 7

The structure of bacterial cells

A typical bacterial cell is composed of a capsule, a cell wall, a cell membrane, cytoplasm containing nuclear material and appendages such as flagella and pili (fimbriae). Certain species of bacteria can produce forms termed endospores or spores, which are resistant to environmental influences. Some of the structural features of pathogenic bacteria which are important in the production of disease or may be useful for the laboratory diagnosis of infection are reviewed in Chapters 10 and 13. The principal structural components of bacterial cells are presented in Table 7.1 and illustrated in Fig. 7.1.

Capsule

Bacteria can synthesize extracellular polymeric material which is usually described as glycocalyx. In some bacterial species this polymeric material forms a capsule, a well defined structure closely adherent to the cell wall. A slime layer is formed when the polymeric material is present as a loose meshwork of fibrils around the cell. Most capsules are composed of polysaccharides; *Bacillus* species such as *B. anthracis* produce polypeptide capsules. Defined capsules can be visualized by light microscopy using negative staining techniques. Bacteria with well defined capsular material produce mucoid colonies on agar media. However, the capsules of most species of bacteria can be demonstrated only by electron microscopy or by immunological methods using antisera specific for the capsular (K) antigens. The main function of capsular material appears to be protection of the bacterium from adverse environmental conditions such as desiccation. In the body, capsules of pathogenic bacteria may facilitate adherence to surfaces and interfere with phagocytosis.

Cell wall

The tough, rigid cell walls of bacteria protect them from mechanical damage and

osmotic lysis. As cell walls are non-selectively permeable, they exclude only very large molecules. Differences in the structure and chemical composition of the cell walls of bacterial species account for variation in their pathogenicity and influence other characteristics including staining properties. Peptidoglycan, a polymer unique to prokaryotic cells, imparts rigidity to the cell wall. This polymer is composed of chains of alternating subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by short tetrapeptide side chains and peptide cross-bridges.

Based on their colour when stained by the Gram method, bacteria can be divided into two major groups, Gram-positive and Gram-negative. This colour reaction is determined by the composition of the cell wall. Gram-positive bacteria, which stain blue, have a relatively thick uniform cell wall which is composed mainly of peptidoglycan and teichoic acids. In contrast, Gram-negative bacteria, which stain red, have cell walls with a more complex structure, consisting of an outer membrane and a periplasmic space containing a comparatively small amount of peptidoglycan (Fig. 7.2). The Gram-staining procedure consists of a primary stain (crystal violet), a mordant (Gram's iodine), alcohol as a decolorizer and a counterstain (carbol fuchsin). The crystal violet penetrates the cell wall of both Gram-positive and Gram-negative bacteria. Following addition of the iodine, a complex with the primary dye is formed. The alcohol dehydrates the peptidoglycan of Gram-positive organisms making them less permeable to the crystal violet complex than Gram-negative bacteria. Thus the dye–iodine complex is retained in Gram-positive organisms whereas it is removed in Gram-negative organisms. Both Gram-positive and Gram-negative organisms absorb the counterstain but its pink colour is visible only in Gram-negative organisms as the deep purple colour of the crystal violet masks the pink colour in Gram-positive organisms.

Table 7.1 Structural components of bacterial cells.

Structure	Chemical composition	Comments
Capsule	Usually polysaccharide; polypeptide in *Bacillus anthracis*	Often associated with virulence; interferes with phagocytosis; may prolong survival in the environment
Cell wall	Peptidoglycan and teichoic acid in Gram-positive bacteria. Lipopolysaccharide (LPS), protein, phospholipid and peptidoglycan in Gram-negative bacteria	Peptidoglycan is responsible for the shape of the organism. LPS is responsible for endotoxic effects. Porins, protein structures, regulate the passage of small molecules through the phospholipid layer
Cytoplasmic membrane	Phospholipid bilayer	Selectively permeable membrane involved in active transport of nutrients, respiration, excretion and chemoreception
Flagellum (plural, flagella)	Protein called flagellin	Filamentous structure which confers motility
Pilus (plural,		Also known as fimbria (plural, fimbriae). Thin, straight, thread-like

pili)	Protein called pilin	structures present on many Gram-negative bacteria. Two types exist, attachment pili and conjugation pili
Chromosome	DNA	Single circular structure without nuclear membrane
Ribosome	RNA and protein	Involved in protein synthesis
Storage granules or inclusions	Chemical composition variable	Present in some bacterial cells; may be composed of polyphosphate (volutin or metachromatic granules), poly-beta-hydroxybutyrate (reserve energy source), glycogen

The outer membrane of Gram-negative bacteria is a protein-containing asymmetrical lipid bilayer. The structure of the inner surface of the membrane resembles that of the cytoplasmic membrane, whereas that of the outer surface is composed of lipopolysaccharide (LPS) molecules. Low molecular weight substances such as sugars and amino acids enter through specialized protein channels, known as porins, in the outer membrane. The outer membrane LPS, the endotoxin of Gram-negative bacteria, is released only after cell lysis. The major components of LPS molecules are core polysaccharides bound to lipid A and long external polysaccharide side chains. The polysaccharide side chains of the LPS molecules stimulate antibody production and correspond to the somatic (O) antigens used for serotyping of Gram-negative cells. Lipid A is the molecular component in which endotoxic activity resides. On account of its composition, the outer membrane excludes hydrophobic molecules and renders Gram-negative bacteria resistant to some detergents which are lethal to most Gram-positive bacteria. Comparative features of the cell walls of Gram-positive and Gram-negative bacteria are illustrated in Fig. 7.3.

The mycoplasmas comprise an important group of bacteria without cell walls. Conventional bacteria, exposed to the action of antibiotics such as penicillin, or other substances which interfere with the synthesis of peptidoglycan, cannot produce cell walls and are termed L forms.

Cytoplasmic membrane

The cytoplasmic membranes of bacterial cells are flexible structures composed of phospholipids and proteins. They can be observed only by electron microscopy and are structurally similar to the plasma membranes of eukaryotic cells. However, bacterial cytoplasmic membranes, with the exception of those present in mycoplasmas, do not contain sterols. The inner and outer faces of cytoplasmic membranes are hydrophilic while the interior is hydrophobic, forming a barrier to most hydrophilic molecules. Only a limited range of small molecules such as water, oxygen, carbon dioxide and some lipid-soluble

compounds can enter bacterial cells by passive diffusion. Two major functions of the cytoplasmic membrane, the active transport of nutrients into the cell and the elimination of waste metabolites, require the expenditure of energy. The energy required by permeases and other carrier molecules for active transport of nutrients derives from adenosine triphosphate. The cytoplasmic membrane is also the site of electron transport for bacterial respiration, of phosphorylation systems and of enzymes and carrier molecules that function in the biosynthesis of DNA, cell wall polymers and membrane lipids.

Figure 7.1 Structures commonly found in a bacterial cell.

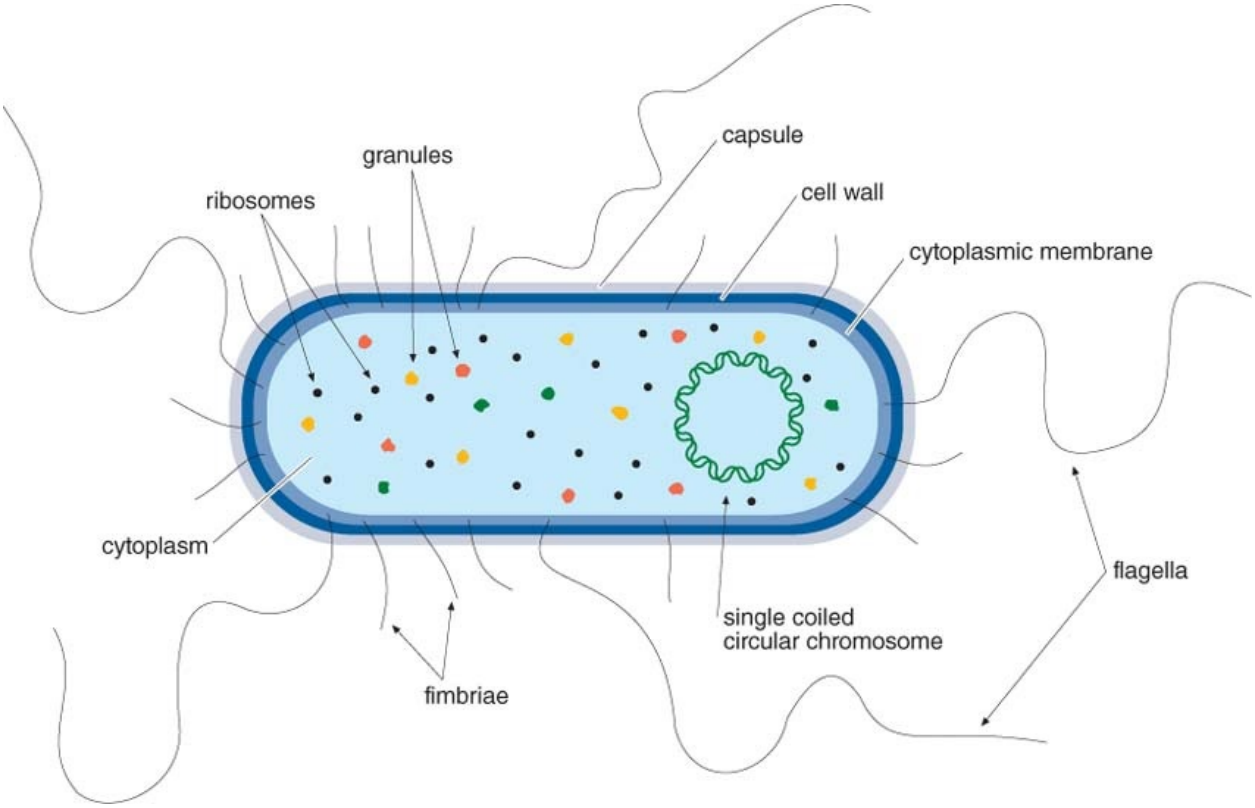


Figure 7.2 Comparison of the capsule, cell wall and cell membrane of a Gram-positive and a Gram-negative bacterium. Structures of importance in staining, virulence and toxicity, antigenicity, and susceptibility to antibiotics are illustrated. Details of the structural features of lipopolysaccharide are shown in the inset.

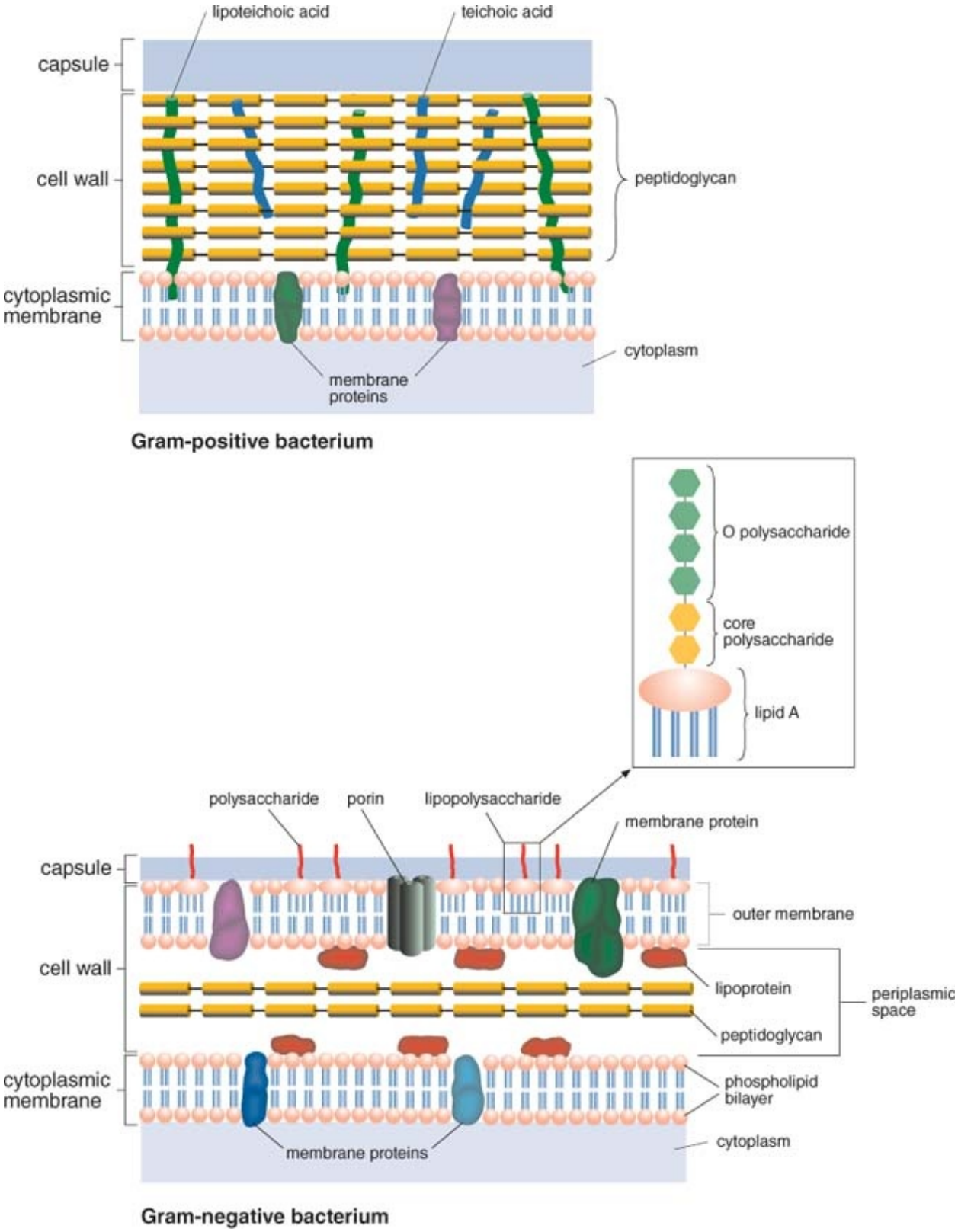
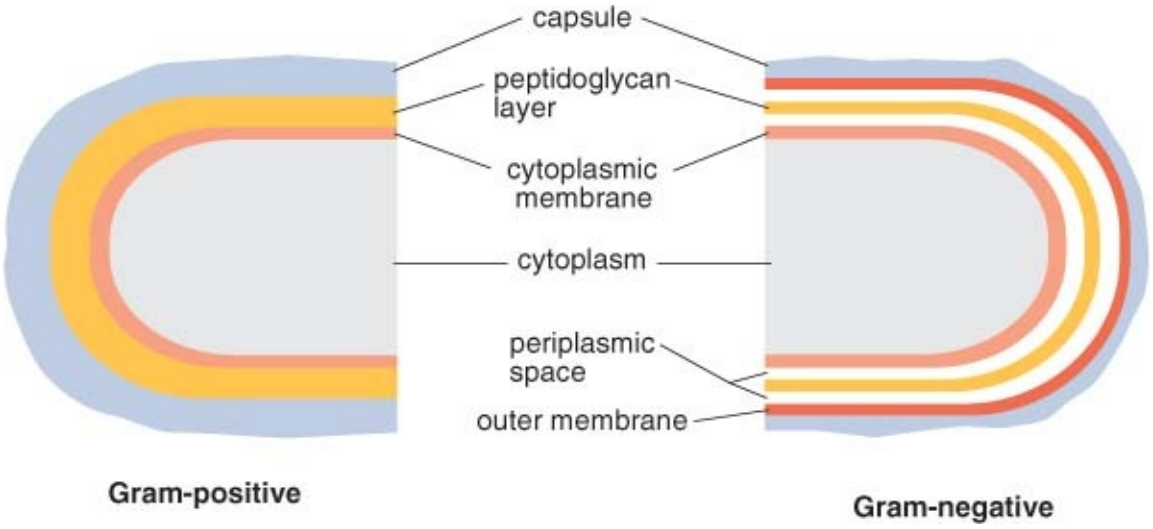


Figure 7.3 Comparative features of the cell walls of Gram-positive and Gram-negative bacteria.



Cytoplasm

The cytoplasm, which is enclosed by the cytoplasmic membrane, is essentially an aqueous fluid containing the nuclear material, ribosomes, nutrients and the enzymes and other molecules involved in synthesis, cell maintenance and metabolism. Storage granules may be present under certain environmental conditions, usually those unfavourable for bacterial growth. These granules, which may be composed of starch, glycogen, polyphosphate or other compounds, can often be identified using particular dyes.

Ribosomes

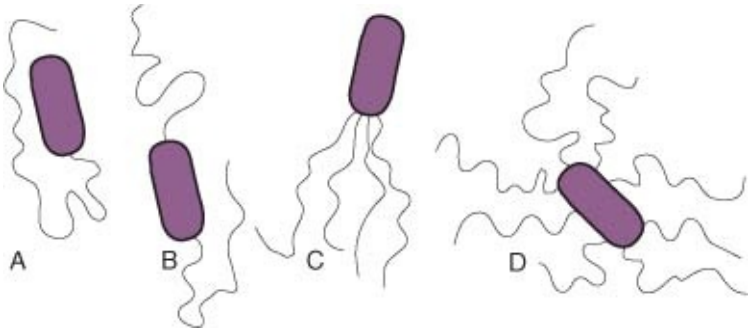
All protein synthesis takes place on ribosomes. These structures are composed of ribonucleoproteins and are up to 25 nm in size. They consist of two subunits, a larger 50S subunit and a smaller 30S subunit. The Svedberg (S) unit is a measure of sedimentation rate, which is dependent on both the size and shape of a particle. Ribosomal ribonucleic acid (rRNA) is complexed with many different proteins and accounts for about 80% of the RNA of the cell. Smaller amounts of transfer RNA (tRNA) and messenger RNA (mRNA) account for the remaining cellular RNA. Ribosomes may be present either in the cytoplasm or associated with the inner surface of the cytoplasmic membrane. During active bacterial growth and rapid protein synthesis, individual ribosomes are joined by mRNA into long chains known as polysomes.

Nuclear material

The bacterial genome is usually composed of a single haploid circular chromosome containing double- stranded DNA. However, some bacteria such as *Burkholderia* species have two circular chromosomes, and others, including *Borrelia* species, have a linear chromosome. Small amounts of protein and RNA are also associated with nuclear material. The genes in the bacterial chromosome encode all the vital functions of the cell. Bacterial genomes vary in size depending on the species. Because of its length, the bacterial chromosome is extensively folded to form a dense body which can be seen by electron microscopy. The nuclear material can also be demonstrated by light microscopy when stained by the Feulgen method which is specific for DNA. During replication, the DNA helix unwinds and both daughter cells, produced by binary fission, receive a copy of the original genome.

Plasmids, small circular pieces of DNA which are separate from the genome, are capable of autonomous replication. Several different plasmids may be present in individual bacterial cells. Copies of plasmids can be transferred from cell to cell during binary fission or through conjugation (see Chapter 9). Plasmid DNA may code for characteristics such as antibiotic resistance and exotoxin production.

Figure 7.4 Bacterial flagella. A, Monotrichous (polar) flagellum; B, amphitrichous flagella; C, lophotrichous flagella; D, peritrichous flagella.



Flagella

Bacteria that possess flagella are motile. Many species of Gram-negative bacteria have flagella. Although they are rarely present in cocci, some species of enterococci and the zoospores of *Dermatophilus congolensis* possess flagella. Flagella are usually several times longer than the bacterial cell and are composed

of a protein called flagellin. They consist of a filament, hook and basal body. The hook functions as a universal joint between the filament and the basal body. The basal body is anchored to the cell wall and to the cytoplasmic membrane. The positions at which flagella are inserted into the bacterial cell vary and may be characteristic of a genus or family (Fig. 7.4). Motile bacteria can move into suitable microenvironments in response to physical or chemical stimuli.

Flagella can be demonstrated by electron microscopy, by light microscopy using special methods and by serology using antibodies specific for flagellar antigens. Motility can be confirmed in young broth cultures using the hanging drop technique or in a semisolid motility medium containing tetrazolium salts.

Pili

Fine, straight, hair-like appendages called pili or fimbriae, composed of the protein pilin, are attached to the cell wall of many bacteria. The number of pili on each bacterial cell varies widely. They are most common on Gram-negative bacteria and they may have different functions. They frequently function in adhesion to host tissues with the adhesin located at the tips of fimbriae. The pili of Gram-negative bacteria are classified into four groups based on the pathways through which they are assembled (Proft and Baker, 2009):

- *Pili assembled through the chaperone–usher pathway*. The principal pili in this group are the Type 1 pili, found in the *Enterobacteriaceae* and some other bacterial species, and the P pili of uropathogenic *E. coli*. These pili are formed by linear, unbranched polymers of pilus subunits. They function in adhesion and have been implicated in biofilm formation.
- *Type IV pili*. These pili are found in many Gram-negative bacteria and in two Gram-positive genera, including *Clostridium* species which are important causes of disease in humans and animals. Type IV pili are composed of long flexible fibres which frequently aggregate, forming bundles. They function as adhesins and in biofilm formation but have additional roles in DNA uptake during transformation, in phage transduction and in a form of movement known as twitching motility.
- *Curli pili*. These are proteinaceous coiled fibres made of repeating subunits of the protein curlin or CsgA. They have been described principally in *Salmonella* species and *E. coli*. Like some other pili, they are thought to play a role in adhesion and biofilm formation, but appear to be important in

induction of the inflammatory response also.

- *CS1 pilus family*. These pili are associated with enterotoxigenic *E. coli* (ETEC).

Pili are also found in many Gram-positive genera such as *Corynebacterium*, *Actinomyces*, *Enterococcus* and *Streptococcus* species. Two types of pili have been identified in Gram- positive bacteria: short thin rods and much longer flexible pili. As in Gram-negative bacteria, these pili function in adhesion to host tissues.

A unique type of pilus, the F (sex or conjugation) pilus, functions in male or donor cells of Gramnegative bacteria as a conduit for the transfer of DNA to female or recipient cells. This pilus is constructed by a type IV secretion system and involves the formation of a pilus which contains a channel through which DNA travels from the donor to the recipient cell. Conjugation is discussed further in Chapter 9.

Biofilms

Biofilms can be defined as bacterial populations which are adherent to each other and/or surfaces and are enclosed in a bipolymer matrix. Bacteria exist in both planktonic (free) or sessile (attached) forms in natural environments and biofilms form when organisms switch to a sessile mode of growth. The first step in biofilm formation involves the attachment of bacteria to a surface and the formation of a monolayer of cells. This is followed by clustering of cells and the formation of microcolonies. The biofilm then begins to mature with the production of extracellular polymers and the surrounding of the cell clusters by a hydrated exopolymer matrix. Nutrients can reach the bacteria through open water channels within the glycocalyx matrix of the biofilm.

Quorum sensing is the regulation of gene expression in response to fluctuations in cell-population density, and molecules involved in quorum sensing facilitate the cell-to-cell signalling required for biofilm development. Quorum sensing is thought to be important in determining biofilm thickness. Once maximum thickness is reached, cell dispersion occurs which involves release of planktonic cells from the biofilm which may then go on to colonize new areas. Biofilm formation is discussed further in Chapter 13.

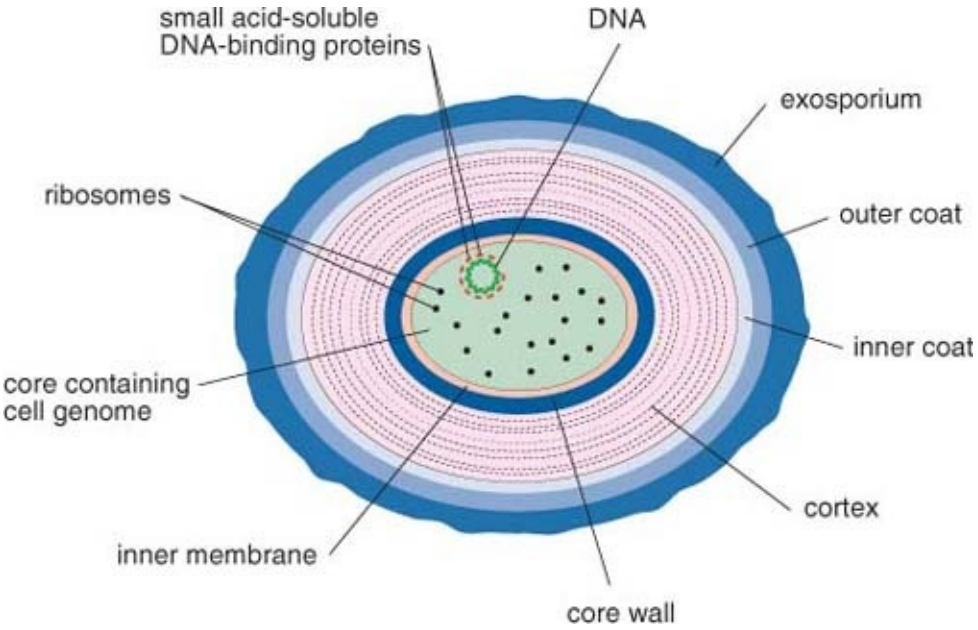
Protein secretion systems

Bacteria require mechanisms to transport proteins from the bacterial cytoplasm through the cell wall and the cell membrane into their surroundings, and, in the case of pathogenic bacteria, into the host cell. Gram-positive bacterial proteins are secreted directly. In Gram-negative bacteria, proteins must traverse a more complex cell wall and these bacteria have evolved secretion pathways known as secretion systems for this purpose. The proteins which are transported are known as effectors and modulate a variety of cell functions. Examples of effector proteins include enzymes such as proteases or lipases, and proteins which act as cytotoxins or promote apoptosis. Five protein secretion systems or pathways, Types I to V, are described in Gram-negative organisms. These systems are of major importance in the pathogenesis of infections caused by certain animal and human pathogens. One example is the Type III secretion system of *Salmonella* Typhimurium. This system, which is well characterized, has a needle-like structure and one of the functions of its effector proteins is promotion of cytoskeletal rearrangements and uptake of bacteria into the host cell.

Endospores

Dormant highly resistant bodies termed endospores are formed by some bacteria to ensure survival during adverse environmental conditions. The only genera of pathogenic bacteria that contain endospore-forming species are *Bacillus* and *Clostridium*. Endospores, which are produced inside the bacterial cell, show species variation in shape, size and position within the mother cell. Because of the resistance and impermeability of the spore coat, special staining procedures which employ heat are required to demonstrate endospores. The resistance of endospores is attributed to their layered structure, their dehydrated state, their negligible metabolic activity, their high levels of small acid- soluble proteins (SASPs) and their high content of dipicolinic acid (Fig. 7.5). Dipicolinic acid, which is not found in vegetative cells, occurs in the core of the spore in combination with large amounts of calcium.

Figure 7.5 Structural features of a mature bacterial endospore.



The high calcium content may explain the extended survival times of endospores in calcium-rich soils. In areas of low soil calcium or in acidic soils, calcium may be leached from spores, shortening their survival times. Small acid-soluble proteins bind to the DNA in the core and protect it from damage due to desiccation, dry heat and ultraviolet radiation. Because spores are thermostable, they can be destroyed with certainty only by moist heat at 121°C for 15 min.

When an endospore is reactivated, germination occurs in three stages, namely activation, initiation and outgrowth. Activation may occur in response to factors such as brief exposure to heat, abrasion of the spore coat or environmental acidity. If other environmental conditions including the presence of adequate nutrients are favourable, initiation of germination will occur. The spore cortex and coats are degraded, water is absorbed, calcium dipicolinate is released and outgrowth develops. Outgrowth is a period of active biosynthesis and terminates with division of the new vegetative cell. The spores produced by some of the filamentous actinobacteria are different from endospores in that their function is mainly concerned with reproduction rather than survival.

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Chapter 8

Cultivation, preservation and inactivation of bacteria

Appropriate conditions of moisture, pH, temperature, osmotic pressure, atmosphere and nutrients are required for bacterial growth. Bacteria increase in number by binary fission (Fig. 8.1). The generation time, that is, the length of time required for a single bacterial cell to yield two daughter cells, is influenced by both genetic and nutritional factors. *Escherichia coli*, a common enteric organism, has a generation time of approximately 20 minutes under optimal nutritional conditions. Bacterial pathogens have generation times ranging from 30 minutes to 20 hours. Long-term preservation of microorganisms usually involves freezing procedures. Heat treatment or chemicals can be used to inactivate bacteria.

Bacterial growth

Following inoculation of bacterial cells into fresh broth medium, the growth curve of the culture exhibits lag, exponential and stationary phases, and a final decline phase (Fig. 8.2). The lag phase is characterized by active metabolism of the cells as they acquire various essential constituents prior to division. Binary fission of the young cells results in an exponential increase in numbers. A straight line relationship is obtained when the logarithmic number of viable cells is plotted against incubation time. Exponential growth in a broth culture is limited and eventually ceases because essential nutrients are depleted and toxic metabolic products accumulate in the medium. During this stationary phase, no increase in bacterial numbers occurs; slow growth and division of a few bacteria are balanced by death of others. As the bacterial population enters the decline phase, old cells die rapidly followed eventually by the younger cells. The resulting rate of cell death is exponential. Abnormally shaped cells, known as involution forms, may be seen in stained smears from cultures in the decline phase. When maintenance of bacteria in the exponential growth phase is

required, a chemostat consisting of a growth chamber connected to a reservoir of fresh medium is used. As fresh medium enters the growth chamber, bacteria are harvested and exhausted medium and waste products are removed.

The size of bacterial populations is usually expressed either as the number or the density of the cells present. Cell numbers can be determined either as a total cell count or as a viable cell count. The standard methods for counting bacterial cell numbers are presented in Table 8.1. Bacteria may be counted by direct microscopy, by colony counting, by membrane filtration or by electronic methods. Accurate cell counts may be required for specific purposes such as vaccine preparation and for bacteriological testing of water.

Bacterial nutrition

Bacteria acquire nutrients from their immediate environment. Most are chemoheterotrophs, using organic chemicals as sources of energy and carbon. Small molecules may be metabolized rapidly or utilized to synthesize macromolecules. Nutrient media for the isolation of pathogenic bacteria are formulated to supply particular growth factors for specific groups of organisms.

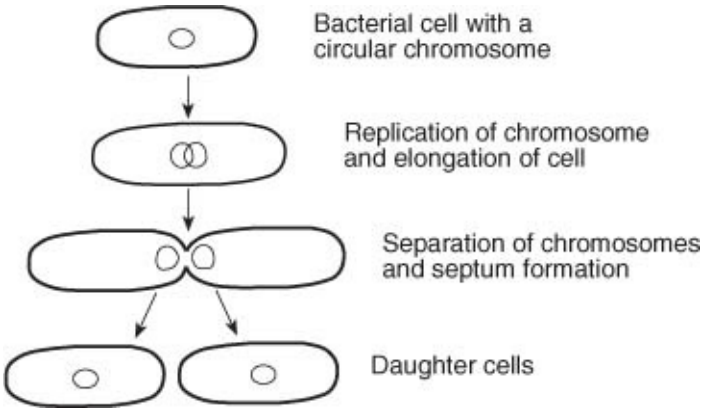
Most bacteria require carbon and nitrogen in relatively large amounts. In culture media, peptones are usually the main source of nitrogen. Peptones, which are mixtures of peptides and amino acids obtained by the digestion of meat and other sources of protein, frequently supply other essential nutrients such as phosphate, sulphate, potassium, magnesium, calcium and iron. Phosphates are essential for the production of nucleic acids and molecules containing energy-rich bonds. Sulphates are required for the synthesis of sulphur-containing amino acids, and magnesium, potassium, calcium and iron are important co-factors for certain enzymes. Trace elements and certain growth factors such as vitamins are also essential for bacterial growth.

Physical and chemical factors which influence growth

In addition to nutritional factors, growth of bacteria is influenced by genetic factors and by chemical, physical and other environmental factors. Awareness of those factors that limit growth is essential for the successful culture and long-term preservation of microorganisms. Growth of bacteria in culture is influenced

by temperature, hydrogen ion concentration, availability of moisture, atmospheric composition and osmotic pressure. Most pathogenic bacteria can be grown aerobically on a nutrient medium at 37°C, close to normal body temperature. Although the optimal temperature for growth of these bacteria, termed mesophiles, is 37°C, they can grow at temperatures between 20°C and 45°C. In contrast, many environmental bacteria grow at temperatures outside this range. Those with an optimal incubation temperature of 15°C are termed psychrophiles and those with an optimal incubation temperature close to 60°C are termed thermophiles (Fig. 8.3).

Figure 8.1 Bacterial replication by binary fission. The time required to produce two daughter cells in rapidly growing bacteria is referred to as the generation time.



As most bacteria grow optimally at neutral pH, it is standard practice to buffer culture media close to pH 7. Bacteria require water for growth, and species vary widely in their susceptibility to desiccation. The ability to tolerate desiccation is determined by the cell wall composition and the surrounding microenvironment. Moreover, the cell wall composition accounts for the ability of bacteria to withstand changing osmotic pressures. Change in the cell wall composition, induced by the action of lysozyme or of antibiotics such as penicillin, results in protoplast formation. These spherical structures lack rigidity and are susceptible to osmotic change. In the animal body, pathogenic bacteria without cell walls (L forms) can replicate, causing chronic or persistent infections. Bacterial cells in the environment are usually present in hypotonic solutions and, provided that the cell wall is intact, they remain in a state of turgor and do not lyse. In hypertonic solutions, bacterial cells undergo shrinkage.

Figure 8.2 The pattern of growth and decline of viable bacterial cell numbers in liquid medium.

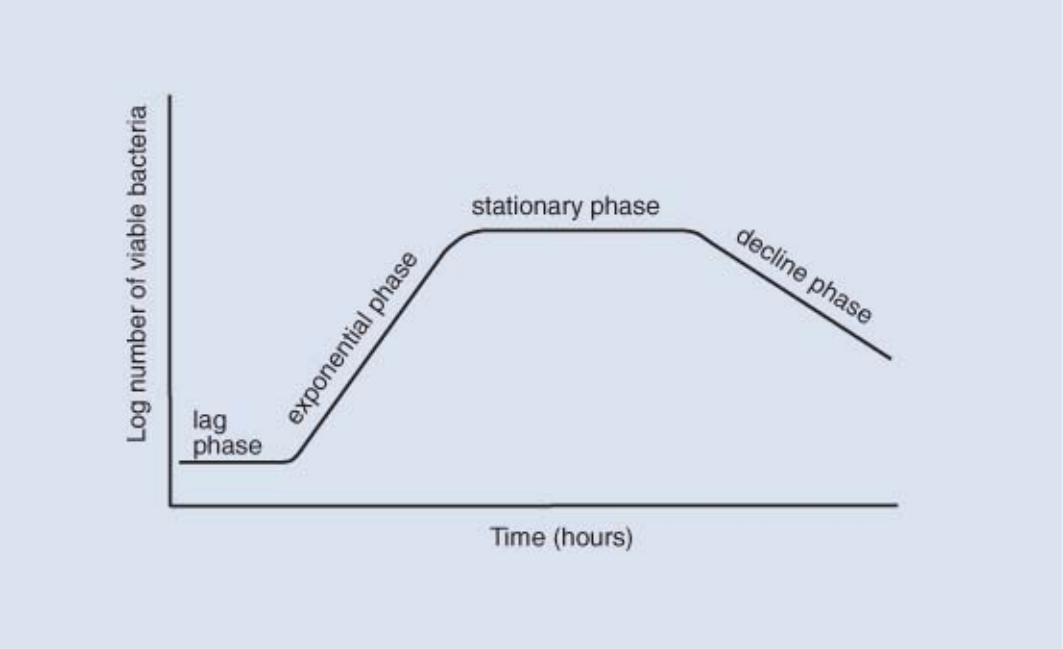


Table 8.1 Methods for counting bacteria.

Method	Technique	Comments
Microscopic counting		
Direct smear (Breed's method)	Counts carried out on a fixed and stained smear prepared from a defined volume of fluid; 50 microscopic fields counted	Traditional method for counting bacteria in milk. Slow and unreliable. Cannot differentiate viable from non-viable bacteria
Counting chamber	Counts carried out on a fixed volume of bacterial suspension using a calibrated slide	Does not differentiate viable from non-viable bacteria
Colony counting		
Spread plate	Following serial ten-fold dilution of a bacterial suspension, a fixed volume of each dilution is spread on the surface of agar plates and incubated for 24 to 48 hours	Colony counts are carried out on plates with 30 to 300 colonies after incubation. The number of viable organisms in the suspension is calculated and expressed as colony-forming units (CFU)/ml of suspension
Pour plate	Following serial ten-fold dilution as in the spread plate technique, 0.1 ml of each dilution is placed in Petri dishes and approximately 20 ml of molten agar at 45°C to 48°C is added and thoroughly mixed	Colony counting is carried out as in the spread plate technique and results are expressed as CFU/ml of suspension
Miles-Misra	Following serial ten-fold dilution, 0.02 ml of each dilution is placed on a sector of an agar plate, five dilutions per plate	Colony counting is carried out as in the spread plate technique and the average colony count from the five drops is used for calculating the number of viable bacterial cells expressed as CFU/ml of fluid
Membrane filtration	Following filtration of a known volume of fluid through a filter of 0.22 µm pore size, the filter, placed on the surface of an agar plate, is incubated for 24 to 48 hours	The number of viable bacteria is expressed as CFU/ml of fluid
Additional counting methods		
Opacity tubes	The bacterial suspension is matched visually with McFarland's opacity standard tubes	Tables indicating the total bacterial cell numbers/ml equivalent to matching opacities are supplied with the standard tubes
Most probable number	The number of organisms in a sample is calculated following serial dilution of the sample in broth. A number of tubes of each dilution are prepared, usually between three and ten. The number of organisms in the original sample is estimated using the sample dilution where some	Tables are available indicating the total viable bacteria/ml equivalent to the number of tubes showing growth.This method is suitable for bacteria that cannot be grown on solid media and for those that require selective enrichment

method	of the tubes show growth (turbidity) following incubation and some remain clear	for detection such as *Salmonella* species in food or environmental samples
Electronic counting	Electronic counting instruments, such as the Coulter counter, when carefully calibrated, give accurate rapid results	Reliability of results is dependent on rigorous quality control. Provides total cell count only
Real-time PCR	The number of bacterial cells in a sample can be quantified based on detection of DNA concentration. For detection of total viable cells, real-time RT–PCR can be used	The rapidity of this technique is a major advantage. As commercial kits become available, this method is likely to replace conventional counting techniques in many instances

Figure 8.3 Categories of bacteria based on temperature ranges at which they can grow. Coloured areas indicate temperature ranges for optimal growth.

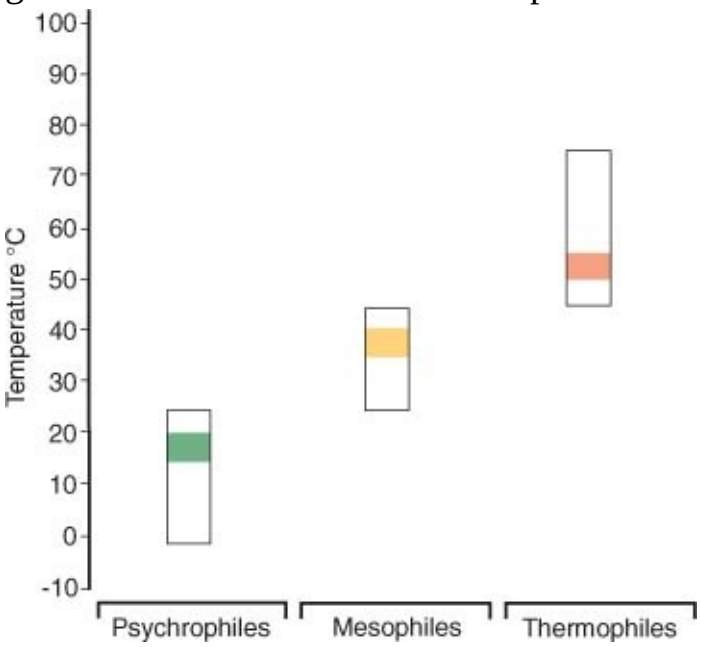
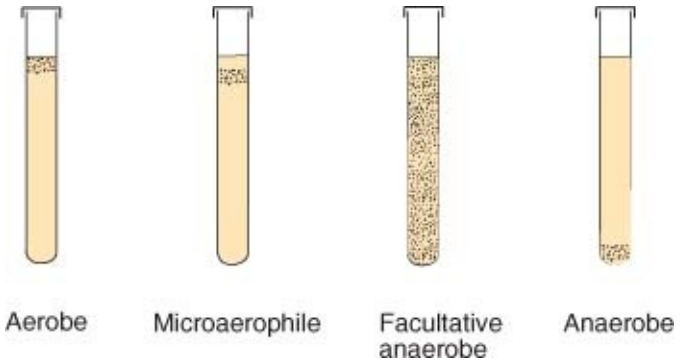


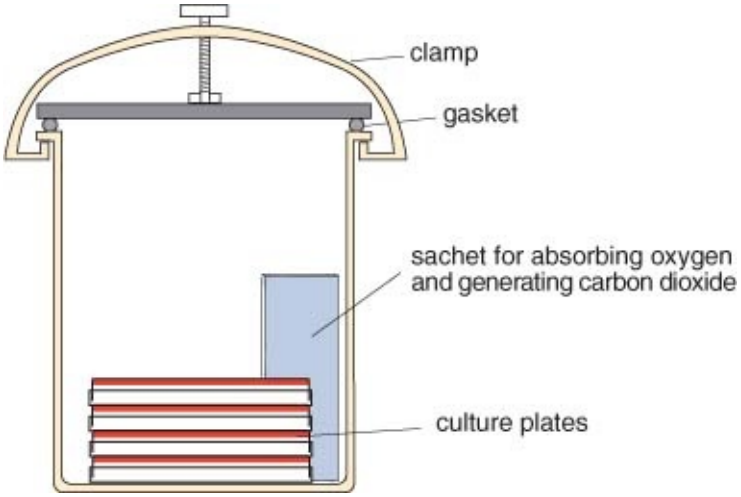
Figure 8.4 The pattern of growth at different depths in semi-solid agar, reflecting the preference of various bacterial species for aerobic, microaerophilic and anaerobic conditions.



Some bacteria, however, have adapted to hypertonic environments and can grow in solutions with high salt concentrations. *Staphylococcus aureus*, an important pathogen of humans and animals, can grow in media containing up to 7.5% sodium chloride.

Based on their preference for particular levels of oxygen, bacteria can be assigned to four main groups, namely aerobes, anaerobes, facultative anaerobes and microaerophiles (Fig. 8.4). Capnophiles, a fifth group, are aerobic bacteria with a requirement for carbon dioxide. Because they utilize metabolic pathways in which oxygen is the final electron acceptor, aerobic bacteria require oxygen for growth and they are incubated in air. In contrast, anaerobic bacteria are unable to grow in an atmosphere containing oxygen. These organisms use fermentative pathways in which organic compounds serve as final electron acceptors. As they lack enzymes such as superoxide dismutase

Figure 8.5 Jar for culturing anaerobic bacteria. When the porous sachet containing ascorbic acid is placed in the jar, which is then tightly sealed, oxygen is absorbed and carbon dioxide is generated. Anaerobic growth is enhanced by the released carbon dioxide.



and catalase, obligate anaerobes survive only briefly in the presence of oxygen. Facultative anaerobes are bacteria which have the ability to grow well under both aerobic and anaerobic conditions. Microaerophilic bacteria require a reduced oxygen concentration for growth.

The cultivation of bacteria other than aerobes requires special laboratory techniques. Strict anaerobes are cultured in tightly sealed jars in an atmosphere from which free oxygen has been removed. One commercially available system employs a gas-producing envelope. On the addition of water to the envelope, hydrogen and carbon dioxide are released into the jar. A palladium catalyst, either in the jar or attached to the envelope, accelerates the reaction of the hydrogen with free oxygen in the jar to form water. In addition, the release of carbon dioxide enhances the growth of anaerobes. A more convenient alternative

system, in which oxygen is removed by reacting with ascorbic acid contained in a porous envelope, has been developed (Fig. 8.5). This system, which eliminates the need to generate hydrogen, releases carbon dioxide into the jar (Brazier and Hall, 1994). Moreover, this method is suitable for culturing strict anaerobes. Anaerobic bags may be used as an alternative to jars, and a number of such anaerobic pouches are commercially available and are suitable for use with small numbers of plates. Other methods for the cultivation of anaerobic bacteria include the use of specially designed anaerobic chambers which are particularly useful when large numbers of specimens are being processed. Media, such as thioglycollate broth and cooked meat broth, with low redox potentials may be used also but are unsuitable as the only isolation medium for clinical specimens which frequently contain mixed flora. Facultative anaerobes may over-grow the anaerobes in the broth, making identification of the predominant pathogen difficult.

For the cultivation of microaerophiles, reduced oxygen levels are required. A gas-producing envelope, which delivers up to 10% carbon dioxide into a sealed jar, is available commercially. This system is also suitable for the cultivation of capnophilic bacteria.

Preservation of microorganisms

In order to produce modified-live vaccines and to maintain stock cultures of the bacteria and fungi used in teaching and research, microorganisms must be preserved. Preservation should ensure viability, freedom from contamination and genetic stability. Subculturing can be used for the short-term preservation of bacteria. Limitations of this procedure include death of some cells and a risk of contamination and mutation. Long-term methods of preservation include freeze-drying (lyophilization), ultra-freezing in liquid nitrogen at −196°C and freezing at −70°C. If properly used, these preservation methods can maintain organisms in a hypobiotic state for more than 30 years and they ensure that the organisms remain unchanged and uncontaminated. However, because freezing can harm microorganisms, chemicals must be employed to minimize the damage and ensure that the majority of organisms remain viable. Cryoprotective agents such as dimethyl sulphoxide or glycerol can minimize the negative effects of freezing on the viability of cells. Young actively growing cultures are less affected by freezing than older cultures. Small vials containing 20–30 porous polypropylene beads in approximately 1 ml of a cryopreservative broth are commercially

available for freezing bacteria at −70°C. This system removes the need for repeated freezing and thawing of stored cultures. A single colony of the organism to be stored is inoculated into the vial. Following mixing and holding for a short period of time to allow the bacteria to adhere to the beads, the cryopreservative liquid is removed by aspiration and the vial placed in the freezer. For recovery of the organism, a single bead is rapidly removed from the vial under aseptic conditions and rolled across the surface of a suitable non-inhibitory medium such as blood agar. When freeze-drying is used as a preservation method, it must be carried out under vacuum as bacteria are easily damaged if desiccation takes place. The microorganisms are subsequently stored in the dark in sealed evacuated ampoules.

Physical methods for inactivating microorganisms

Physical and chemical methods can be used for microbial inactivation or inhibition. Chemical agents include antimicrobial drugs (see Chapter 11), disinfectants (see Chapter 94) and food preservatives. Techniques that either inactivate bacteria or interfere with their metabolism include elevated temperature, low pH values, desiccation and high osmotic pressures. Some of the methods for preventing spoilage or limiting microbial growth in food are presented in Table 8.2. Sterilization is the method employed for the destruction of microorganisms on equipment used in microbiological and surgical procedures. Physical methods for sterilizing equipment or fluids are presented in Table 8.3. Sterilization procedures are effective for the destruction of bacterial, fungal and viral agents. However, unconventional infectious agents such as prions require more rigorous sterilization procedures. When dealing with bacterial endospores, such as those of *Clostridium* species, heating at a temperature of 121°C for 15 minutes is required for inactivation. Factors which may influence the effectiveness of sterilization by heat are listed in BOX 8.1.

Table 8.2 Methods for preventing spoilage and limiting microbial growth in food.

Method	Application	Comments
Refrigeration at 4°C	Prevention of growth of spoilage organisms and pathogenic bacteria	Pathogens such as Listeria *monocytogenes*, *Yersinia* species and many fungal species can grow at 4°C
Freezing at −20°C	Long-term storage of food. Microbial multiplication prevented	Surviving microorganisms can multiply rapidly when thawed food is left at ambient temperature

Boiling at 100°C	Inactivation of vegetative bacteria and fungi in food	Many endospores can withstand prolonged boiling
Pasteurization at 72°C for 15 seconds	Inactivation of most vegetative bacteria	Heat treatment should be followed by rapid cooling. If present in high numbers, some bacteria may survive
Acidification	Adjustment of pH to a low level inhibits bacterial growth	Applicable to a limited range of foods such as vegetables preserved in a fluid phase in jars or cans
Increasing osmotic pressure	Inhibition of microbial multiplication; used for preservation of food	Addition of salts or sugars increases osmotic pressure; applicable to a limited range of foods
Vacuum packing	Packaging of meat and other perishable foods	Removal of oxygen prevents the growth of aerobes
Irradiation	Inactivation of spoilage organisms and pathogenic bacteria	Not permitted in some countries

Table 8.3 Physical methods for sterilizing equipment or fluids and for disposing of contaminated material.

Method	**Comments**
Moist heat (autoclaving) employing steam under pressure to generate 121°C for 15 minutes or 115°C for 45 minutes	Used for sterilizing culture media, laboratory items and surgical equipment. Inappropriate for heat-sensitive plastics and fluids. Prions are not inactivated by this treatment
Dry heat in a hot-air oven at 160°C for 1 to 2 hours	Used for sterilizing metal, glass and other solid materials. Unsuitable for rubber and plastics
Incineration at 1,000°C	Used for destruction of infected carcasses and other contaminated material; environmental pollution a possible disadvantage
Flaming	Used for sterilizing inoculating loops in the naked flame of a Bunsen burner
Gamma irradiation	Ionizing rays used for sterilizing disposable plastic laboratory and surgical equipment. Unsuitable for glass and metal equipment
UV light	Non-ionizing rays with poor penetration. Used in biosafety cabinets
Membrane filtration	Used for filtering out bacteria from heat-sensitive fluids such as serum and tissue culture media. Pore size of filter should be 0.22 μm or less

When a population of microorganisms is exposed to high temperatures, there is an exponential decline in the numbers of viable organisms. Susceptibility to moist heat, used in autoclaving, can be expressed in terms of the thermal death time, which is the time required to kill all bacteria in suspension at a given temperature. The thermal death time is dependent on the initial size of the microbial population. The decimal reduction time (D value) is the time in minutes, at a particular temperature, required to reduce the viable cell population by 90%. The D value is inversely related to temperature and is independent of the size of the initial bacterial population.

BOX 8.1 Factors that influence the outcome of sterilization by heat.

- Temperature and holding time
- Degree of contamination
- Presence of endospores or prions

- Nature and quantity of the material being heat-treated

Biosafety cabinets

Personnel handling hazardous materials require suitable protection. Biological safety cabinets protect operators from aerosols containing microbial pathogens. Different levels of protection can be provided depending on the type of cabinet used. At higher levels of protection, all contact between the operator and infective material is prevented through the use of closed cabinets fitted with rubber gloves. Air extracted from biosafety cabinets is filtered through high efficiency particulate air (HEPA) filters designed to trap particulate matter such as microorganisms.

Reference

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Further reading

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Chapter 9

Bacterial genetics, mechanisms of genetic variation and gene databases

Much of the genetic information in bacteria is contained on a single chromosome located in the cytoplasm of the cell. Bacterial genomes differ in size, a feature which determines an organism's characteristic traits or phenotype. In this chapter an outline of the essential features of a bacterial gene is provided along with the biochemical pathway involved in gene expression. The consequences arising from alterations in the gene sequence are presented. Extrachromosomal DNA elements are defined and their contribution to unique traits of a bacterium are explained. The final part of this chapter deals with recombinant nucleic acid technology and the development of gene databases that can be used for therapeutic and other purposes.

Properties of a bacterial cell, including those of veterinary interest such as antimicrobial resistance and virulence, are determined by the microbial genome. The genomic structure consists of three types of genetic information: the chromosome, plasmids and bacteriophages (the latter two structures provide additional genetic information and are transient in some instances). Most bacteria are defined as haploid with a single circular chromosome consisting of double-stranded DNA. In *Escherichia coli* K-12, the chromosome is a circular double-stranded DNA molecule of approximately 4.6×10^6 base pairs, containing 157 RNA-encoding genes including ribosomal and transfer RNA along with 4,126 bacterial proteins. Bacterial chromosomes typically encode between 1,000 and 4,000 different genes. Individual genes (Fig. 9.1) consist of a starting point, referred to as the start codon, composed of the nucleotides ATG, an open reading frame (ORF) and a stop codon (TTA, TAG or TGA).

Although the chromosome exists free in the cytoplasm, it is compacted through super-coiling and looping of its structure. The central tenets of genetics consist of the expression of a gene from its locus on the chromosome or on a plasmid through transcription (production of messenger RNA or mRNA synthesis) and finally translation: decoding of the mRNA to produce a

polypeptide (Fig. 9.2). Since the DNA is located in the bacterial cytoplasm, it facilitates the simultaneous transcription and translation of bacterial genes. The gene sequence and its subsequent expression through these biochemical pathways accounts for the phenotypic variation observed among bacteria (Fig. 9.2). Recently, this has given rise to defined areas of research referred to as genomics, functional genomics or transcriptomics and proteomics.

Replication of bacterial DNA

As bacteria replicate by binary fission, the daughter cells produced are usually indistinguishable genetically. Replication of the chromosome in bacteria begins at a specific location referred to as the origin of replication (or origin), at a locus referred to as *ori*. It proceeds at a rate of 1,000 nucleotides per second. During replication, the purine and pyrimidine nucleotides in each DNA strand are accurately copied into two new double-stranded daughter molecules. Each of these molecules is composed of a strand from the parent molecule and a newly synthesized complementary strand, a process termed semi-conservative replication. As the two parent strands of the helical DNA unwind under the influence of the enzyme DNA helicase, each acts as a template for the synthesis of a complementary strand. In this manner, two identical helical DNA molecules are formed through the action of the replicating enzyme, DNA polymerase. The ends of the new, fully formed strands are joined by DNA ligase forming circular chromosomes.

Figure 9.1 A defined segment of bacterial DNA containing a gene and showing the regulatory features associated with transcription and translation. The regulatory features, RNA polymerase binding site, the translational start and stop codons of the gene’s open reading frame (ORF) and the site at which transcription terminates are shown. The mRNA produced from the sense strand of bacterial DNA is shown along with the translated protein, indicating the amino and carboxy-terminal ends.

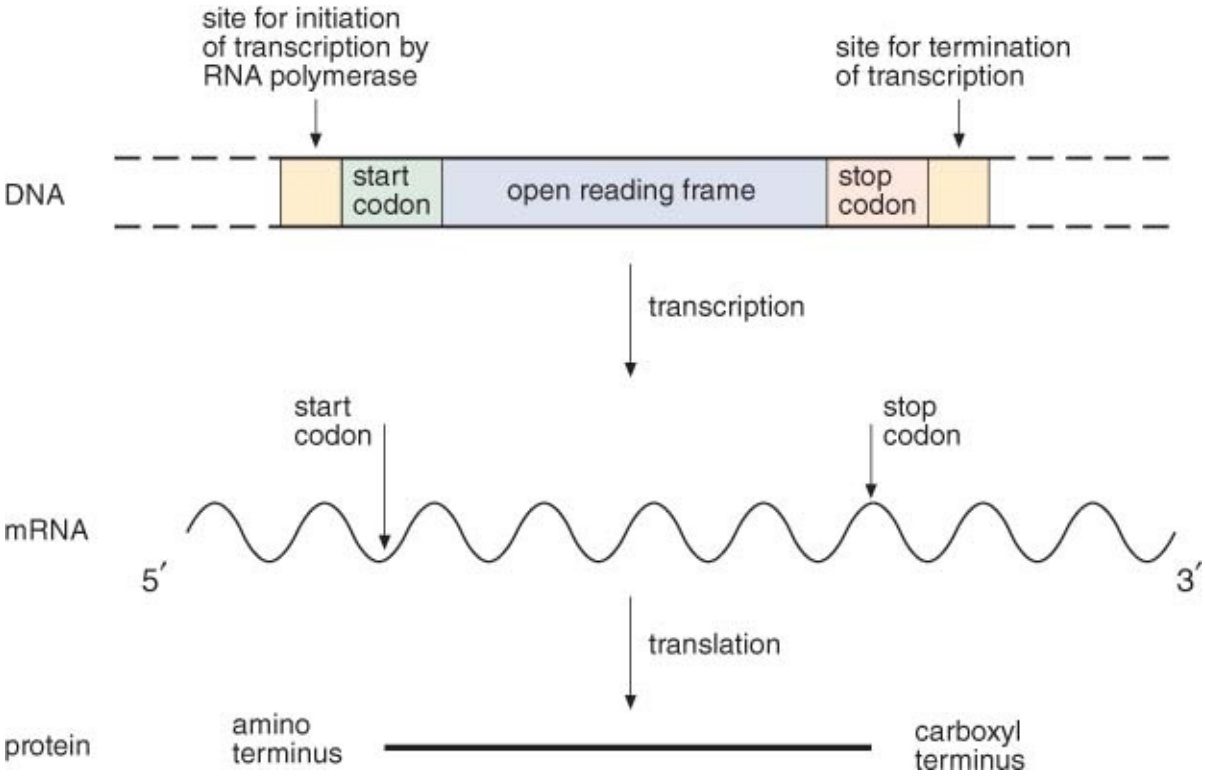
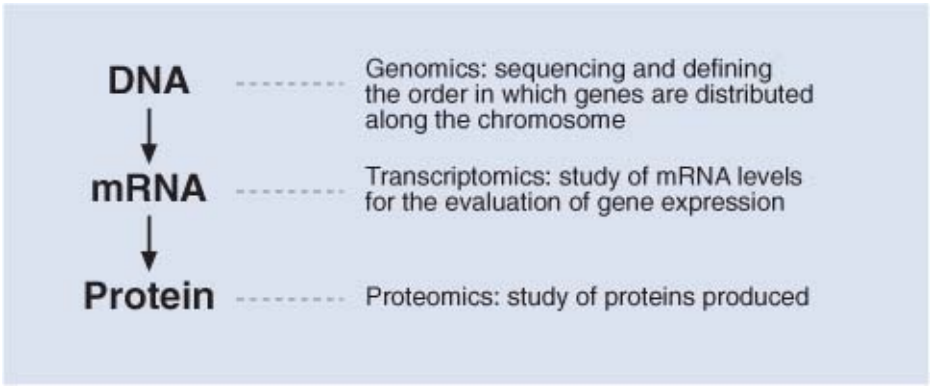


Figure 9.2 Central dogma of genetics showing the flow of genetic information. New scientific areas of genetic research are indicated.



Transcription and translation, the expression of genetic information

During transcription, one strand of DNA, the positive strand, is transcribed forming a messenger RNA (mRNA) molecule. This is mediated by the enzyme DNA-dependent RNA polymerase that binds to the promoter region of a gene, which is composed of two conserved DNA-binding sites referred to as the −35 and −10 promoter sequences. The two strands of DNA are partially unwound

and locally separate following which mRNA is synthesized. Transcription continues until the RNA polymerase enzyme reaches the termination signal. The information encoded in the mRNA is translated into protein on a ribosome through the involvement of transfer RNA (tRNA). Each tRNA molecule has a particular triplet of three bases, the anticodon, which is complementary to a codon on the mRNA. Each triplet of tRNA transfers a specific amino acid to the mRNA on the ribosome where the amino acids are enzymatically joined together, forming a peptide bond and extending the polypeptide chain. Following the linking of two amino acids, the tRNA molecule that carried the first amino acid is released from the ribosome. Synthesis of the protein chain continues until a stop codon on the mRNA is encountered by the ribosome (Fig. 9.1).

Mechanisms contributing to genetic variation

Genetic variation may occur following mutation, in which a change occurs in the nucleotide sequence of a gene, or by recombination, in which new groups of genes are introduced into the genome (Fig. 9.3 and BOX 9.1). The genotype of a cell determines its inheritable potential. However, only a small proportion of the genetic information is expressed under defined environmental conditions. The phenotype represents those recognizable characteristics expressed by the cellular nucleic acid. *Bacillus anthracis*, which causes anthrax, has a capsule that is expressed only *in vivo* and not when it is growing in laboratory media. Thus, both the genotype of an organism and its environment can influence phenotypic expression.

Mutation

A stable inheritable alteration in any genome is termed a mutation. A bacterium carrying a mutation is referred to as a mutant. When the original parent and mutant are compared, their genotypes differ and their phenotype may also differ depending on the nature of the mutation. Spontaneous mutations are the result of rare mistakes in DNA replication and occur at a frequency of about one in every 10^6 cell divisions. Because a gene with altered base pairs may code incorrectly for an amino acid in a protein, the mutation introduced may result in a phenotypic change (Fig. 9.4). Mutational changes may be beneficial or damaging for the organism. Under defined environmental conditions, selected mutations may provide growth advantage for the mutant over the parent or wild-

type bacterium. Mutations can also be experimentally induced by physical, chemical or biological mutagens.

Many viruses that infect animals have RNA genomes and these may also undergo mutation. The spontaneous mutation rate associated with these genomes is approximately 1,000-fold higher than that occurring in the host chromosome. Proofreading systems help to maintain the fidelity of DNA genomes, but similar systems do not exist to correct mutations in RNA genomes when they occur, leading to higher mutation rates.

Mutations can be defined as transition mutations wherein a purine/pyrimidine base is substituted for another purine/pyrimidine, or transversion mutations, wherein a purine/pyrimidine is substituted with a pyrimidine/purine base. Types of mutation that may occur in bacteria are listed in BOX 9.1. Point mutations involving one base pair or a limited number of base pairs may not result in phenotypic changes. In contrast, mutations in which many base pairs are deleted or inserted can result in the formation of nonfunctional proteins. Extensive changes that affect protein synthesis influence bacterial viability.

DNA may become damaged following contact with mutagenic chemicals, through exposure to UV irradiation and by other means. In general, the types of DNA damage that can arise include damage to a base in the DNA structure; single-strand damage requiring the removal and re-synthesis of a new strand using the complementary strand to guide the process, and double-stranded breakages which are more serious. Different mechanisms are available within the cell to manage the repair of damaged DNA, and the choice depends on the type of damage to be corrected. Repair of damage to an individual base or a single strand is virtually error-free, whereas repair of double-stranded breakages may require an error-prone mechanism. An example of an error-prone repair mechanism is the SOS system from which mutations arise. Once the damage has been repaired, the SOS system is repressed and the further introduction of mutations ceases.

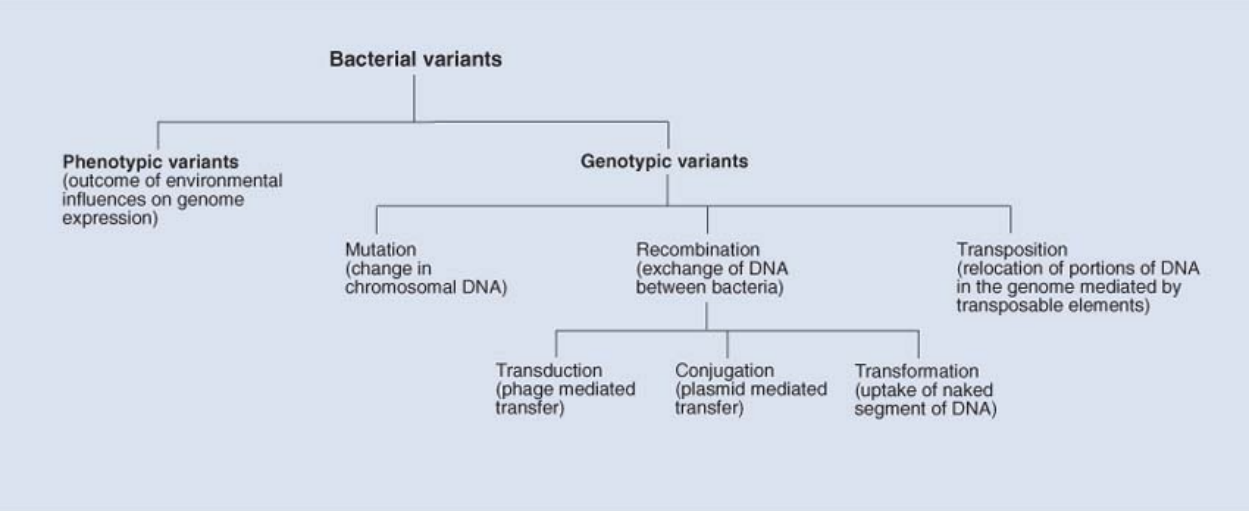
Genetic recombination

Recombination occurs when sequences of DNA from two separate sources are integrated. In bacteria, recombination induces an unexpected inheritable change due to the introduction of new genetic material from a different cell. This new genetic material may be introduced by conjugation, transduction or transformation.

Conjugation

The transfer of genetic material during conjugation is a complex process that has been extensively studied in the enteric bacterium *Escherichia coli*. These studies have shown that two strains of *E. coli*, F^+ (male) and F^- (female), participate in the process. The F^+ strains are the source of donor DNA, the fertility (F) plasmid, whereas F^- strains, having no F plasmid, are recipient cells. The F plasmid is a circular double-stranded DNA molecule 99.2 kbp in size. This plasmid contains genes required for plasmid replication and the *tra* genes, which are involved in mating pair formation. In addition, there are several insertion sequence elements. During conjugation, F^+ bacteria synthesize a modified pilus, the F or sex pilus. This pilus, through which genetic material can be transferred, can attach to F^- bacteria (Fig. 9.5). One strand of F plasmid DNA is unwound and is passed to the recipient F^- bacterium in which a complementary strand is then synthesized. Once a new F plasmid is formed, the recipient is converted into an F^+ bacterium.

Figure 9.3 The basis of bacterial variation. A bacterium that has acquired DNA from another bacterial cell by recombination is termed a recombinant cell.



BOX 9.1 Mutations observed in bacteria.

- Base-substitution or point mutation producing
  – silent mutations: code for the same amino acid
  – mis-sense mutations: code for a different amino acid
  – nonsense mutations: code for a stop, resulting in a truncated protein

- Microinsertions or microdeletions of base pairs
  – frame shift mutations: +1 or −1 frameshifts
- Reversions
  – back mutations reversing point mutations (base-pair substitution)
- Deletions of multiple base pairs
- Insertions during recombination resulting in errors
- Translocation of DNA segments within the genome
- Inversions
  – inverted orientation of a segment of DNA within the chromosome

Because of the complexity of conjugation, conjugative plasmids are relatively large, with genes occupying 30-kbp or more. During conjugation, plasmid DNA is the genetic material transferred. However, chromosomal DNA can sometimes be transferred, especially when the F plasmid is integrated into the bacterial genome. The F plasmid integrates at specific sites in the bacterial chromosome via insertion sequence elements in a process called homologous recombination. Upon integration, a high frequency recombination (Hfr) strain is formed wherein the F plasmid is contained within the chromosome at a specific site. These sites represent regions of homology between the chromosomal DNA of the bacterium and that of the plasmid. During conjugation of Hfr strains, the genes closest to the F factor origin of replication are transferred first. The potential exists for transfer of the entire chromosome. However, this is an unlikely occurrence because transfer can take up to 100 minutes and is usually interrupted before completion. Typically, the DNA strand being transferred breaks, resulting in only part of the chromosome being transferred to the recipient bacterium. The partial segment transferred must recombine with the recipient chromosome in order to be maintained. This step may give rise to a new phenotype gained by the recipient bacterium cell. Although Hfr strains can transfer at high-frequency, the recipient cell does not become a F^+ or a Hfr cell, as the transfer of the entire co-integrated plasmid is not achieved.

Figure 9.4 Examples of mutations that occur following the insertion of a base (indicated as a G) into a gene sequence, resulting in a +1 frame shift and the deletion of a base (indicated as a C), producing a −1 frame shift. Alterations in the resulting polypeptide segment arising from changes in the amino acid sequence are also shown.

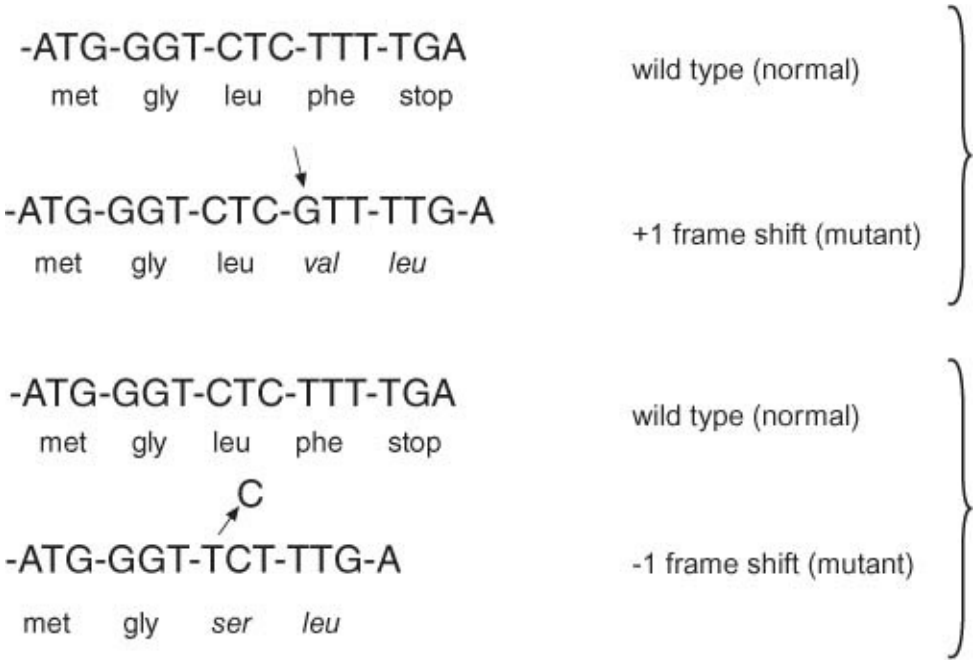
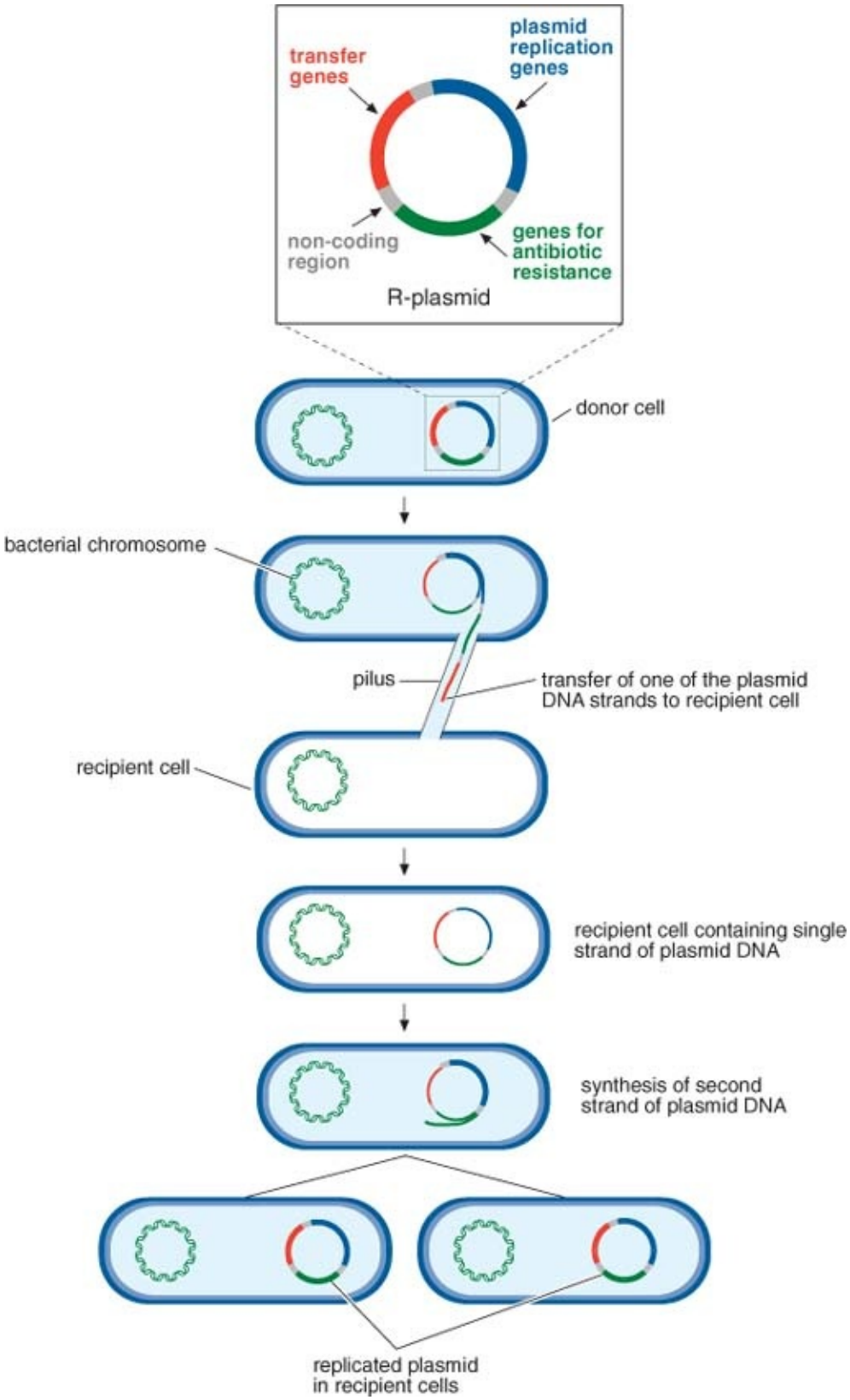


Figure 9.5 A genetic map of a typical resistance (R) plasmid, showing the genes for antibiotic resistance, the transfer genes and the genes required for plasmid replication. The steps involved in the transfer of an R plasmid from a donor bacterium to a susceptible recipient strain are shown. This process is termed conjugation and involves direct cell-to-cell contact via the sex pilus. A single strand of the plasmid moves through the pilus into the recipient, where replication of the second strand is later completed. This process gives rise to an altered bacterium containing a copy of the R plasmid.



Although conjugation is most frequently associated with Gram-negative

bacteria, it can also occur in Gram-positive organisms. A sex pilus is not formed in Gram-positive bacteria; plasmid DNA can be transferred directly through the cell walls when the bacteria are in close contact. Plasmid transfer by conjugation has important ecological significance, particularly where antibiotic resistance-encoding genes are concerned. A plasmid containing an antibiotic resistance gene in a bacterial cell can, under appropriate conditions, convert the entire bacterial population into similar plasmid-containing bacterial cells (Fig. 9.5). This is one mechanism by which antibiotic-resistant bacteria can be selected, through the inappropriate use of therapeutic agents. Plasmid transfer can also occur naturally in the gastrointestinal tracts of animals, giving rise to commensal bacteria with altered phenotypes.

Transduction

DNA acquired either from the original bacterial chromosome or plasmid in a previously infected bacterial cell can be incorporated into phage nucleic acid and transferred by progeny of the phage to susceptible recipient cells in a process called transduction. During a lytic cycle, the phage acts as a vector so that DNA derived from any part of the host genome may be transferred. In temperate phages, transduction affects only those bacterial genes adjacent to the prophage when a lytic cycle is induced. Transduction which takes place during a lytic cycle is termed generalized transduction and it occurs at a low frequency of one cell in 10^6 to 10^8 transduced, for a particular bacterial characteristic or genetic marker. Specialized transduction may occur when a prophage is induced to undergo a lytic cycle by exposure to mutagens. This type of transduction can result in the transfer of bacterial genes to many other cells because the bacterial genes are copied to all phage progeny. A small number of bacterial genes are excised with the prophage and some phage genes remain integrated in the bacterial chromosome when lysis occurs. In these circumstances, the phage progeny are defective because some phage genes are missing.

Transformation

This process involves the transfer of free or ‘naked’ DNA containing genes on a segment of chromosomal or plasmid DNA from a lysed donor bacterium to a competent recipient. Natural transformation is uncommon and occurs in only a few bacterial genera. Where transformation occurs in particular bacterial cells, such cells are termed ‘competent’. The competent cells can bind naked DNA

which is then transported into the cell. A specific protein binds to the DNA and protects it from intracellular nucleases; the DNA subsequently integrates into the bacterial genome.

Transformation can be chemically or electrically induced in some bacterial cells under laboratory conditions. This approach is often used to introduce recombinant DNA molecules into bacteria.

Examples of mobile genetic elements

Plasmids

Although most bacteria carry all of the genes necessary for survival on their chromosome, many bacteria contain small additional genetic elements, termed plasmids, which are also located in the cytoplasm and can replicate independently of the host chromosome. Many different plasmids are known in Gram-positive and Gram-negative bacteria. Most are closed circular double-stranded DNA molecules (Fig. 9.5) but some linear plasmids have been identified in bacteria. Depending on their genetic content, the size of a plasmid can vary from 1 kbp to more than 1 Mbp. Plasmids can carry genes that confer a wide variety of properties on the host bacterial cell. Most are not essential for normal survival, but they may offer a selective advantage under certain conditions such as the ability to conjugate and transfer genetic information, resistance to antibiotics and ability to produce bacteriocins, proteins inhibitory to other bacteria (Table 9.1). All plasmids carry the genes required for their stable maintenance. In some pathogenic bacteria, plasmids encode virulence factors and antibiotic resistance.

Thousands of plasmids have now been described, and these differ in size and genetic content and in their incompatibility types. By definition, those plasmids that can coexist in the same host bacterium are referred to as compatible, whereas those that cannot are defined as incompatible. Incompatibility (Inc) group typing of plasmids has identified several different incompatibility groups in the *Enterobacteriaceae*. Plasmids belonging to the same Inc group cannot exist in the same bacterial host cell, whereas those of different Inc types can coexist together. Inc typing schemes have also been developed for bacteria such as staphylococci and pseudomonads. The IncFI incompatibility group plasmids (encoding resistance to ampicillin, aminoglycosides, chloramphenicol,

sulphonamides and tetracycline) in non-typhoidal *Salmonella* and IncH plasmids are associated with the development of clusters of resistance. Stable coexistence of plasmids in bacteria provides the opportunity for the reassortment of plasmid-containing genes leading to the emergence of new antibiotic resistant structures.

Table 9.1 Virulence factors of pathogenic bacteria mediated by defined genetic elements.

Pathogen	**Virulence factors / Genetic elements**
Bacillus anthracis	Toxins, capsule / plasmid
Clostridium botulinum, types C, D and E	Neurotoxins / bacteriophages
Escherichia coli	Shiga-like toxin / bacteriophage Adherence factors, enterotoxins / plasmids
	Heat-stable toxin, siderophore production / transposons
Salmonella Dublin	Serum resistance factor / plasmid
Staphylococcus aureus	Enterotoxins (A,D,E), toxic shock syndrome factor-1 / bacteriophages
	Coagulase, exfoliating toxins, enterotoxins / plasmids
Yersinia pestis	Fibrinolysin, coagulase / plasmid

The number of copies of a plasmid may vary, with some present in high numbers. Plasmids may use host cell enzymes for replication, and some plasmids can replicate and be maintained in a small number of species. In contrast, other plasmids of the IncP and IncQ incompatibility groups are described as promiscuous, surviving in many bacterial hosts. Replication of most plasmids, however, is not directly related to replication of the host bacterium.

Distribution of plasmids between daughter cells is random. Plasmids in the bacterial cytoplasm may be transferred not only during replication but also by conjugation and by transformation, as outlined above.

The broad host range of some plasmids, together with their ability to conjugate, contributes to wide dissemination, a fact that accounts for the spread of antibiotic resistance among bacterial strains. Emergence of bacteria resistant to one or more antibiotics is significant in veterinary medicine. This is correlated with the increasing use of drugs for growth promotion in some instances and to treat infectious diseases in animals. Importantly, in some cases this may impact on human health where resistant zoonotic bacteria such as *Salmonella* and *Campylobacter* may be transferred to humans via the food chain.

Figure 9.6 Schematic representation of a DNA bacteriophage (T2 phage).

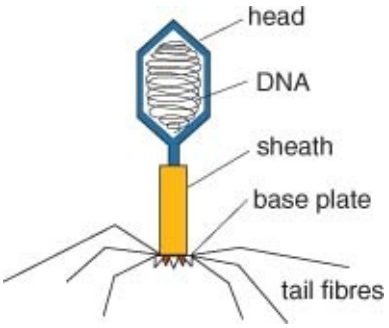
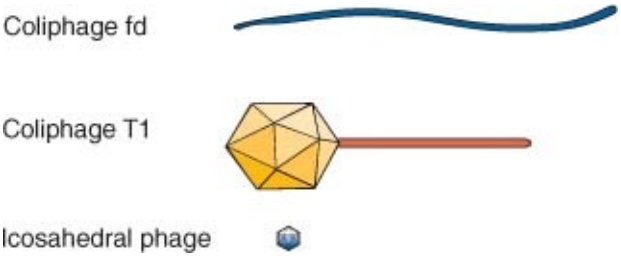


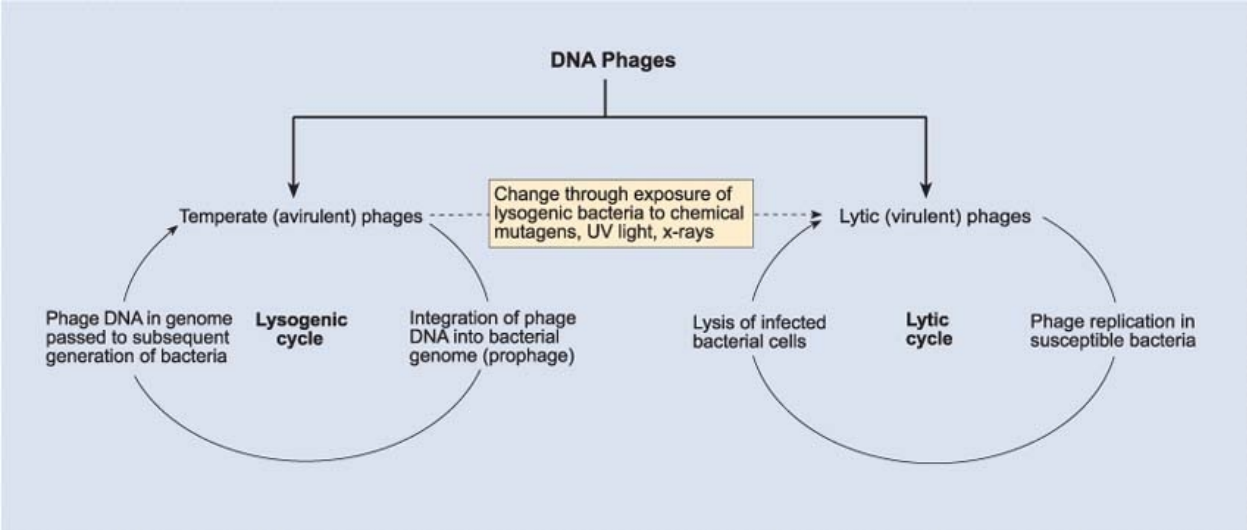
Figure 9.7 Morphology of bacteriophages. The majority contain double-stranded DNA, but some contain single-stranded DNA, double-stranded RNA or single-stranded RNA.



Bacteriophages

Viruses that infect bacteria are termed bacteriophages or phages (Fig. 9.6). There is considerable morphological diversity among bacteriophages. Some are filamentous with helical symmetry and others have icosahedral or pentagonal heads with tails of different lengths. Structural features of phages are illustrated in Fig. 9.7. Phages may be either virulent or temperate, depending on their method of replication (Fig. 9.8). Most phages attack only a small number of strains of related bacteria, and therefore can be described as having a narrow and specific host range. Virulent phages undergo a lytic cycle in bacteria, culminating in the production of phage progeny with lysis of host cells. Temperate phages or prophages are usually dormant and are integrated into the bacterial genome but they may also be present as circular DNA in the cytoplasm like plasmids. Prophages can also express some of their genes which confer additional properties on the host cell. Temperate phages can also undergo a lytic cycle, either as a rare natural event or when exposed experimentally to UV light or to other mutagens. A prophage in a bacterial cell may be responsible for changes in phenotypic characteristics, a phenomenon referred to as lysogenic conversion. The production of neurotoxins by certain types of *Clostridium botulinum* is associated with lysogenic conversion of host cells (Table 9.1).

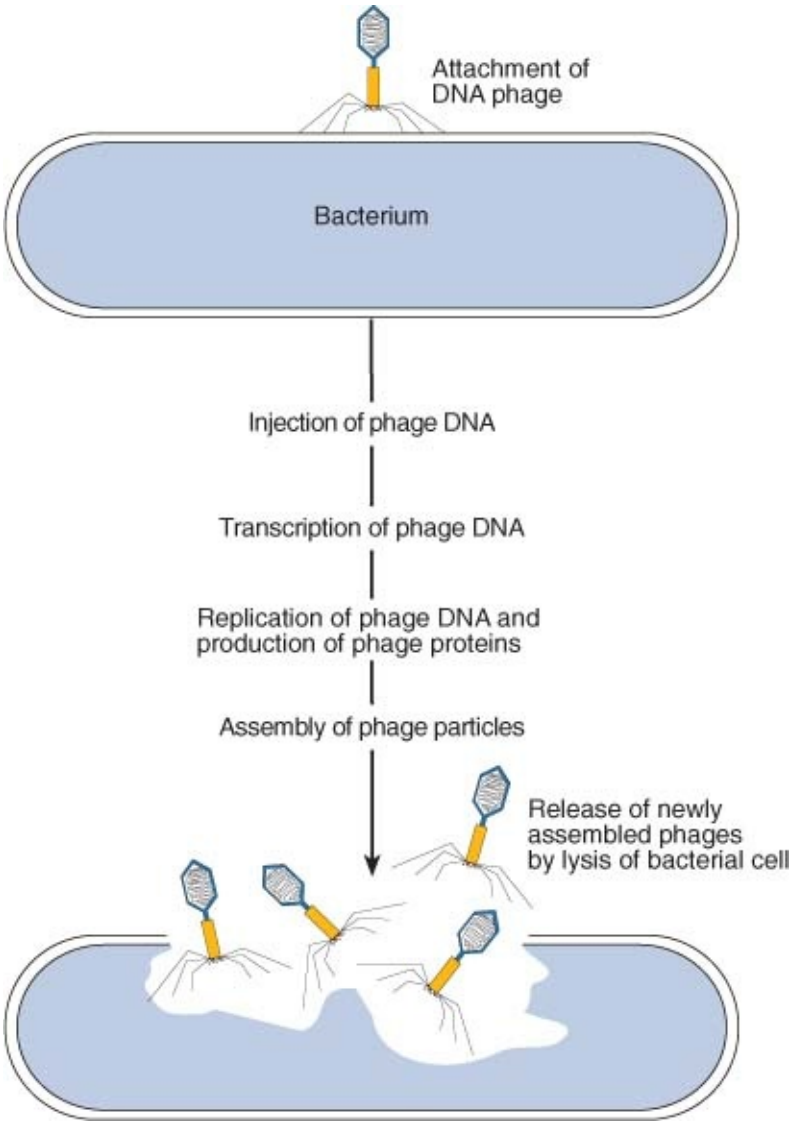
Figure 9.8 Lysogenic and lytic cycles of bacteriophages illustrating sporadic conversion of phages in the lysogenic cycle to lytic phages.



The phage genome may be composed of either single-stranded or double-stranded DNA or RNA. Replication of phages is similar in many respects to that of animal viruses (Fig. 9.9). However, the protein capsid usually remains outside the bacterial cell after introduction of phage nucleic acid into the cytoplasm. The host specificity of phages relates to chemical affinity between attachment structures on the phages and specific receptor sites on the bacterium. A repressor protein, encoded by a temperate phage, is synthesized following entry, and this repressor blocks the production of virion proteins. The DNA of temperate phages is incorporated into host genomes, usually at specific integration sites, and is transmitted to progeny bacteria during binary fission.

Insertion sequences and transposons

Figure 9.9 Replication of a double-stranded DNA phage. Attachment of phage to a specific receptor is followed by injection of phage DNA. Mature phages are released after lysis of the host cell.



Transposons are genetic elements that can move as a single unit from one replicon (chromosome, plasmid or bacteriophage) to another. This process is referred to as transposition. Transposons (Fig. 9.10) do not possess an origin of replication and only replicate as the host replicon into which they are inserted replicates. Transposons encode the necessary features to promote self-translocation but do not possess their own origin of replication. The frequency of transposition varies from 1 in 10^3 to 1 in 10^7 transposable elements per generation. Mechanisms involved differ from classical recombination in that there is little sequence homology between the transposon and the replicon into which they become inserted. Transposition could be described as a type of specific recombination as unique DNA sequences on the transposon, the inverted repeats located at the extremities of the structure and the target site for insertion,

are recognized by the transposase enzyme. An example of a simple transposon is an insertion sequence element denoted as IS (Fig. 9.10a), that contains only a transposase-encoding gene required for insertion into new locations. This gene of approximately 1,000bp is flanked by direct or inverted repeats. Several IS elements are known and these differ in the numbers of nucleotides they possess. Many bacteria possess multiple IS copies inserted at different locations throughout their genomes.

Some transposons consist of a gene encoding resistance to an antibiotic such as kanamycin in Tn5, as illustrated in Fig. 9.10b, flanked by two IS*50* elements, IS*50L* and IS*50R*. The former IS element is defective. Other transposons also contain antibiotic resistance genes as illustrated by Tn3 which encodes a β-lactamase gene (Fig. 9.10c) along with other transposase genes (*tnpA* and *tnpR*) required to catalyse the insertion and subsequent resolution events. Another example of an important complex transposon is Tn*1546* which encodes genes conferring resistance to the glycopeptide antibiotics vancomycin, teichoplanin and the formerly used growth promoter, avoparcin.

Insertion of a transposon into a gene essential for bacterial survival results in cell death.

Integrons and gene cassettes

Integrons (Fig. 9.11) are derived from transposon Tn*21* and these elements can capture antibiotic resistance encoding genes via an integron-encoded integrase (a member of the bacterial integrase superfamily) that catalyses a site-specific recombination. These integrons possess a conserved structure (CS) on the proximal end (known as the 5′-CS) containing an integrase gene (*int1*), a recombination site (*att1*) and a promoter (P_{ant}), along with a conserved distal region (3′-CS) containing a *qacEΔ1* conferring resistance to quaternary ammonium compound(s), which are used as disinfectants, and a *sul1* determinant conferring resistance to sulphonamides. These CS regions flank a variable central locus into which gene cassettes are recombined. Gene cassettes are composed of one or more open reading frames (orf) encoding antibiotic resistance gene(s) and a 59 base recognition sequence located at their 3′-end.

Figure 9.10 A schematic illustration showing three examples of mobile DNA elements. (a) An insertion sequence (IS) element, where the yellow colour of the bar represents the transposase-encoding gene and the open bars at each end depict flanking repeat sequences (direct in the upper case and indirect in the

lower as indicated by arrows). (b) An example of a transposon 5 (Tn*5*) showing a central kanamycin resistance gene flanked by two IS elements, denoted as IS*50*L and IS*50*R. (c) Tn*3* containing a *bla*$_{\text{TEM-1}}$ encoding gene along with *tnpA* transposase and *tnpR* resolvase genes.

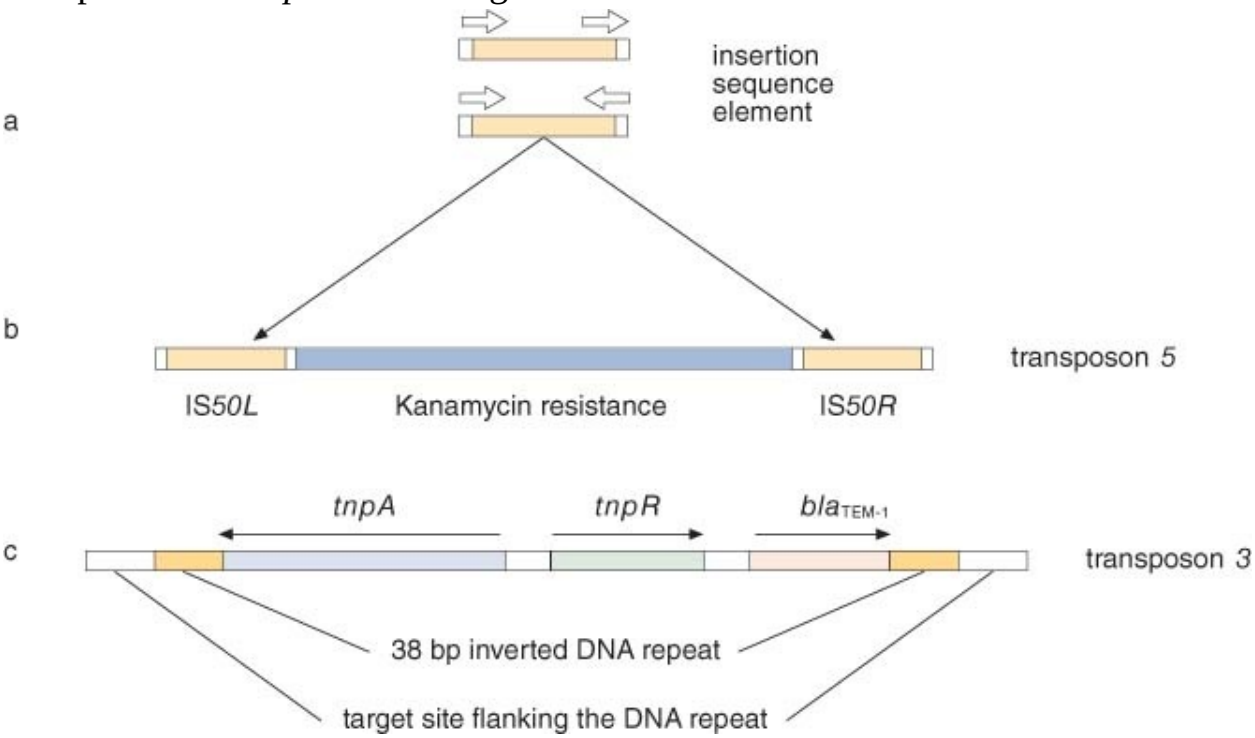
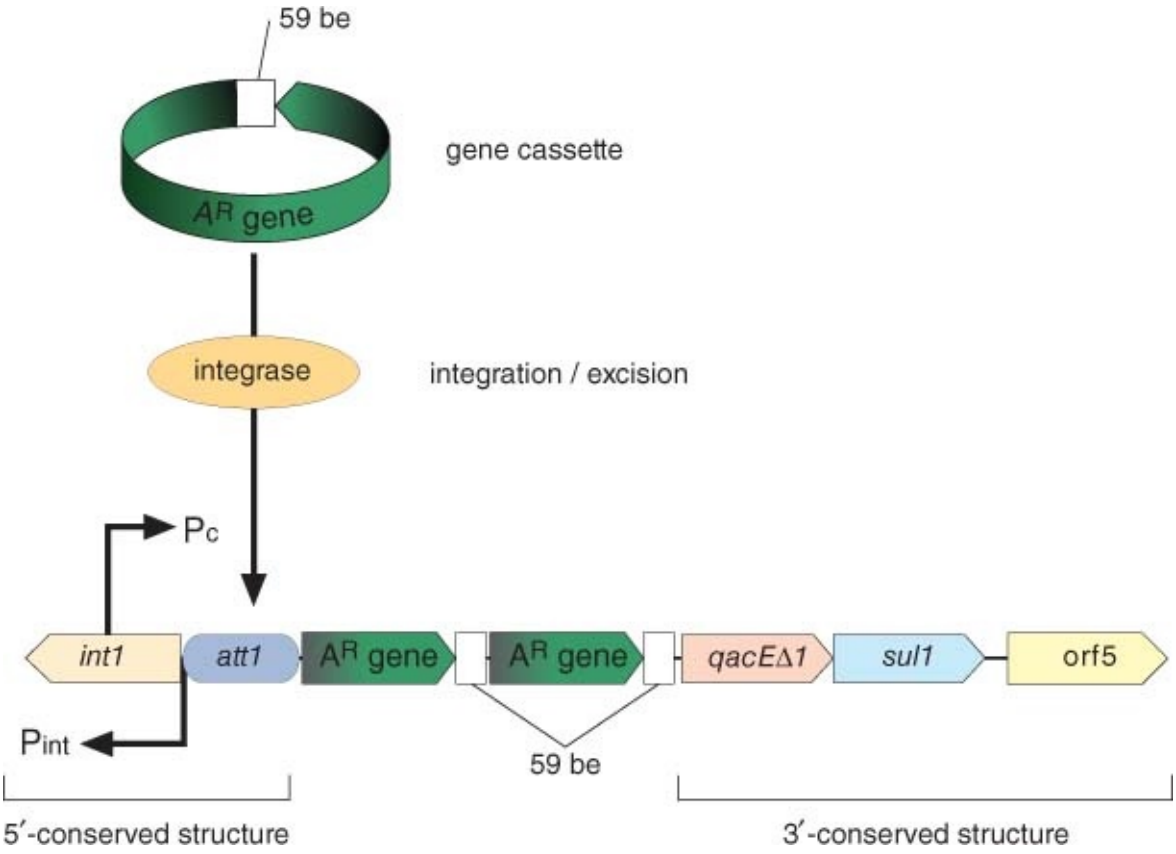


Figure 9.11 Structure of a typical class 1 integron. The 5′-conserved structure (CS) containing the *int1*-encoding integrase gene along with the *att1* site-specific recombination site are shown. Within the 3′-CS, the *qacEΔ1* and *sul1* genes are indicated. Located between these conserved regions is the gene cassette region, which shows two antibiotic resistance-encoding ORFs, denoted as A^R gene, along with associated 59 base elements (59 be) required for recombination at the *attI* site. An individual gene cassette is also shown as it undergoes site-specific recombination catalysed by *int1*.



Integrons are capable of capturing a variety of genes encoding resistance to antibiotics such as aminoglycosides, β-lactamases, chloramphenicol, erythromycin, rifampicin and trimethoprim, and contributing to their mobilization in response to environmental selective pressure. More than 60 different gene cassettes have been identified, with some integrons possessing multiple gene cassettes arranged in a classical ‘head- to-tail’ orientation. As these resistance determinants are under the control of a single strong upstream promoter (located towards the 3′ end of *int1*), all recombined gene cassettes are co-expressed. Therefore, selective pressure imposed by the use of a particular antibiotic can co-select for another resistance determinant located within an adjacent gene cassette. Additionally, exposing integron-containing bacteria to sub-inhibitory levels of biocides may co-select for antimicrobial resistance.

Genetic engineering of bacteria in the laboratory

Useful genetic characteristics encoded by genes in the DNA of a naturally

occurring organism can be cloned into a host bacterium in the laboratory, in a process referred to as genetic engineering. These genes can be inserted into plasmids forming recombinant plasmids. They can then be introduced into bacterial cells (usually by transformation) and propagated. The DNA fragments carrying the genes that are selected are produced by either cleaving the donor DNA containing them, using suitable restriction endonuclease enzymes or through direct amplification by the polymerase chain reaction (see Chapter 6). Restriction enzymes, which cleave the nucleic acid asymmetrically, produce DNA fragments with cohesive or 'sticky' ends. If recipient plasmid DNA is cut using the same restriction endonuclease as that used for donor DNA, the cohesive ends of the donor and plasmid DNA will be complementary. The donor DNA fragment can be incorporated using DNA ligase into the cleaved plasmid, and in the process the plasmid is then restored to its circular form (Fig. 9.12).

Propagation of the host bacterial cell produces a population of identical cells, known as a clone, in which each cell contains a copy of the new recombinant genetic material. The plasmid used to introduce the new genes is termed the cloning vector. Recombinant plasmids containing the gene of interest express the desired trait. Plasmids are used as cloning vectors because they replicate independently without integration into the bacterial chromosome. Some suitable bacteriophages can also be used as cloning vectors.

Genetic engineering is currently used for the production of vaccines, hormones and other pharmaceutical products (see Chapter 5). Vaccines produced in this manner are potentially safer than conventional vaccines. The genes that code for the vaccine antigens can be cloned separately from genes encoding replication of the parent organism. Genetically engineered vaccines may therefore stimulate an effective immune response without the risk of introducing a pathogen capable of replicating in the vaccinated animals.

Genetic databases and bioinformatics

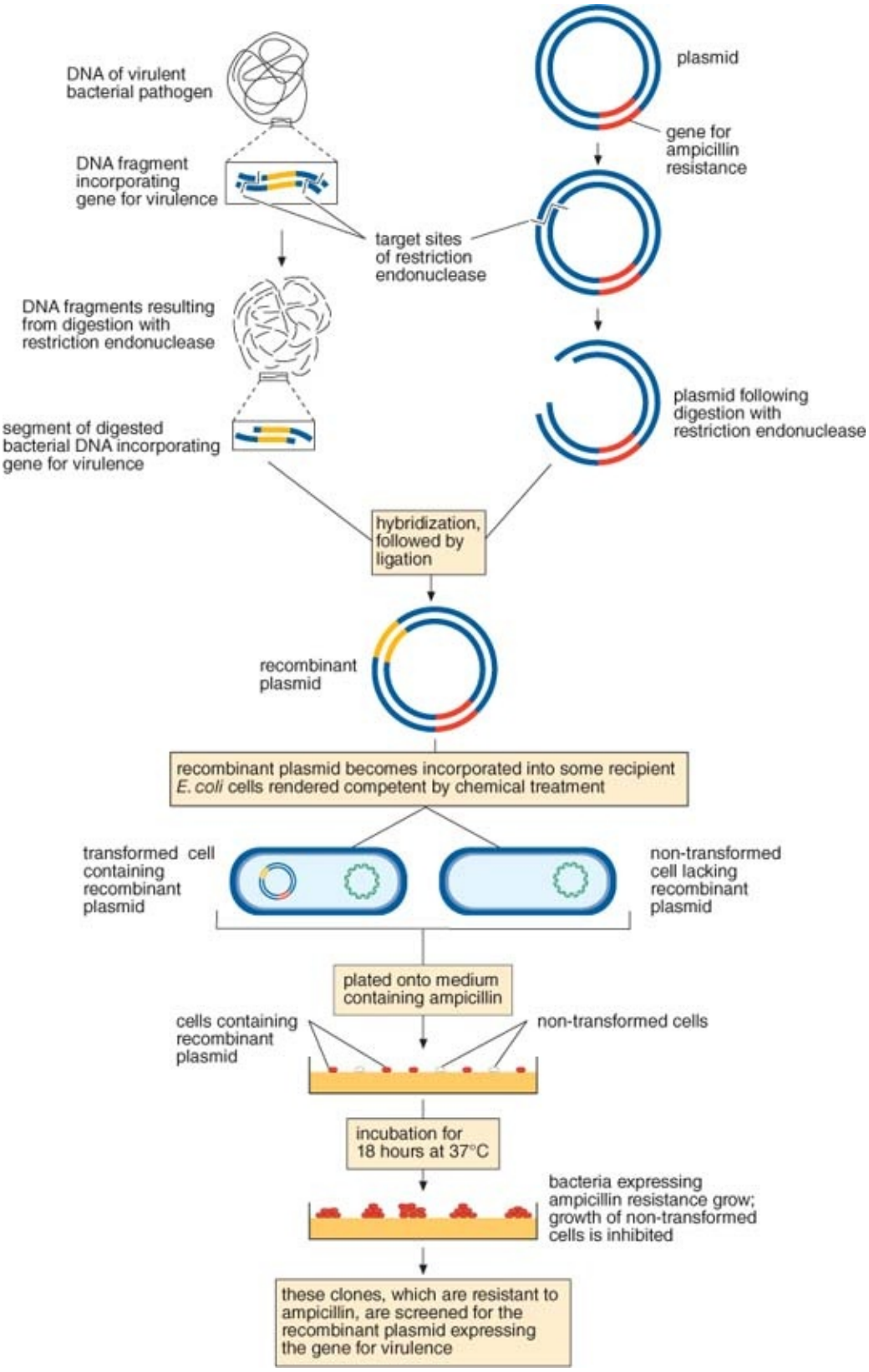
In 1977 the entire DNA sequence of the phage Φχ174 was first published. Since that time there has been an exponential increase in DNA sequence information submitted to gene databases around the world. With increasing volumes of data entries, including whole- genome sequences for bacteria and other microorganisms, the first of which was *Haemophilus influenzae* (1.8 Mbp) completed in 1995, it has become impractical to analyse this information by manual methods. This has necessitated the development of specific

computational tools to analyse DNA information and identify genes and their corresponding polypeptide sequences along with regulatory features at a molecular level.

Bioinformatics is a new scientific discipline that relates to the development of computer algorithms and statistical techniques for analysing and managing genetic information. These tools facilitate the rapid annotation of genome sequences with identification of the position of genes within the genome leading to the identification of genes involved in disease production.

Companies involved in the manufacture of pharmaceutical and diagnostic reagents often use bioinformatics to ‘data mine’ genomes, in an attempt to identify new therapeutic agents or useful diagnostic markers.

Figure 9.12 Recombinant nucleic acid technology. Steps required to clone a gene and create a recombinant DNA molecule. Plasmid DNA is cleaved with a restriction endonuclease. Foreign DNA, containing the gene of interest (in this instance a gene for virulence), is similarly digested. The digested molecules, plasmid and gene of interest are mixed or hybridized, and the gaps in the DNA molecules are sealed with DNA ligase, forming the recombinant DNA molecule. This recombinant molecule is subsequently incorporated into a bacterium and a colony of the clone containing the recombinant DNA is formed during culture.



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Chapter 10

Laboratory diagnosis of bacterial disease

Laboratory investigation of bacterial disease is necessary for identifying the aetiological agent and, sometimes, for determining the antimicrobial susceptibility of pathogens. A full clinical history including the age, sex, species and number of animals affected and treatment administered should accompany the specimens, together with a tentative clinical diagnosis. In the absence of adequate clinical information, procedures for the detection of relevant pathogens may not be carried out.

Selection, collection and transportation of specimens

The accuracy and validity of the results of laboratory examinations are largely influenced by the care taken in the selection, collection and submission of samples to the laboratory. The following points should be considered when dealing with clinical specimens:

- Ideally, specimens should be obtained from live animals before administration of antimicrobial therapy. Samples from dead animals should be collected, if possible, before autolytic or putrefactive changes occur.
- Specimens from a site most likely to yield a pathogen should be collected using procedures that minimize contamination.
- In warm weather, refrigeration of samples may be required. Samples for bacterial culture should not be frozen.
- Samples must be submitted in separate leak-proof containers. Each container should be labelled with the identity of the animal, the type of specimen and the date of collection. Specific regulations for transport of diagnostic specimens by public and private companies are in place in most jurisdictions and must be implemented. Secondary and tertiary packaging in addition to the specimen container is usually required.

- For particular procedures, specimens collected must be suitable for the technique to be carried out: for example, formalin-fixed sections are generally unsuitable for fluorescent antibody staining.

Identification of pathogenic bacteria

The presence of pathogenic bacteria can be confirmed by examination of stained smears, cultural and biochemical characteristics and detection by immunological and molecular methods.

Examination of stained smears

Staining methods routinely used in diagnostic bacteriology are presented in Table 10.1. Gram-stained smears from tissues or exudates are useful rapid procedures for demonstrating bacteria present in large numbers. The contrast between Gram-positive bacteria and tissue debris is marked, rendering them easier to detect in smears than Gram-negative organisms. The Ziehl-Neelsen stain is used to detect pathogenic mycobacteria. *Coxiella burnetii*, *Brucella* species, *Nocardia* species and chlamydiae can be demonstrated in smears using the modified Ziehl-Neelsen stain. The fluorescent antibody staining method gives rapid, specific identification of bacterial pathogens in smears and cryostat tissue sections. Although this technique is suitable for identifying many bacterial species, it is particularly useful for pathogens such as *Clostridium chauvoei*, spirochaetes, *Campylobacter fetus* and *Lawsonia intracellularis*, which are difficult to culture.

Table 10.1 Routine staining methods for bacteria.

Method	Comments
Gram stain	Widely used for the routine staining of bacteria in smears. The crystal violet, which is retained in cell walls despite decolorization, stains Gram-positive bacteria blue. In contrast, Gram-negative bacteria which do not retain the crystal violet are counterstained red
Giemsa stain	Useful for demonstrating *Dermatophilus congolensis*, rickettsiae and *Borrelia* species which stain blue
Dilute carbol fuchsin	Especially useful for recognizing *Campylobacter* species, *Brachyspira* species and *Fusobacterium* species which stain red
Polychrome methylene blue	Used for the identification of *Bacillus anthracis* in blood smears. The organisms stain blue with distinctive pink capsules
Ziehl-Neelsen stain	Hot concentrated carbol fuchsin which penetrates mycobacterial cell walls is retained after acid-alcohol decolorization. The red-staining bacteria are described as acid-fast or Ziehl-Neelsen positive
Modified Ziehl-Neelsen stain	Unlike the Ziehl-Neelsen stain, this method employs dilute carbol fuchsin with decolorization by acetic acid

Cultural and biochemical characteristics

The selection of culture medium, atmospheric conditions and other features essential for isolation are determined by the pathogenic bacterium suspected. Routine isolation of many pathogens involves inoculation of blood agar and MacConkey agar plates followed by incubation for 24 to 48 hours at 37°C.

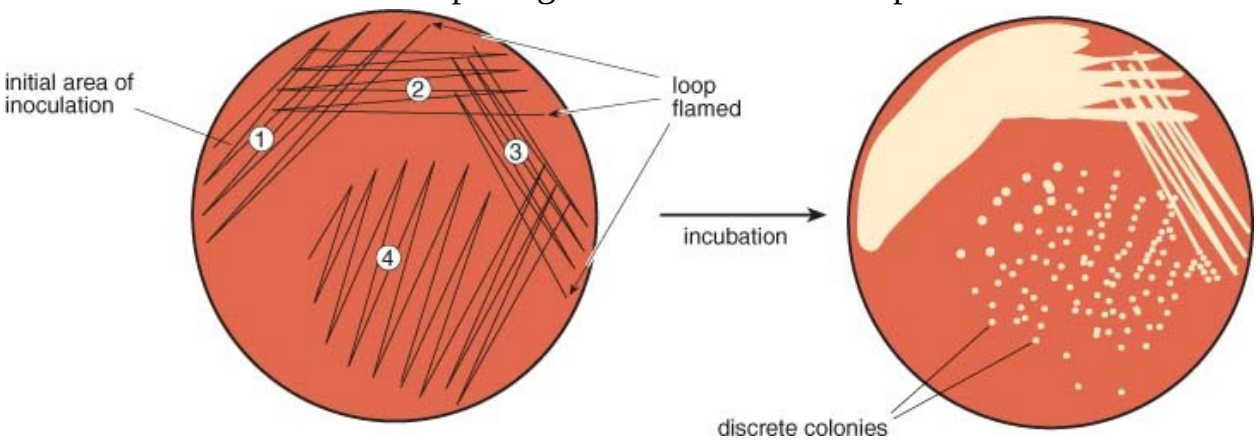
Media used in diagnostic bacteriology are indicated in Table 10.2. Nutrient agar is a basic medium which supplies essential nutrients for the growth of non-fastidious bacteria. However, nutrient agar is unsuitable for the primary isolation of fastidious pathogenic bacteria. The growth characteristics and reactions on blood agar and MacConkey agar form the basis for the preliminary identification of many bacterial pathogens. Blood agar, which supports the growth of most pathogens, is appropriate for routine primary isolation. Selective media may be used for particular organisms. Some media are designed to give a presumptive identification of bacterial colonies on the basis of bio-chemical reactions. MacConkey agar, which contains bile salts, is selective for many Gram-negative bacteria. This medium contains lactose with neutral red as a pH indicator. If an organism growing on this medium ferments lactose, the acidic byproducts turn the medium pink. Non-lactose fermenting bacteria metabolize the peptones in the medium, generating alkaline byproducts which impart a yellowish coloration to the medium and the colonies.

Table 10.2 Laboratory media used for the isolation and presumptive identification of bacterial pathogens.

Medium	Comments
Nutrient agar	A basic medium on which non- fastidious bacteria can grow. Suitable for demonstrating colonial morphology and pigment production; also used for viable counting methods
Blood agar	An enriched medium which supports the growth of most pathogenic bacteria and is used for their primary isolation. Allows the recognition of bacterial haemolysin production
MacConkey agar	A selective medium containing bile salts which is especially useful for isolation of enterobacteria and some other Gram-negative bacteria. Allows differentiation of lactose fermenters and non-lactose fermenters. Colonies of lactose fermenters and the surrounding medium are pink
Selenite broth, Rappaport-Vassiliadis broth	Selective enrichment media used for the isolation of salmonellae from samples containing other Gramnegative enteric organisms
Edwards medium	A blood agar-based selective medium used for the isolation and recognition of streptococci
Chocolate agar	Heat-treated blood agar which supplies special growth requirements (X and V factors) for the isolation of *Haemophilus* species and for the culture of *Taylorella equigenitalis*
Brilliant green agar	An indicator medium for the presumptive identification of *Salmonella* species. Salmonella colonies and surrounding medium have a red colour
Buffered peptone water	A non-selective enrichment medium often used for isolation of pathogens when present in low numbers in samples collected from foods and environmental sources

Figure 10.1 Plate inoculation technique for obtaining isolated colonies on an

agar medium. With a sterile inoculating loop, a sample of the specimen (the inoculum) is spread over a small area at the edge of the plate, the ‘well’ (1). The inoculum is spread from the well sequentially over three contiguous areas of the plate (2, 3, 4). The loop is sterilized by flaming before inoculation of each area. When carried out carefully, the procedure results in a reduction in bacterial numbers at each step. In area 4, discrete bacterial colonies can be recognized after incubation. The loop should be sterilized by flaming on completion of inoculation to ensure that no pathogens survive on the loop.



Plates should be inoculated using a streaking technique which facilitates growth of isolated colonies (Fig. 10.1). This is an essential step for the identification of pathogens in clinical specimens which may contain microbial contaminants. Such contaminants may derive from the normal flora or from environmental sources. Definitive identification of a potential pathogen involves subculture of an isolated colony to obtain a pure growth which can then be subjected to biochemical or other tests.

Morphological characteristics and biochemical tests allow presumptive identification of a bacterial pathogen (BOX 10.1). Additional features, which may aid identification, include pigment and odour production on both blood agar and MacConkey agar, and the production of haemolysis on blood agar. Definitive identification of bacteria is usually based on biochemical or molecular tests and serology. Additional tests can be used to aid identification of particular organisms (Table 10.3).

Biochemical techniques

Catalase, an enzyme produced by many aerobes and facultative anaerobes, causes the breakdown of hydrogen peroxide to oxygen and water. A positive oxidasetest indicates the presence of cytochrome oxidase C in the bacterial cell.

Reactions in oxidation–fermentation medium can be used to identify the atmospheric requirements of certain pathogens (Fig. 10.2).

BOX 10.1 Criteria for the presumptive identification of bacterial pathogens.

- Colonial morphology and colour
- Presence or absence of haemolysis on blood agar
- Appearance when stained by the Gram method
- Motility
- Ability to grow on MacConkey agar
- Reaction in the oxidation–fermentation test
- Reactions in catalase and oxidase tests

Biochemical tests relate to the catabolic activities of bacteria, and an indicator system is usually employed to demonstrate the utilization of a particular substrate (Table 10.4). Because the range of sugars utilized by individual bacterial species is usually limited, catabolism of different sugars is frequently used for identification. Several commercial companies produce miniaturized versions of biochemical tests for the identification of bacteria. These usually consist of a strip of plastic cupules containing the requisite reagents for each test to which a suspension of the bacterium for identification is added. The identity of the organism can be deduced from the pattern of the reactions in the cupules. Strips are available for different categories of bacteria including the enterobacteria, non-enteric Gram-negative organisms, anaerobes, staphylococci and streptococci.

Table 10.3 Tests used in the identification of particular bacterial pathogens.

Test	Pathogens	Comments
CAMP reaction	*Streptococcus agalactiae*	Haemolysis caused by *Staphylococcus aureus* is enhanced by pathogenic bacteria growing close to staphylococcal colonies
	Rhodococcus equi	
	Actinobacillus pleuropneumoniae	
	Listeria monocytogenes	
Pitting of Loeffler's serum slope	*Arcanobacterium pyogenes*	Proteolytic digestion of the medium around colonies
Haemagglutination	*Bordetella bronchiseptica*	Agglutination of suspended ovine red blood cells by the bacteria

Nagler test	*Clostridium perfringens*	Breakdown of lecithin in egg yolk agar by alpha toxin (lecithinase) produced by the organism. Surface application of antitoxin inhibits the alpha toxin activity

Figure 10.2 The possible reactions produced by bacteria in oxidation–fermentation medium which has a green colour before inoculation (indicator: bromothymol blue).

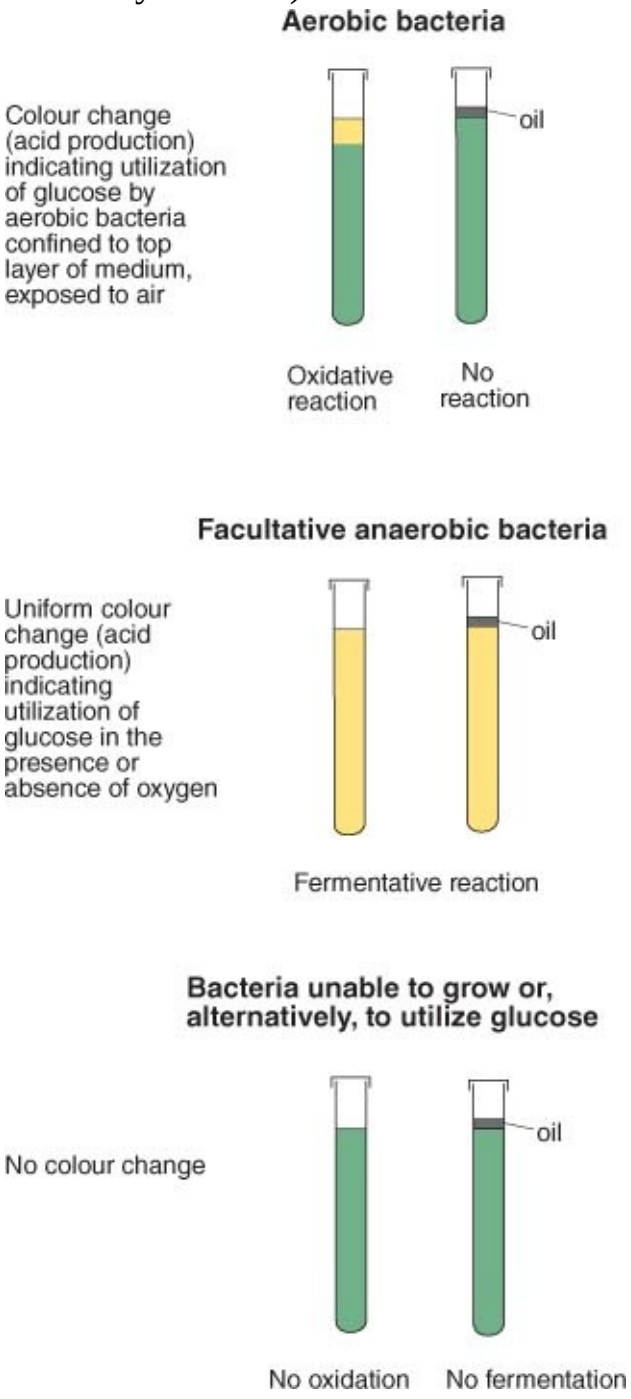


Table 10.4 Biochemical tests used for the presumptive identification of bacterial pathogens.

Test	Indicator	Comments
Sugars in peptone water	Andrade's	Used for differentiating *Streptococcus* species
Triple sugar iron	Phenol red	Used for presumptive identification of *Salmonella* species
Hydrogen sulphide production	Iron or lead compounds	Employed in tests for *Salmonella* and *Brucella* species
Decarboxylase	Bromocresol purple	Used for presumptive identification of enterobacteria
Urease	Phenol red	Used for the presumptive identification of *Proteus* species and *Corynebacterium renale*
Indole test Methyl red test Voges-Proskauer test Citrate utilization	Kovac's reagent Methyl red Oxidation of acetoin Bromothymol blue	Used for identification of enterobacteria; collectively known as IMViC tests

Immunological techniques

Serotyping is based on the immunological identification of surface antigens on pathogens such as *Escherichia coli* and other members of the *Entero-bacteriaceae*, *Listeria monocytogenes, Pasteurella multocida* and *Actinobacillus pleuropneumoniae*.

Immunological techniques such as fluorescent antibody staining can be used for identifying bacterial

pathogens. Antigen capture and direct enzyme-linked immunosorbent assays have been developed for some bacterial pathogens and require the immobilization of specific antibody on a solid phase. The bacterial agent, if present in the diagnostic specimen, is bound by the specific antibody and can be demonstrated by an enzyme-labelled antibody. Techniques using immune reactions may be combined with other methods for improving detection of pathogens. Immunomagnetic separation, in which magnetic particles coated with antibodies to the particular pathogen bind the organism, combines physical and immunological methods. Immunomagnetic separation is usually followed by either cultural identification or molecular characterization of the organism.

Phage typing

Techniques employing a standardized set of well characterized lytic phages can be used for the further identification of human and veterinary pathogens. The fact that a particular phage is lytic for a limited number of susceptible strains of bacteria allows differentiation by phage typing. The pattern of susceptibility of a bacterial isolate, tested against a panel of typing phages, establishes its phage type, such as phage type DT104 in the case of the multi-drug resistant *Salmonella* Typhimurium. Some phages, which lyse all members of a bacterial species, can be used to identify organisms to species level. More often, phages

infect only certain strains of a bacterial species and they can be used to characterize organisms at subspecies level.

Phage typing is frequently used on isolates of *Staphylococcus aureus*, *Salmonella* Typhimurium and *Salmonella* Enteritidis to provide additional information for the identification and tracing of sources of infection in outbreaks of food-poisoning.

Molecular techniques

Selected molecular techniques can be used for the detection and enumeration of pathogenic bacteria. These techniques, along with phage typing and serotyping, may also be of use in epidemiological investigations. In addition, molecular techniques assist in determining the virulence of an isolate by identifying genes associated with pathogenic properties.

The main molecular techniques for pathogen detection are described in Chapter 6 and include nucleic acid hybridization and the polymerase chain reaction (PCR). In nucleic acid hybridization, synthetic nucleic acid probes, specific for a particular pathogen, are applied either to prepared clinical specimens or to genetic material extracted from the pathogen. Probes can be designed to detect DNA or RNA. However, the usefulness of RNA probes is limited by the lability of the RNA molecule. Nevertheless, diagnostic tests based on the detection of RNA can be particularly useful in specific areas such as food microbiology because they allow discrimination of viable from dead microorganisms. Probes can be designed to detect all members of a particular genus or to detect strains of organisms within a species. For example, a probe for the detection of the gene which encodes 16S ribosomal RNA can often detect all members of a genus because it is highly conserved in the species within a genus. In contrast, intergenic regions display more variability and are useful for designing probes to discriminate between different strains within a species.

Assays based on the direct detection of DNA or RNA are relatively insensitive because they usually require large numbers of bacteria (10^4 to 10^5) in the specimen. For specimens containing small numbers of bacteria, amplification of the nucleic acid of the target organisms by PCR can be used. After amplification of a specific fragment of DNA, using either a DNA or RNA template, the PCR product can then be identified by its electrophoretic pattern using appropriate size- marker molecules.

Methods employed for epidemiological investigations are described in detail in

Chapter 6. The selected technique must be convenient to use and must discriminate between closely related strains by detecting genetic differences of epidemiological significance. Restriction endonucleases can be used to cleave chromosomal or plasmid DNA to generate fragments which can then be separated by gel electrophoresis. Analysis of the resulting electrophoretic patterns allows comparison of isolates. Restriction enzymes which cleave DNA in only a few places produce large fragments which can be separated using pulsed-field gel electrophoresis, a method frequently used in epidemiological studies.

Serology

Many potentially pathogenic bacteria are present as part of the normal flora of a host or are common in the environment. As animals are frequently exposed to such bacteria, they may produce antibodies to these organisms. Antibodies demonstrable in a serum sample are therefore evidence of exposure to an infectious agent but they do not necessarily confirm an aetiological role for that agent in the animal sampled. Despite these limitations, serological tests are used extensively for confirming infection with particular pathogens in susceptible animals. Collection of two serum samples approximately 2 weeks apart and demonstration of a four-fold rise in antibody titre are

indicative of recent exposure to an infectious agent and, together with relevant clinical signs, are frequently accepted as confirmation of infection with a particular pathogen.

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Chapter 11

Antibacterial agents

Antibiotics are low molecular weight microbial metabolites which can kill or inhibit the growth of susceptible bacteria. The term ‘antibiotic’ refers to drugs used to treat infectious diseases in animals, humans and plants, and these substances may be naturally occurring, semi-synthetic or synthetic.

Development of chemotherapy for bacterial infections

The convincing experimental work of Louis Pasteur and the meticulous experiments carried out by Robert Koch in the 1880s eclipsed the prevailing notions that infectious diseases were caused by disturbances in the upper atmosphere, chemical poisons or unspecified evil influences. The germ theory of disease, promoted by Pasteur and Koch, stimulated an interest not only in new studies on immunity to infectious agents but also on the treatment of infectious diseases caused by bacteria. Progress was slow and only a small number of scientists took up the challenge of investigating chemicals for the treatment of bacterial infections. Of these, Paul Ehrlich, a German medical scientist with considerable experience in immunology, undertook a detailed evaluation of hundreds of chemicals as potential therapeutic agents. In the field of immunology, Ehrlich had already proposed the side-chain theory of antibody production which was a revolutionary concept for the early 1900s. Ehrlich reasoned that there must be chemicals, which he referred to as ‘magic bullets’, with the ability to attach to specific microorganisms but not to host cells. The 606th compound that Ehrlich synthesized, the famous drug, arsphenamine (‘Salvarsan’), was an arsenical compound with selective toxicity: it was active against the bacteria causing syphilis in rabbits, monkeys and humans but was relatively non-toxic for recipients. Arsphenamine proved difficult to use, however, because of its instability and the need to administer it intravenously. Despite these limitations, arsphenamine pointed the way towards more effective

chemotherapeutic agents.

A period of pessimism followed Ehrlich's work on syphilis, and many scientists were of the opinion that antibacterial chemotherapy was an unrealistic expectation. Further significant developments in chemotherapy did not occur for almost 30 years after Ehrlich's pioneering experiments. In 1927, a German biochemist, Gerhard Domagk, continuing Ehrlich's investigation of chemicals (especially dyes), made an important discovery. A new dye for staining leather, Prontosil Red, was non-toxic for mice and protected them against streptococcal infections. The dye failed, however, to inhibit cultures of bacteria growing on laboratory media. The results of Domagk's experiments were published in 1935 and that same year French scientists showed that Prontosil Red was converted in the body to sulphonilamide. It was shown subsequently that sulphonilamide was active against bacteria in the body and also against bacterial cultures growing on laboratory media. The discovery of sulphonilamide stimulated widespread research on related compounds with therapeutic activity and, by 1945, thousands of derivatives of sulphonilamides, collectively called sulphonamides, had been evaluated for their toxicity and antibacterial activity. Many of these sulphonamides, which were less toxic and more effective than sulphanilamide, are still used for treating bacterial infections.

Arsphenamine and sulphonamides are examples of synthetic chemotherapeutic agents made by chemists. A second category of chemotherapeutic agents, naturally occurring compounds produced by microorganisms, had been recognized as early as the 1870s. These natural chemotherapeutic agents produced by microorganisms inhibit the growth of other microorganisms and are referred to as antibiotics. The first antibiotic used therapeutically was penicillin. The discovery of penicillin in 1929 by Alexander Fleming, a Scottish physician, heralded a new era in chemotherapy, the age of antibiotics. Although penicillin was actually discovered in 1896 by a French medical student, Ernest Duchesne, his work was forgotten. Fleming's rediscovery of penicillin, produced by the fungus *Penicillium*, proved to be highly significant. This naturally occurring compound was effective against many pathogenic bacteria in culture and was much less toxic than sulphonamides. Despite the importance of his observation, Fleming's subsequent experiments suggested to him that penicillin would not remain active for a sufficient length of time after injection into the body to destroy pathogens. He discontinued his research on penicillin in 1931. The major problem with the evaluation of penicillin was the difficulty of obtaining sufficient quantities of the antibiotic in a purified form for clinical trials.

With the outbreak of World War II, the need for treatment of wound infections in Allied soldiers intensified and the potential of penicillin as a chemotherapeutic substance was gradually realized. Through the cooperative efforts of Fleming with Ernest Chain, Howard Florey and co-workers, large-scale production, purification and testing of penicillin proceeded apace. With the participation of the US pharmaceutical industry, large quantities of penicillin became available for human use in the early 1940s. The dramatic success of this newly discovered antibiotic for the treatment of infections in soldiers and civilians was received with enthusiasm by the general population and stimulated interest in the search for other antibiotics. In 1944, Selman Waksman announced that he and his associates had found a new antibiotic, streptomycin, produced by the actinomycete, *Streptomyces griseus*. This discovery resulted from screening thousands of bacteria and fungi present in soil for their ability to produce antibiotics. Over the succeeding decade, microorganisms producing tetracyclines, neomycin and chloramphenicol were isolated (Table 11.1 and Fig. 11.1). The search for new and more effective antibiotics continued into the 1960s and subsequently entered a period of decline. This decline was attributed in part to the pharmaceutical industry's response to the negative influence of drug pricing and also to the exacting criteria set by regulatory authorities to ensure efficacy and safety of new antibiotics before their release on to the market.

Table 11.1 Antimicrobial agents produced by microorganisms.

Microorganism	**Antimicrobial agent**
Paenibacillus polymyxa (var. *colistinus)*	Colistin (polymyxin E)
Paenibacillus polymyxa	Polymyxin B
Bacillus subtilis	Bacitracin
Cephalosporium species (F)	Cephalosporins
Chromobacterium violaceum	Monobactams
Micromonospora echinospora	Gentamicin
Penicillium notatum (F) and other species	Penicillin G
Penicillium griseofulvin (F)	Griseofulvin (anti-fungal activity only)
Streptomyces species	Spectinomycin, tetracyclines
Streptomyces cattleya	Carbapenems
Streptomyces fradiae	Neomycin
Streptomyces griseus	Streptomycin
Streptomyces. kanomyceticus	Kanamycin
Streptomyces lincolnensis	Lincomycin
Streptomyces venezuelae	Chloramphenicol
Streptomyces nodosus	Amphotericin B (anti-fungal activity only)
Saccharopolyspora erythraea	Erythromycin
Amycolatopsis orientalis	Vancomycin

Amycolatopsis mediterranei	Rifamycin

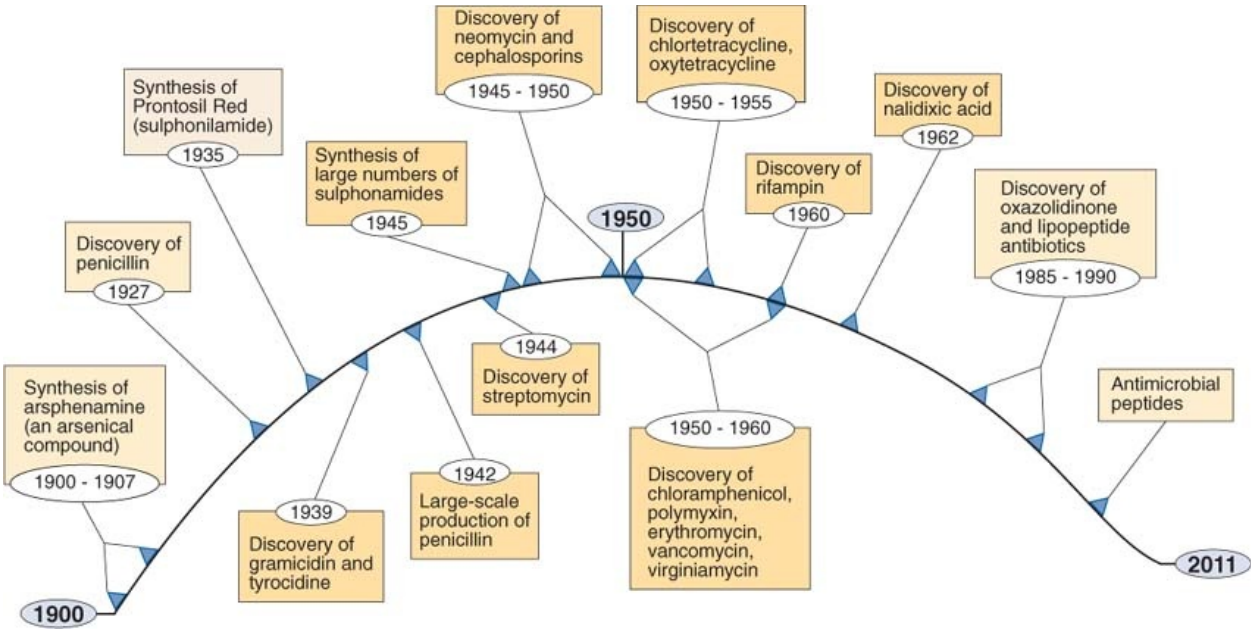
(F), fungus.

It was widely considered that, by the 1970s, antibiotic therapy had signaled the end of bacterial infections as a significant cause of mortality in human and animal populations. However, this premature optimism was dispelled by the emergence of antibiotic resistance in many bacterial pathogens, an intractable problem resulting from the widespread use of these drugs.

Mode and site of action

The therapeutic use of antibiotics depends on their selective toxicity: these drugs can either kill (*bactericidal*) or inhibit (*bacteriostatic*) bacterial pathogens without direct toxicity for animals receiving treatment. The basis of the selective toxicity of many antibiotics is poorly understood. However, biochemical differences in cellular structures and/or metabolic pathways between mammalian and bacterial cells frequently account for selective antibacterial toxicity. Penicillin, for example, inhibits cell wall synthesis by acting on peptidoglycan, a component unique to bacterial cell walls. Only a small percentage of the large number of known antibiotics exhibit sufficient selective toxicity to be therapeutically useful. Individual antibacterial agents are not effective against all pathogenic bacteria. Some are active against a narrow range of bacterial species while broad-spectrum antibiotics such as the tetracyclines and chloramphenicol are active against many species.

Figure 11.1 Major developments relating to the synthesis of antibacterial compounds and the discovery of antibiotics, from 1900 onwards.



In order to interfere with bacterial cell growth, antibacterial agents must interact with a vital structure or block a metabolic pathway. The modes and sites of action of antibacterial drugs are indicated in Fig. 11.2. Bacteriostatic agents inhibit the growth of bacteria, allowing host immune defences to eliminate the infection. If this type of therapeutic agent is not maintained at effective concentrations in the tissues, dissociation of the drug/cell structure complex can occur, permitting bacterial survival. In contrast, bactericidal agents cause irreparable damage and bacterial cell death by binding irreversibly to target structures. At high concentrations, some bacteriostatic agents can be bactericidal. Antibacterial agents may inhibit the synthesis of cell walls, nucleic acids or proteins. In addition, they may disrupt cell membrane function. The major classes of antibacterial drugs and their modes of action are listed in Table 11.2.

Inhibition of cell wall synthesis

Because peptidoglycan is a unique component of bacterial cell walls, antibacterial agents that prevent cross- linking of peptidoglycan chains inhibit cell wall synthesis and are selectively toxic for bacteria. The penicillins and cephalosporins comprise the largest and most important class of antibacterial drugs that inhibit cell wall synthesis. Their bactericidal activity relates to this effect in actively growing cells. The basic structure of β-lactam antibiotics is illustrated in Fig. 11.3. Semi-synthetic penicillins and cephalosporins can be produced by incorporating various chemical side chains into the basic molecules.

Differences in side chains of the particular antibiotic influence their spectrum of activity, stability and resistance to β- lactamases. The mode of action of β-lactam antibiotics involves binding to cell receptors known as penicillin binding proteins (PBPs). In addition to interfering with transpeptidation, many of these drugs promote autolysin activity causing cell lysis.

Because β-lactamases cleave the β-lactam ring rendering the antibiotic ineffective, bacteria that produce these enzymes are resistant to β-lactam antibiotics. These enzymes may be plasmid-mediated as in staphylococci, or they may be chromosomally encoded as in many Gram-negative bacteria. Tolerance to β-lactam antibiotics exhibited by some bacteria may relate to an inability of the antibiotic to induce autolysin activity. In these circumstances, although the cell wall is damaged and growth is inhibited, the bacteria survive. Differences in the structure and composition of the cell walls of Gram-positive and Gram-negative organisms determine their susceptibility to β-lactam antibiotics. Because some antibacterial agents cannot penetrate the outer membrane of Gramnegative cells, their antimicrobial spectrum is confined to Gram-positive bacteria.

Figure 11.2 Modes and sites of action of antibacterial drugs.

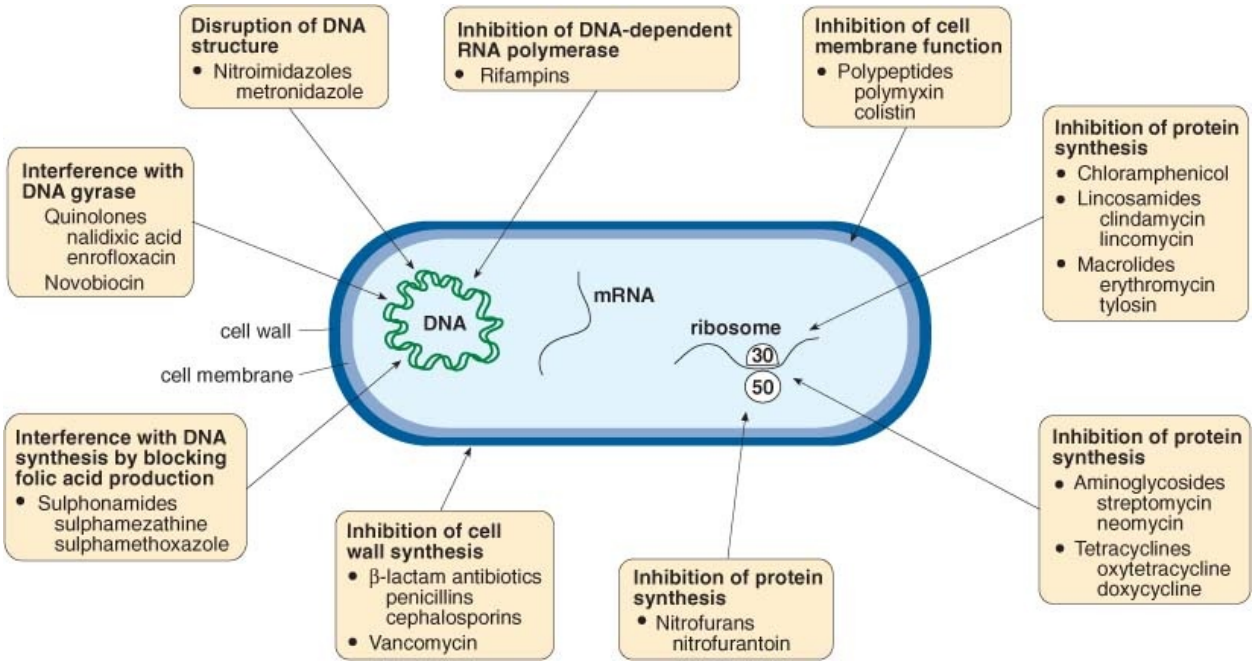
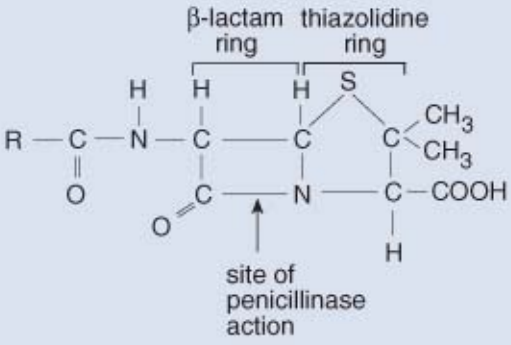


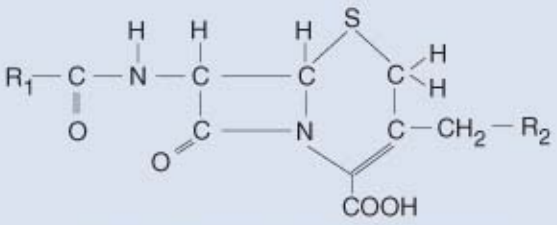
Table 11.2 Major classes of antimicrobial drugs and their modes of action.

Antimicrobial drug	Mode of action	Effect	Comments
β-Lactam antibiotics Penicillins Cephalosporins	Inhibition of cell wall synthesis	Bactericidal	Low toxicity. Many are inactivated by β-lactamases
Glycopeptides Vancomycin Teicoplanin	Inhibition of cell wall synthesis	Bactericidal	Used against methicillin-resistant *Staphylococcus aureus*
Polypeptides Polymyxin Colistin	Inhibition of cell membrane function	Bactericidal	Resistance slow to develop. Potentially nephrotoxic and neurotoxic
Nitrofurans Nitrofurantoin	Inhibition of protein synthesis	Bacteriostatic	Synthetic agents with broad-spectrum activity. Relatively toxic
Aminoglycosides Streptomycin Neomycin	Inhibition of protein synthesis. Block 30S ribosomal activity	Bactericidal	Active mainly against Gram-negative bacteria. Ototoxic and nephrotoxic
Tetracyclines Oxytetracycline Doxycycline	Inhibition of protein synthesis. Block 30S ribosomal activity	Bacteriostatic	Formerly used in feed for prophylactic medication. Development of resistance common
Lincosamides Clindamycin Lincomycin	Inhibition of protein synthesis. Block 50S ribosomal activity	Bactericidal or bacteriostatic	May be toxic in many species. Contraindicated in horses and neonatal animals. Oral administration is hazardous in ruminants
Macrolides Erythromycin Tylosin	Inhibition of protein synthesis. Block 50S ribosomal activity	Bacteriostatic	Active against Gram-positive bacteria. Some macrolides active against mycoplasmal pathogens
Quinolones/fluoroquinolones Nalidixic acid Enrofloxacin	Inhibition of nucleic acid synthesis by blocking DNA gyrase	Bactericidal	Synthetic agents used for treating enteric infections and for intracellular pathogens
Novobiocin	Inhibition of nucleic acid synthesis by blocking DNA gyrase	Bactericidal or bacteriostatic	Often used along with other compatible drugs for treatment of mastitis
Rifampins	Inhibition of nucleic acid synthesis by blocking DNA-directed RNA polymerase	Bacteriostatic	Antimycobacterial activity; used in combination with erythromycin for treating *Rhodococcus equi* infections
Sulphonamides Sulphamezathine Sulphamethoxazole	Inhibition of nucleic acid synthesis by competitive blocking of para-aminobenzoic acid (PABA) incorporation into folic acid	Bacteriostatic	Synthetic structural analogues of PABA, active against rapidly growing bacteria
Trimethoprim	Inhibition of nucleic acid synthesis by combining with the enzyme dihydrofolate reductase	Bacteriostatic	Usually administered with sulphamethoxazole. This combination, referred to as a potentiated sulphonamide, is bactericidal
Nitroimidazoles Metronidazole	Disruption of DNA structure and inhibition of DNA repair	Bactericidal	Particularly active against anaerobic bacteria; also active against some protozoa

Figure 11.3 Basic structure of penicillin and cephalosporin molecules. Biological activities of different penicillins and cephalosporins are influenced by their side-chain structures (R).



Basic structure of penicillins



Basic structure of cephalosporins

Glycopeptides are effective antibiotics against Gram-positive bacteria only, as their outer membrane confers intrinsic resistance on Gram-negative organisms. Resistance to glycopeptide antibiotics such as vancomycin emerged in recent years. Vancomycin binds covalently to the ubiquitous *N*-acyl-D-Ala-D- Ala structure found in bacterial cell walls. Five hydrogen bonds are formed as a result. Formation of this complex blocks the transglycosylation and transpeptidation steps required to complete the cell wall polymer.

Inhibition of cell membrane function

If the functional integrity of the cell membrane is disrupted, macromolecules and ions escape from the cell, leading to cell damage and death. Comparatively few antibacterial agents act on the cell membrane; those that target it are usually bactericidal. Because antibacterial agents with this activity are more toxic for animal cells than other classes of antibiotics, their use is generally limited to topical application.

Inhibition of protein synthesis

A number of classes of antibacterial agents inhibit protein synthesis. The selective toxicity of some antibiotics relates to the difference in structure between prokaryotic (70S) and eukaryotic (80S) ribosomes. Such antibiotics bind to receptors on the 30S or 50S subunits of bacterial ribosomes. Aminoglycosides bind to 30S ribosomal subunits and affect a number of different steps in protein synthesis. This results in the formation of non-functional proteins. Resistance to aminoglycosides may be intrinsic due to lack of a specific receptor on the subunit. Extrinsic resistance is conferred by plasmids, which may encode one or more of three classes of aminoglycoside-modifying enzymes capable of inactivating these antibacterial drugs. In some bacteria, particularly anaerobes, the active transport system essential for the intake of aminoglycosides may be lacking.

Tetracyclines enter cells by an active uptake process and bind to receptors on the 30S subunit. They block attachment of tRNA molecules to acceptor sites preventing the addition of amino acids to the polypeptide chain. Chloramphenicol, an antibiotic which binds to the 50S subunit, also prevents the linking of amino acids to growing polypeptide chains. The antibacterial activity of both of these classes of drugs is diminished if effective concentrations are not maintained for the required period.

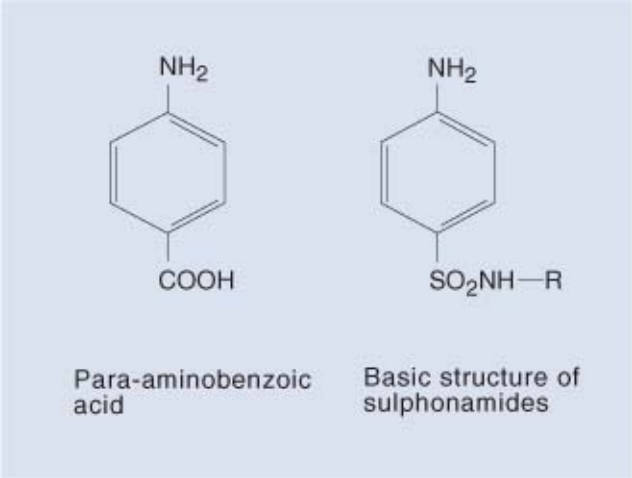
Macrolide antibiotics also inhibit protein synthesis by blocking 50S subunit activity. Although these antibiotics are bacteriostatic, at high concentrations they can be bactericidal. Resistance to macrolide antibiotics, which is transferred through chromosomes or plasmids, involves alteration of the binding site on the 50S ribosomal subunit.

Inhibition of nucleic acid synthesis

Many antibacterial agents including quinolones, novobiocin, rifampin, nitroimidazoles and sulphonamides inhibit nucleic acid synthesis (Table 11.2). The quinolones and novobiocin act on enzymes which separate the strands of DNA during bacterial replication such as DNA gyrase (in Gram-negative bacteria) and topoisomerase IV (more commonly in Gram- positive organisms). Although novobiocin is active against staphylococci and streptococci, because of its toxicity its use is limited to local intramammary therapy. Rifampin, by interfering with the activity of DNA-dependent RNA polymerase, prevents RNA synthesis. This antibiotic is active against Gram- positive bacteria including

mycobacteria. It is usually used in combination with other antibacterial agents because of the rapid development of resistant organisms. Metronidazole, the most commonly used drug of the nitroimidazole class, causes breaks in DNA strands and is particularly effective against obligate anaerobic bacteria such as clostridia.

Figure 11.4 Sulphonamides, analogues of para-aminobenzoic acid, competitively inhibit the enzyme dihydropteroate synthetase preventing folate production, an essential step in the production of bacterial DNA. This type of activity is known as competitive inhibition.



Sulphonamides interfere with the formation of folic acid, an essential precursor for nucleic acid synthesis. Their action relates to their structural similarity to para-aminobenzoic acid (PABA) as illustrated in Fig. 11.4. When present at sufficient concentrations, sulphonamides are utilized by the enzyme dihydropteroate synthetase instead of PABA, forming nonfunctional analogues of folic acid. The synthetic pyrimidine derivative, trimethoprim, inhibits the activity of dihydrofolate reductase, a later step in the synthesis of folic acid by bacteria. When used in combination, the action of each drug is potentiated resulting in enhanced activity against bacteria. Potentiated sulphonamides are selectively toxic for bacteria because animals can absorb preformed folic acid from their feed.

Combined antibacterial therapy

When antibacterial drugs are combined for the treatment of disease, the outcome is influenced by the particular combinations used. An additive effect is produced

when the combined action of the drugs is equivalent to the sum of the actions of each drug when administered separately. A synergistic effect results when the combined action of two drugs is significantly greater than the sum of effects of each drug used separately. Indifference is defined as lack of an enhancement effect when two drugs are administered in combination. Antagonism describes the reduced effectiveness of combined antibacterial therapy when compared with the effectiveness of each drug alone.

These effects, which can be demonstrated *in vivo* and *in vitro*, must be considered when selecting drugs for combined treatment of infected animals. If a bacteriostatic drug is combined with a bactericidal drug, antagonism may occur. Bactericidal drugs, particularly the ß-lactam antibiotics, are effective against actively dividing cells. If they are combined with a bacteriostatic drug, which inhibits bacterial growth, their bactericidal activity may be abolished. Drugs that act synergistically include sulphonamides and trimethoprim, which act at two different sites in the folic acid pathway, and clavulanic acid and penicillin combinations, in which clavulanic acid inhibits ß-lactamase activity, preventing inactivation of penicillin.

Factors influencing antibacterial activity

The activity of antibacterial agents is influenced *in vivo* by the site and rate of absorption, the site of excretion and the tissue distribution and metabolism of a particular agent. In addition, antibacterial activity can be affected by interactions between pathogen and drug and between host and pathogen.

Drug–pathogen interactions

The response of a bacterial pathogen to exposure to a drug *in vivo* may differ considerably from that *in vitro*. The environment *in vitro* tends to be constant whereas pathogens may encounter different microenvironments in various organs and tissues of a host. Following therapeutic administration, the distribution and concentration of a drug can vary widely. For example, some drugs can cross the blood–brain barrier while others are concentrated in the urine during excretion. If pathogens are quiescent in the presence of bactericidal drugs such as penicillin, they may survive and multiply later producing clinical disease. Because of their location, intracellular bacteria tend to be resistant to chemotherapeutic agents. A drug bound to proteins and other tissue components

may have reduced effectiveness. Moreover, products of inflammatory reactions such as pus and necrotic debris may adsorb antibacterial agents. The acidic environment in necrotic tissue can also inhibit the activity of some antibacterial drugs.

Host-pathogen interaction

Antibacterial drug administration can alter the host's immune response and may change the normal flora, particularly on the skin and in the intestinal tract. Disturbance of the normal intestinal flora following therapy for salmonellosis may allow the development of a prolonged carrier state. In addition, major disturbance of the normal flora may permit overgrowth of resistant organisms leading to disease. In horses treated orally with antibiotics, *Clostridium difficile* overgrowth can cause acute colitis. Many inflammatory responses may be modified by drug administration. Acute responses can become chronic if drug therapy suppresses the growth of a pathogen while permitting its survival.

Further reading

Arias, C.A. and Murray, B.E. (2009). Antibiotic-resistant bugs in the 21st century – a clinical super-challenge. *New England Journal of Medicine*, **360**, 439–443.

Giguère, S., Prescott, J.F., Baggot, J.D., Walker, R.D. and Dowling, P.M. (2006). *Antimicrobial Therapy in Veterinary Medicine*. Fourth Edition. Iowa State University Press, Ames, Iowa.

Chapter 12

Antibacterial resistance

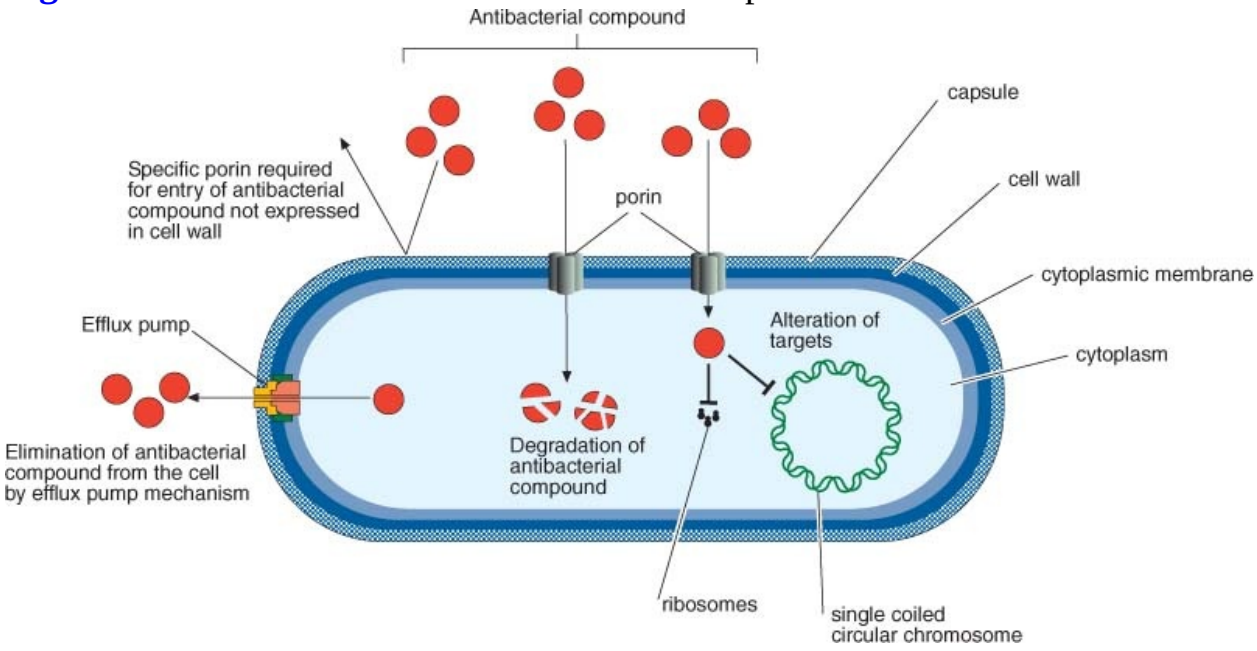
Drug-resistant microorganisms have become a global health problem, resulting in a reduction in the efficacy of many common antibiotics. Emergence of resistance to these antimicrobial drugs is an inevitable consequence of the selective pressure arising from their use (sometimes indiscriminate) in animal husbandry and human medicine. Resistance to antibacterial drugs is an increasingly important problem in both humans and animals. In general terms, resistance to antibiotics occurs as a result of drug inactivation, drug-target modification and decreased intracellular accumulation associated with reduced membrane permeability (following a reduction in the expression of outer membrane-spanning porin proteins) or increased drug efflux (Fig. 12.1). Some of the underlying genetic mechanisms involved include: mutations in housekeeping-structural genes (such as *gyrA* which encodes the A-subunit of the DNA gyrase enzyme) and in regulatory genes (such as *acrR*, which encodes the regulator of the *acrAB-tolC* tripartite efflux pump) or alternatively through the horizontal acquisition of foreign genetic material. Occasionally, resistance may manifest itself through several mechanisms, as is the case with tetracycline resistance.

In broad terms, resistance for an organism can be defined as either innate (intrinsic) or acquired (extrinsic). Innate resistance is chromosomally encoded and relates to the general physiology of an organism arising from its existing properties such as cell wall complexity, efflux mechanisms or enzymatic inactivation of an antibiotic. In contrast, acquired resistance can arise from a mutation in a resident gene or the transfer of genetic material encoding resistance genes via plasmids, bacteriophages carrying resistance genes or transposons containing integron sequences. The latter is a common method for developing a resistance phenotype and includes the acquisition of genes for ß-lactamase enzymes which degrade ß-lactam antibiotics (penicillins and cephalosporins), the recently described *qnr* genes conferring resistance to fluoroquinolones, and plasmid-encoded efflux pumps.

When selection is maintained by drug use, resistant bacteria become the predominant species in a population and they may also transfer some of their genetic material to susceptible bacteria which then become resistant. There are several mechanisms that bacteria can use to transfer resistance including conjugation (sexual pairing between plasmid-containing (male) and plasmid-free bacterial cells (female)), transformation (uptake of naked DNA from the environment) and transduction (mediated by bacteriophages).

Resistance to an antibacterial agent often results in cross-resistance to other agents in the same class. This form of resistance is encountered with sulphonamides, tetracyclines, aminoglycosides and macrolides. As an example, the use of tylosin for the treatment of pigs can be associated with cross-resistance to erythromycin. Carriage of several resistance genes by plasmids and transposable elements often enables bacteria to become resistant to a number of drugs from different classes. This type of resistance can be transferred rapidly between different bacterial genera and species. It is particularly common among members of the *Enterobacteriaceae*, *Pseudomonas* species and anaerobes colonizing the intestinal tract.

Figure 12.1 Mechanisms involved in the development of antibiotic resistance.



Resistant strains of non-pathogenic or commensal *E. coli* were found in healthy children in cities in the USA, Venezuela and China (Marshall *et al*., 2009). The realization that non-pathogens can acquire resistance to antibacterial compounds is a cause of concern. High levels of resistance in the endogenous

bacterial flora of healthy human populations increases the risk of this resistance transferring to pathogenic organisms also.

Resistance mechanisms

Increasing numbers of bacteria showing resistance to one or more antibiotics have been reported in recent years. As a consequence of this, some of these drugs have been rendered therapeutically useless. Several mechanisms account for bacterial resistance to antibiotics. The front line of resistance is the external structure of a bacterial cell, which provides a physical barrier to the entry of many antibiotics. Other mechanisms contributing to resistance to antibacterial drugs include production of enzymes by bacteria which destroy or inactivate the drug, such as ß-lactamases and aminoglycoside kinases, and reduction of bacterial cell permeability by altering porin production. Bacteria may also develop alternative metabolic pathways to those inhibited by the drug as exemplified by vancomycin resistance. An antibiotic may be eliminated from the cell through the action of a range of membrane-bound efflux pumps, or the target site of the drug may be structurally altered, such as mutations in *gyrA* and *parC* in the case of fluoroquinolones, *rpoB* for rifampin and 23S rRNA for linezolid. Alteration of the target site and enzymatic destruction of the agent are probably the most common mechanisms whereby resistance can occur. A summary of resistance mechanisms in particular bacteria is presented in Table 12.1 and Fig. 12.1.

Multiple drug resistance

Multiple drug resistance (MDR) is of particular concern, especially in zoonotic and nosocomial pathogens. *Salmonella* Typhimurium DT104 is one of the most common causes of human food poisoning in the developed world (Glynn *et al*., 1998). This strain emerged in the 1980s as a global health problem and was involved in animal and human diseases. *Salmonella* Typhimurium DT104 is characterized by a penta-resistance phenotype ACSSuT (conferring simultaneous resistance to *a*mpicillin, *c*hloramphenicol/ florphenicol, streptomycin/spectinomycin, *su*lfonamide and *t*etracycline). A genomic island identified in this epidemic strain, the *Salmonella* Genomic Island, SGI1, was described by Boyd *et al*. (2000). SGI1 is an integrated, mobilizable island 43 kbp

in size and located on the chromosome of some *Salmonella* serotypes. On the right-hand side of this structure is a 13-kbp region containing all of the resistance genes corresponding to the ACSSuT phenotype (Fig. 12.2). SGI1 is incapable of independent existence outside the chromosome; however, it can be transmitted to other *Salmonella* serotypes and to *E. coli* aided by a 'helper plasmid', which provides the mating apparatus (Doublet *et al.*, 2005). Identification of SGI1 in other *Salmonella* serotypes from both animal and human sources provides evidence of its wide distribution and of increasing numbers of organisms displaying a multi-drug-resistant phenotype. Other genetic arrangements containing antibiotic resistance genes in *Salmonella* have also been described and these involve mobile genetic elements.

Table 12.1 Targets of antibacterial drugs and the genetic basis of resistance in bacterial pathogens belonging to the *Enterobacteriaceae* and in some other bacterial species.

Drug	Target	Examples of resistant bacteria / Genetic basis	Comments
Fluoroquinolones	DNA gyrase	*Enterobacteriaceae* / Plasmid-mediated or chromosomally-based	Mutation results in structurally altered enzyme
	Cell membrane	*Enterobacteriaceae* / Chromosomally-based	Decreased permeability
Rifampin	DNA-dependent RNA polymerase	*Enterobacteriaceae* / Chromosomally-based	Mutation results in alteration of enzyme
Erythromycin	Ribosomal protein	*Staphylococcus aureus* / Chromosomally-based	Due to structural change, ribosomes unaffected by drug action
Streptomycin	Ribosomal protein	*Enterobacteriaceae* / Chromosomally-based	Mutation results in altered ribosome
Tetracycline	Ribosomal protein	*Enterobacteriaceae* / Plasmid-mediated	Protective proteins block action of drug on ribosomes
Chloramphenicol	Peptidyltransferase	*Staphylococcus* species, Streptococci / Plasmid-mediated or chromosomally-based	Inactivation of drug by a specific acetyltransferase
Sulphonamides	Dihydropteroate synthetase	*Enterobacteriaceae* / Plasmid-mediated or chromosomally-based	Altered biosynthetic pathway employing sulphonamide-resistant enzyme
β-Lactam antibiotics	Penicillin-binding proteins (PBP)	*Staphylococcus aureus* / Chromosomally-based	Decreased affinity of PBP for drug
	Penicillin-binding proteins	*Enterobacteriaceae* / Chromosomally-based	Outer membrane of most Gram-negative bacteria inherently impermeable to these drugs
	Penicillin-binding proteins	*Staphylococcus aureus*, *Enterobacteriaceae* / Plasmid-mediated or chromosomally-based	Enzymatic degradation of these drugs by β-lactamases

Membrane-bound efflux pumps, common in prokaryotic organisms, are known

to expel a wide range of structurally dissimilar organic compounds including antibiotics, bile salts, dyes, detergents, disinfectants and other substances. These systems transport several classes of antibiotics consistent with a multi-drug-resistant phenotype and thereby contribute to the intrinsic resistance of an organism. Five families of efflux pumps are known and are associated with multi-drug resistance in bacteria. Features of these pumps are summarized in Table 12.2 and their corresponding structures are illustrated in Fig. 12.3. Classification of efflux pumps is based on their number of structural components, the number of transmembrane spanning regions and the energy source used. Bacteria can express a range of efflux pumps. *Escherichia coli, Salmonella* Typhimurium and *Campylobacter jejuni* express an RND-type pump (known as *acrAB-tolC* in the former and *cmeABC* in the latter). In *Campylobacter*, CmeABC is responsible for high-level resistance to fluoroquinolones. Major Facilitator Superfamily (MFS) transporters *floR* and *tetG* are located within SGI1 of *S.* Typhimurium (Fig. 12.2).

Figure 12.2 A linear representation showing the distal part of the SGI1 island which was originally identified in the multi-drug resistant *Salmonella* Typhimurium DT104. The diagram shows a genetic cluster of the antibiotic-resistant genes which account for the ACSSuT phenotype. This structure consists of a complex integron In104 showing inverted repeats (IR) flanking the two class 1 integrons, with the 5′-conserved structure (CS) containing the *int1* integrase gene, a single gene cassette and 3′-CS containing the *qacEΔ1* and *sul1* genes (see Chapter 9 for further details). The gene cassettes encoding the *aadA2* (streptomycin/spectinomycin resistance) and *pse1* (ampicillin resistance) are shown within the respective integrons. The *floR* and *tet*(G) genes encoding florphenicol/chloramphenicol and tetracycline resistances are indicated. A functional *sul1* gene encoding sulphonamide resistance is located within the 3′-CS structure of the class 1 integron on the right side of the figure. Additional genes are also indicated in this region of the SGI1 genomic island as follows: *tnpR* encodes a transposon resolvase, orf1 encodes a putative *lysR*-type transcriptional regulator, orf2 encodes a transposase-like gene and orf5 and orf6 are open reading frames of unknown function, IS*6100* is an insertion sequence (see Chapter 9) and S044 is a hypothetical protein.

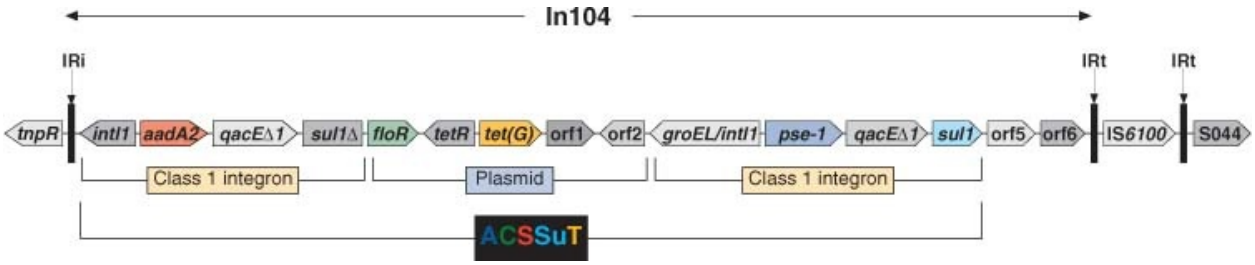


Table 12.2 Structural features of major efflux pumps, molecules transported and the bacterial genera and species in which they occur.

Efflux pump family	Features of pump structure	Molecules transported	Bacterial genera in which pump has been identified
Major facilitator superfamily (MFS)	12 to14 membrane-spanning regions	Antibiotics, quaternary ammonium compounds, basic dyes and phosphonium ions	*Escherichia* *Vibrio* *Mycobacterium*
Small multidrug resistance (SMR) family	Approximately 100 to 120 amino acids in primary structure containing four helices	Antibiotics, quaternary ammonium compounds, tetraphenyl phosphonium and ethidium bromide	*Escherichia* *Staphylococcus* *Mycobacterium*
Multidrug and toxic compound extrusion (MATE) family	12 putative membrane-spanning regions	Aminoglycosides, dyes and fluoroquinolones	*Bacillus* *Vibrio* *Haemophilus*
Resistance/nodulation/cell division (RND) family	Multi-component segments associated with inner and outer membranes	Antibiotics, basic dyes, detergents and fatty acids	*Escherichia coli* *Pseudomonas* *Salmonella*
ATP-binding cassette (ABC) transporters	Multiple membrane-spanning helices, ATP-binding cassette region	Alkaloids, ethidium bromide, phospholipids and ionophores	*Escherichia* *Staphylococcus* *Mycobacterium*

Arising from their broad specificity, the physiological role of efflux pumps is the elimination of host-derived molecules such as bile salts, thereby allowing the bacterium to survive in the host. In addition to the export of antibiotics in some bacterial species, these pumps also transport virulence determinants, including adhesins, toxins and other proteins (Table 12.3).

Some organisms which are resistant to multiple antimicrobial compounds may cause disease in hospitalized humans and animals, where the selection pressure is high. These nosocomial pathogens are sometimes referred to as ‘superbugs’ and generally fall into one of two categories (Wright, 2007): (i) widely recognized pathogens such as methicillin-resistant *Staphylococcus aureus*, which have acquired resistance to multiple antimicrobial agents and (ii) environmental organisms such as *Acinetobacter baumannii* or *Pseudomonas aeruginosa*, which cause opportunistic infections and are intrinsically resistant to many drugs.

Figure 12.3 Efflux pump families identified in bacteria.

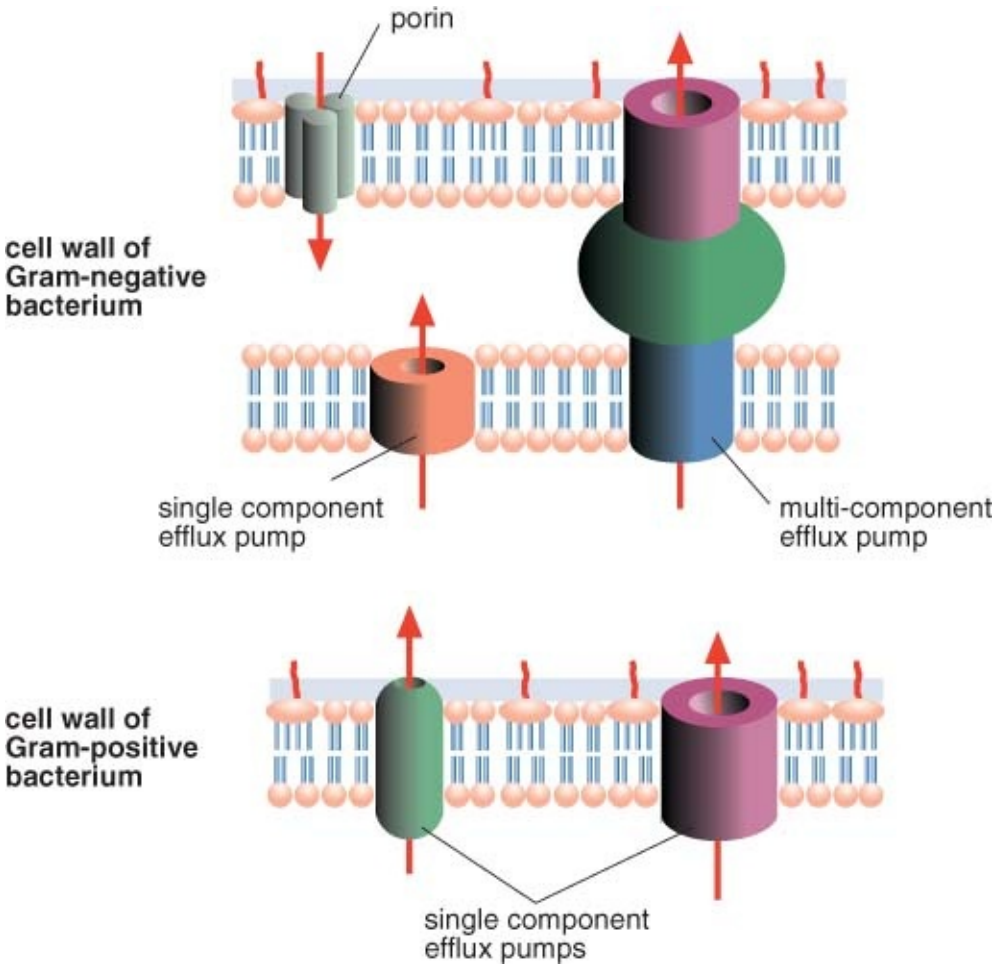


Table 12.3 Features associated with resistance/nodulation/cell division (RND) efflux pumps.

RND efflux pump	Bacterial species in which pump has been identified	Characteristics of bacteria which express these pumps	Naturally occurring molecules transported
AcrAB-TolC	*Salmonella* Typhimurium	Adherence to and invasion of host cells; colonization of enterocytes and persistence in avian intestine	Bile salts
	E. coli	Considered to have characteristics similar to *Salmonella* Typhimurium	Bile salts
MexAB-OprM	*Pseudomonas aeruginosa*	Invasion of host cells; causes opportunistic infection in many animal species	Not determined
CmeABC	*Campylobacter jejuni*	Colonization of enterocytes and persistence in avian intestine	Bile salts

Strategies for limiting antibacterial resistance

Antibacterial resistance is widespread and control measures in a country may be

rendered ineffective as a result of importing resistant bacteria in food or in the normal flora of animals or humans from countries in which controls are less stringent. Health professionals and the general public should be aware of the risks associated with resistance so that realistic control measures can be implemented. It is probable that measures to restrict antibiotic usage, combined with the control of bacterial contamination, may reduce the occurrence and dissemination of resistant organisms.

Recommendations for dealing with resistance to antibacterial drugs are contained in a number of publications including The Copenhagen Recommendations (Rosdahl and Pedersen, 1998), recommendations issued by expert committees in the UK (Anon., 1998, 1999) and the USA (Cohen, 1998) and more recently in a report issued by the FAO/WHO/OIE (2008). Effective surveillance systems for collection of data on resistant organisms should be established at local, national and international levels. The supply and use of antibacterial drugs should be closely monitored to allow evaluation of the risks and benefits of therapy. Prescription of antimicrobial drugs should be based on sound medical and veterinary therapeutic principles. Ideally, antibiotic therapy should be based on the results of laboratory examinations, and drugs should be administered at the recommended therapeutic dose and for the prescribed period of time. There should be strict adherence to drug withdrawal periods following treatment of food- producing animals. Antimicrobial agents should not be used for growth promotion and greater reliance should be placed on improved hygiene measures, disinfection and vaccination for the prevention and control of infectious disease.

Antimicrobial susceptibility monitoring systems, such as the National Antimicrobial Resistance Monitoring System (NARMS) for Enteric Bacteria in the United States and the Danish integrated antimicrobial resistance monitoring and research programme (DANMAP) in Denmark, provide valuable information on resistance traits among food-borne bacteria. DANMAP is unique in that it collects data on both antimicrobial consumption and resistance in bacteria from animals, food and humans and provides a temporal relationship between usage and resistance (Bager, 2000).

Antibacterial susceptibility testing

Tests to determine the most suitable antibiotic for the effective treatment in a given disease can be conducted on isolates from clinical cases. However, these

tests which are carried out *in vitro* cannot allow for the various factors that may affect antibacterial activity *in vivo*. The results obtained following treatment may not reflect the susceptibility pattern of an isolate as determined in the laboratory. A number of antibacterial susceptibility tests are available including broth dilution, disc diffusion, agar gradient and some automated methods (Jorgensen *et al*., 1999). The Kirby-Bauer disc diffusion method is a flexible and relatively inexpensive technique that is commonly used in diagnostic laboratories. This standard procedure is based on Clinical and Laboratory Standards Institute protocols (CLSI, 2008) and is used mainly for testing rapidly growing aerobic bacteria. Filter paper discs containing specified amounts of antibacterial agents are placed on agar uniformly seeded with the test bacterium. The procedure and the method of interpretation are indicated in Fig. 12.4. The diameter of each zone of inhibition is measured in millimetres (from three separate directions, taking the average diameter) and the results compared with standards for interpretation of the zone size (CLSI, 2008). Susceptibility to an antibacterial drug indicates that the infection caused by the bacterium may respond to treatment if the drug reaches therapeutic levels at the site of infection.

Determination of the minimum inhibitory concentration

Laboratory procedures for determining the minimum inhibitory concentration (MIC) are illustrated in Figs 12.5 and 12.6. The MIC of an antibacterial agent for a specific bacterium can be determined *in vitro*. The MIC is the highest dilution of an antibacterial agent that inhibits growth of an isolate. The minimum bactericidal concentration (MBC) is the highest dilution of a drug that can kill a particular bacterium (Fig. 12.5). In contrast, MICs can be determined directly using an E-test strip. In this approach the MIC is determined directly at the point of intersection between the strip and the ellipse of the zone of inhibition (Fig. 12.6).

Figure 12.4 An antibiogram of *Escherichia coli* using a lawn of bacteria on Mueller-Hinton-based medium. Following the application of antimicrobial discs, the inoculated plate is incubated at 37°C for 18 hours. The diameters of the zones of inhibition are measured (mm) and compared with internationally accepted measurements to determine the susceptibility or resistance of the isolate.

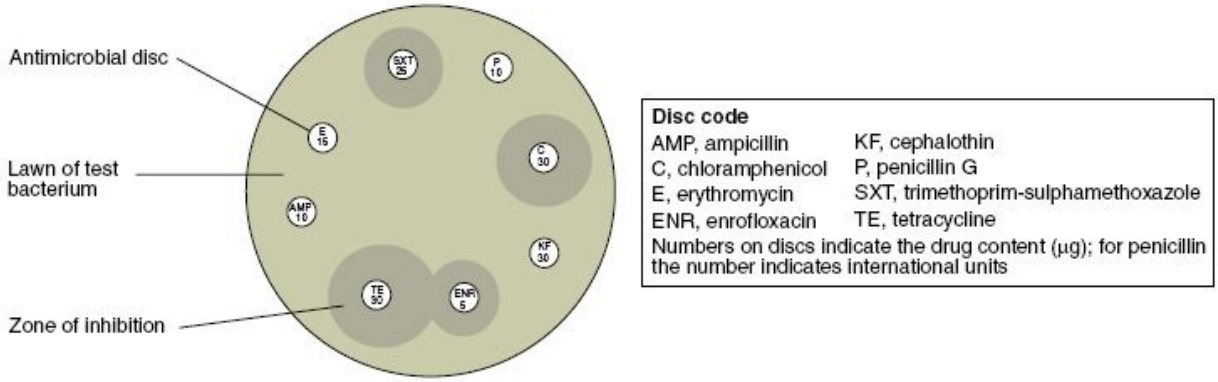
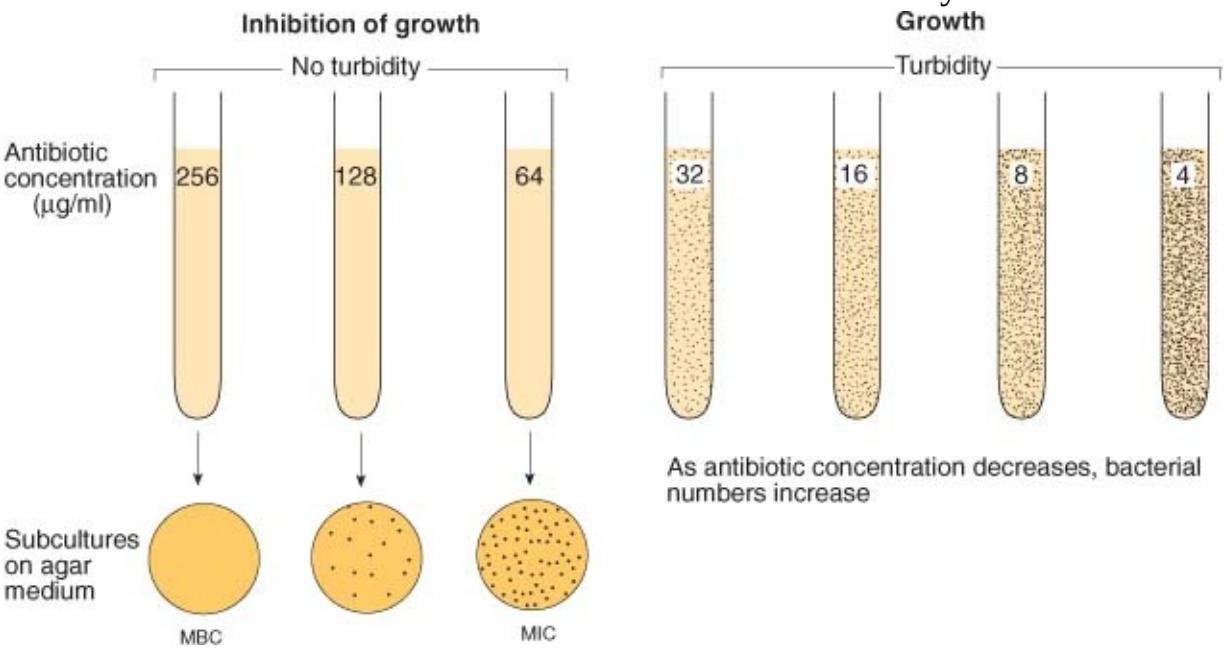


Figure 12.5 Dilution method for determining the minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of an antibiotic for a test bacterium. Doubling dilutions of the antibiotic are made in broth, a standard amount of bacterial inoculum is added to each tube and the test is incubated at 37°C for 24 hours. The MIC is the highest dilution of an antibiotic that inhibits the growth of the test bacterium, indicated by the absence of turbidity in the tube (64 μg/ml in the example given). The MBC is the highest dilution of an antibiotic that kills all the bacterial cells (256 μg/ml in the example given) demonstrated by subculturing the broth on agar. Below the MBC, growth occurs in the subcultures of broth from tubes without turbidity.



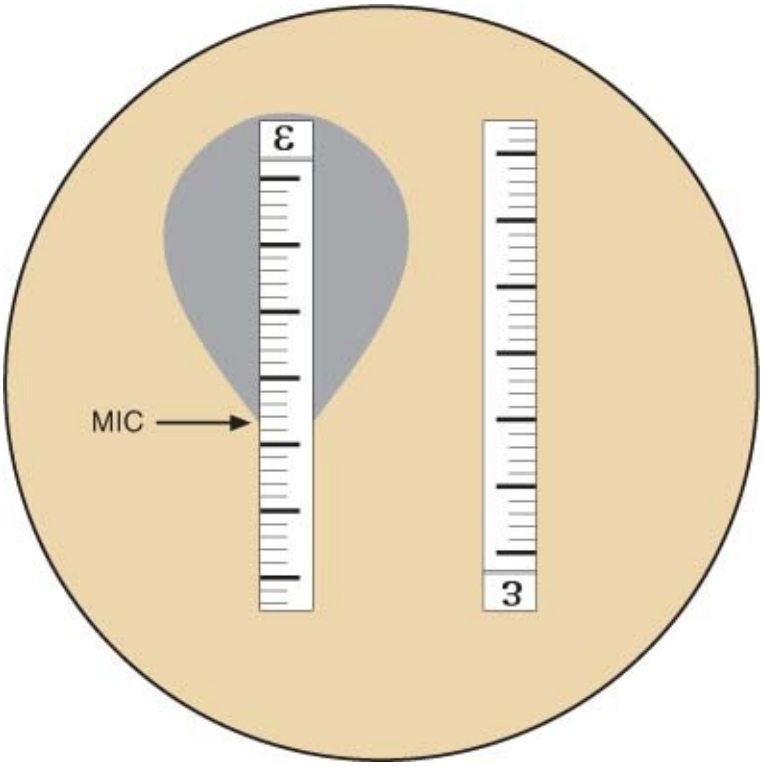
Quantitative methods of susceptibility testing are increasingly used in research and diagnostic laboratories and are now the preferred methods of susceptibility testing. Results can be used in conjunction with pharmacokinetic and pharmacodynamic data to more accurately calculate drug dosage. In addition,

MIC data are more valuable than qualitative data for monitoring trends in antimicrobial resistance.

Molecular methods for antimicrobial susceptibility testing

Although phenotypic methods are more commonly used in diagnostic laboratories for the detection of antimicrobial resistance, methods involving identification of resistance genes have been developed. These methods are usually based on conventional or real-time PCR and can be used to detect the presence of resistance genes in an organism isolated from a clinical specimen or even in the clinical specimen itself. The major advantage of molecular methods is the speed with which results can be achieved, especially for organisms that are difficult to grow. This allows the rapid prescription of antibiotics, which are likely to be effective. The disadvantage of these methods is that the presence of a gene does not necessarily imply its expression phenotypically and thus inaccurate clinical information could be generated using such methods. In addition, if molecular detection of resistance is used directly on clinical specimens, isolates are not available for MIC testing.

Figure 12.6 Agar plate inoculated with a bacterial culture showing a pattern of susceptibility to the antibiotic used on the left and resistance to the one used on the right. The inert plastic strip has a predefined antibiotic gradient. After incubation, the MIC value is read from the scale (arrow) at the point of intersection between the zone edge and the test strip. The MIC, the lowest concentration of antibiotic which inhibits growth, is indicated.



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Chapter 13

Bacterial colonization, tissue invasion and clinical disease

A number of bacterial species can reside in animal hosts, either as commensals or as potential pathogens. Mammals are hosts to an enormous number of resident microbes: for instance, a healthy adult human can harbour up to 10^{14} bacteria, a figure that exceeds the total number of host cells by at least one order of magnitude. The vast majority of resident bacteria are commensals that have a history of co-evolution and harmonious co-existence with the host. In contrast, pathogenic bacteria can interact with host tissues in ways that result in disease (Fig. 13.1).

Commensals

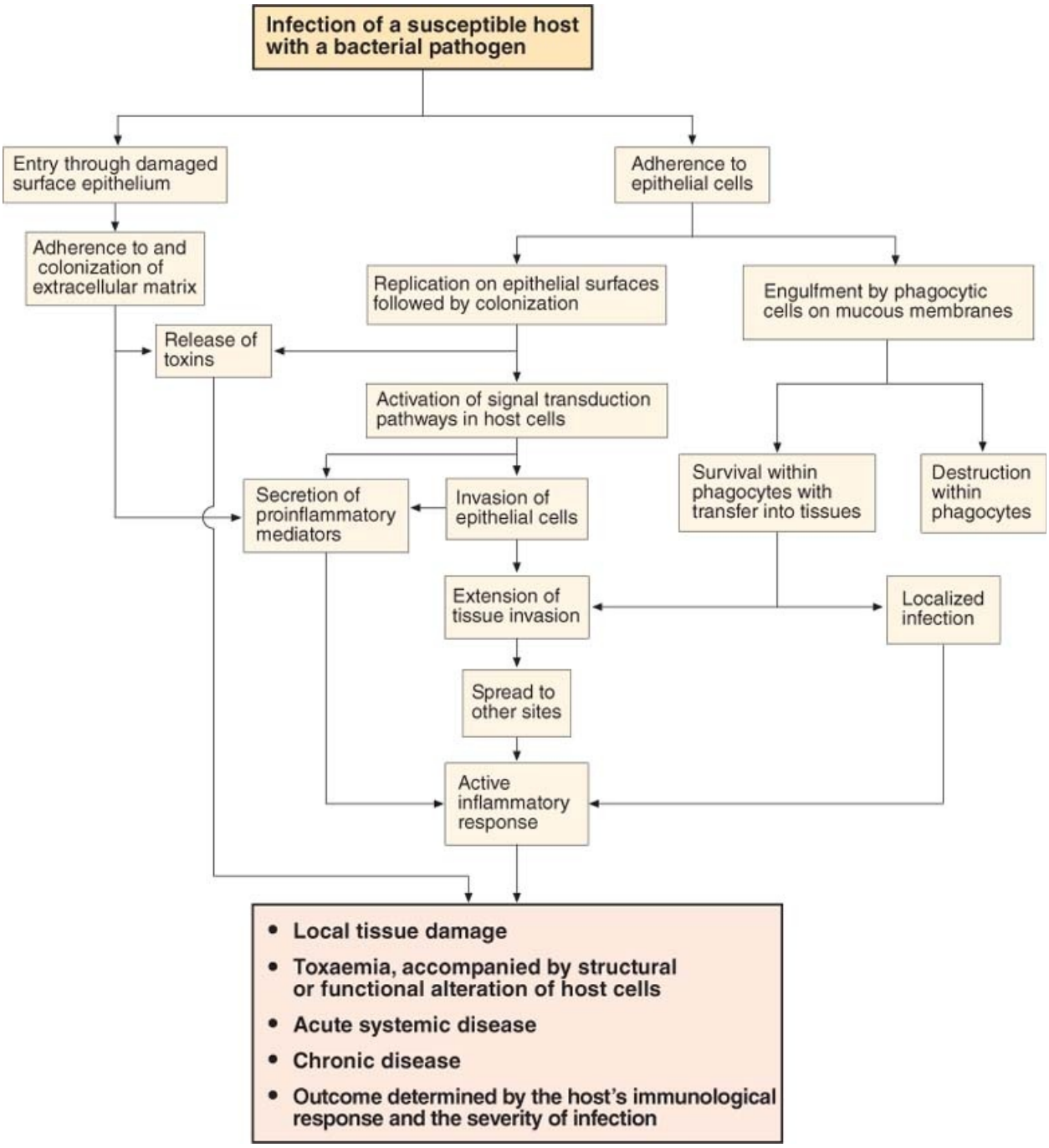
Commensal organisms, acquired soon after birth, are able to adhere to body surfaces. They form stable polymicrobial communities that are present throughout life as ‘normal microflora’ on the skin and in the hollow organs whose surfaces and cavities are open to the environment. The composition of the microbial communities tends to be host-specific and, within hosts, to be organ-specific. In any particular niche, the proportional representation of the several species is controlled by competition for nutrients and for adhesion sites and by antibacterial products such as bacteriocins released by some members of that microbial community.

A stable microflora is beneficial to the host in many ways. The digestive system relies on the normal microflora to degrade ingested material in the rumen of cattle and sheep, in the caecum and colon of horses, and in the colon of pigs. The microflora of the rumen synthesizes vitamin K and some of the vitamin B group, as does the microflora of the intestine in non-ruminants. The normal microflora primes the immune system, facilitating a more efficient host response to challenge by bacterial pathogens. It is suggested that the immune system is

stimulated non-specifically by antigens associated with some commensals (Tannock, 1995). Germfree animals, which are denied this stimulation, have poorly developed secondary lymphoid organs, weak cell-mediated immune responses and lower serum immunoglobulin levels than conventional animals. However, it is believed that the most important beneficial effect of the indigenous microflora is to provide competition for exogenous bacteria attempting to colonize niches in which they must compete with the resident flora for nutrients and for receptors.

When the community of commensals occupying a particular niche is in ecological balance, the bacteria utilize the resources they require from the host without causing any obvious adverse effects. However, when that equilibrium is disturbed or when the host is under severe stress, an indigenous member may escape from the restraining influences of the bacterial community and act as an opportunistic pathogen. This can occur in the gastrointestinal tract following the administration of oral antibiotics. Furthermore, bacteria that are harmless in their usual ecological niche can cause disease in another site: for instance, *Fusobacterium necrophorum* can exist as a commensal in the rumen but when it transfers to the liver of feedlot cattle it can act as a pathogen that causes hepatic abscesses.

Figure 13.1 Possible sequelae following infection of a susceptible animal with a bacterial pathogen.



Pathogens

For a microbe to act as a pathogen, it must find an appropriate niche within a host, it must compete with the normal microflora to gain a foothold in that niche, it must evade or overcome normal host defences, and it must express the genes that encode the factors that cause disease. The ability of a microbe to damage a

host is called pathogenicity; the relative capacity of a pathogen to damage a host is the degree of virulence. The bacterial traits that confer pathogenicity are called virulence factors; these include adhesins, toxins and capsules. The genes for these virulence factors are not expressed constitutively; the metabolic economy of the bacterium dictates that, generally, genes are expressed only when their products are required. The process by which bacteria can switch on or switch off the expression of a gene product is called phase variation, an extremely important attribute of pathogenic bacteria.

During the course of its existence, a pathogenic bacterium may be called upon to adapt to a wide variety of changing environmental conditions, whether in the target host, in the external environment or, perhaps, in an incidental host. The survival of the bacterium depends on its ability to respond to diverse environmental cues by appropriate changes in the expression of subsets of its genes. Even after it encounters a host, the fate of the bacterium is dependent on the facility to up-regulate or down-regulate the expression of different gene products, particularly those recognized as virulence factors. For instance, when a pathogen enters a host, it must express specific gene products that enable it to persist and proliferate in an appropriate location. Later, the gene products that have linked the bacterium to a particular niche within the host may have to be down-regulated in favour of products of other genes that enable the pathogen to move to a new location. As a general rule, gene products that confer an advantage in a particular environment are expressed, while those that are unnecessary or unhelpful at that time in that environment are not expressed. Switching off the expression of unwanted gene products saves metabolic energy; it also reduces the risk of the host mounting an effective immune response against a virulence factor that the pathogen may require later. During the course of an infection, phase variation regulates the expression of virulence factors in response to signals generated by interactions between host and pathogen. Some virulence factors are also subject to random phase variation that produces bacterial populations in which some members express a particular component and other members do not express it. This random phase variation continuously generates subpopulations of diverse phenotypes. The subpopulation best adapted to the prevailing conditions persists.

Genes encoding virulence factors are not uniformly distributed among all the strains of a particular bacterium, as is evident in several bacterial species in which the population structure is clonal. In nature, a bacterial species may exist as a number of discrete genetic lineages, each represented by a clone of cells

descended from a distinct ancestral cell. *Escherichia coli, Staphylococcus aureus* and several *Salmonella* species are recognized as highly clonal organisms, whereas *Pseudomonas aeruginosa* is considered nonclonal. Within a given clonal species, the members of a clone are likely to carry the same determinants of virulence. Some lineages are inherently more virulent than others. It is recognized that most of the diseases caused by a given pathogen are due to a small proportion of the total number of clones of that pathogen. The fact that a large proportion of the several diseases caused by *S. aureus* infections are caused by a limited number of clones implies that clones can differ greatly in relative virulence: some clones can remain as commensals for extended periods; other clones promptly cause devastating disease. A degree of tissue tropism is evident in the relative frequency with which some pathogenic clones cause certain clinical diseases, a trend that has been noted in widely separated geographical areas and in different environmental conditions; for instance, there is evidence that a limited number of clones of *S. aureus* are responsible for many cases of bovine mastitis and that some of these clones are implicated in disease production in Ireland and in the USA (Fitzgerald *et al*., 1997).

Many of the genes that encode virulence factors are associated with mobile genetic elements: bacteriophages, plasmids and pathogenicity islands. Pathogenicity islands occupy relatively large regions of the bacterial genome. They encode clusters of virulence genes which appear to have been acquired during evolution by horizontal transfer. The cluster may be incorporated into the bacterial chromosome or it may be part of a plasmid or of a bacteriophage. The genes encoded in the pathogenicity island are absent from the genome of avirulent strains or of closely related species. Horizontal transfer of a pathogenicity island can convert an avirulent strain into a pathogen. Virtually any of the entire spectrum of bacterial virulence factors – adhesins, invasion factors, secretion systems and toxins – can be encoded in a pathogenicity island. However, a cluster of virulence genes that is present in a pathogenicity island in one pathogen may be on a virulence plasmid in another pathogen. Although pathogenicity islands have been recorded for a wide range of pathogens, they have not been found in *Mycobacterium* or *Chlamydia* species, in spirochaetes or in most streptococci. It has been observed that pathogenicity islands appear to extend the range of habitats that can be colonized by a given bacterial species (Schmidt and Hensel, 2004).

The interplay of genetic factors involved in any infectious disease is part of a continuing dynamic process in which pathogens and hosts have evolutionary

effects on one another: over time, genetic variation in a pathogen is matched by appropriate adaptive change by its host, and vice versa. In this process, many different combinations of microbial genotype and host genotype occur in evolutionary time, generating corresponding changes in virulence. Rather than being a set characteristic of a given pathogen, virulence is variable, a product of responses that are triggered by the contemporary genotypes of pathogen and host and are modulated by the environmental conditions prevailing in the host at that particular time. Although the proximal determining influence, genetic or environmental, can intensify the damage in some pathogen–host encounters, nevertheless, in general, co-evolution has fostered a drift towards a more benign outcome in all but a minority of microbial infections.

Colonization and growth

Animals may be exposed to infection from endogenous or exogenous sources. Endogenous infections arise when bacteria that live on the skin or mucous membranes as harmless commensals take advantage of impaired antimicrobial defences of the host and behave as opportunistic pathogens. This is likely to happen when epithelial barriers are damaged, when immunity is weakened by drugs, radiation or exogenous pathogens, when the ecological balance of the resident microbial community is disturbed by administration of antibiotics, or when the bacteria gain access to sites from which they are usually absent.

Exogenous infections occur after direct or indirect transmission from an infected animal or from the environment. The route of infection determines the site of the initial interaction between pathogen and host and also the organs at greatest risk of infection. Subsequent interactions are driven by microbial genes that express virulence factors and by host genes that are responsible for resistance to pathogens. Although pathogens may enter a host through the skin, the conjunctiva, the umbilicus, or the teat canal, the main portals of entry are the mucosae of the gastrointestinal, respiratory and urogenital tracts. To a large extent, those surfaces are lined by polar epithelial cells linked on adjacent lateral cell membranes by tight junctions that close the intercellular spaces just below the free surface of the epithelium. The tightness of the barrier varies in different locations, from ‘leaky’ epithelium in the intestines, where the intercellular junctions are permeable to small molecules, to ‘tight’ epithelium in segments of the nephron, where the intercellular junctions allow virtually nothing to pass through the paracellular route between adjacent cells. Thus, the layer of

epithelial cells provides a mechanical barrier that serves to protect the underlying tissues from invasion by bacteria. The epithelial cells secrete fluids such as mucus in which bacteria can be entrapped and subsequently expelled. Epithelial cells ‘recognize’ pathogens, activate the innate immune response, and secrete antibacterial peptides and proteins.

Microbes that seek to colonize a niche must attach promptly to intact epithelial cells or to components of the exposed subepithelial extracellular matrix, such as collagen, elastin, fibronectin and laminin. Non-specific attachment can occur as a consequence of electrostatic or hydrophobic forces; specific attachment is achieved by ligand–receptor binding. Typically, the ligand is a protein, an adhesin, on the bacterial cell surface and the receptor is a specific carbohydrate moiety in a glycoprotein or glycolipid constituent of the host cell. Some adhesins are anchored within the bacterial cell membrane (afimbrial or nonfimbrial adhesins), others are present in fimbriae (or pili), filamentous structures that project from the cell membrane. A single bacterium may have genes for a number of different adhesins but it does not always express them: expression of each of the genes may be subject to phase variation. Phase variation of adhesins gives a bacterium the opportunity to ‘slip anchor’ from a primary site of attachment, after which it may use a different adhesin to attach to another location within the host or it may be shed to the exterior, contaminating the environment or transferring to a new host. As surface components are likely to be highly immunogenic targets for protective antibodies, pathogens may use phase variation as a mechanism for immune evasion.

Once bacteria bind to host cell receptors they must replicate to colonize the site and to avoid total elimination when the host cells are desquamated. The new arrivals compete with the indigenous microflora for nutrients. Availability of iron is a limiting factor for the growth of bacteria. Iron, as a component of the cytochromes and the iron-sulphur proteins involved in electron transport, plays a major role in bacterial respiration. Most iron in the animal body is unavailable to bacteria because it is bound by iron-binding proteins such as lactoferrin and transferrin. However, many pathogenic bacteria have evolved mechanisms for obtaining iron from their hosts. These include the production of iron-chelating compounds (siderophores) which can remove iron from transferrin and lactoferrin. Some bacteria can bind iron from these molecules in the absence of siderophores; other bacteria can lyse erythrocytes to obtain iron from haemoglobin. Because inadequate uptake of iron interferes with the ability of bacteria to infect the host, iron- uptake systems can be considered as virulence

factors. If the supply of nutrients is adequate and the environmental conditions are favourable, the newly acquired bacteria replicate. Replication can lead to one of three outcomes: a transient benign colonization; a permanent benign colonization that does not appear to induce any significant changes in either the bacteria or the host; or an infectious process that damages the host.

Bacteria that succeed in establishing a presence in an anatomical niche tend to create a biofilm on the epithelial surface. In the early phase of colonization, the bacteria secrete a polysaccharide matrix in which the bacterial cells are incorporated. The sticky extracellular polysaccharide shields the bacteria from the defence mechanisms of the host and from antimicrobial drugs. Bacteria can adopt the biofilm mode of growth under a variety of different circumstances: for instance, in dental plaque and on indwelling medical devices in humans, in infections of the urinary tract and of the female genital tract in animals and in humans, and in air conditioning systems where *Legionella* species may be present in biofilms. Many of the bacteria in a biofilm produce small signal molecules (pheromones, formerly called auto-inducers) by which they communicate with other bacterial cells of other species as well as of the same species. As the bacteria proliferate, the pheromones accumulate within the biofilm. When the population reaches a defined density (a bacterial quorum), the pheromones attain a critical threshold concentration at which they coordinate the expression of bacterial genes so that the colonizing bacteria act as a community rather than as individuals. This process of quorum sensing is a feature of colonization by bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, S. epidermidis, E. coli* and *Streptococcus* species. The genes expressed by bacteria in the biofilm may be significantly different from those expressed by the colonizing bacteria from which the biofilm has been derived. Bacterial pathogens regulate virulence determinants to ensure that they are expressed only at the appropriate stage of infection. Quorum-sensing pathogens do not waste energy expressing virulence determinants when they are not required; rather, expression is delayed so that recognition by the adaptive immune system of the host is avoided until the bacterial cell population has reached a critical mass that has the capacity to effectively challenge the defences of the host.

Pathogen– host interactions

In bacterial infections, the host may be damaged by bacterial toxins, by the

inflammatory response elicited by the bacteria or their toxins or, commonly, by both toxins and inflammatory reactions. The immune responses of the host may also be a source of tissue damage. This is a feature of the pathogenesis of the chronic inflammatory reactions associated with mycobacterial infections which are related to the immune response of the host. The outcome of pathogen–host interactions can vary from clinically silent infection to fulminating lethal disease. This variability is determined by the virulence of the bacteria and by the effectiveness of the host response. To a considerable degree, the virulence expressed by a pathogen is pertinent to its immediate needs, principally to survive in a particular niche, to propagate its genes and to disseminate those genes to new hosts. Therefore, in general, it is in the interest of the bacterium to take the resources it requires from the host without being excessively destructive.

Virulence factors

Pathogenic bacteria have evolved many virulence factors which enable them to colonize extracellular or intracellular niches in mammalian hosts. Pathogens that are able to survive within host cells are grouped into two categories: strict (obligate) intracellular pathogens and facultative intracellular pathogens. Chlamydiae and rickettsiae are obligate intracellular pathogens. Among the facultative intracellular pathogens, *Mycobacterium* species enter and replicate in phagocytic cells, while *Brucella* species, uropathogenic *E. coli, Salmonella* species, and *Listeria monocytogenes* invade and replicate in non-phagocytic epithelial cells. A wide range of virulence factors is required by pathogens to colonize these diverse niches. The major virulence factors are adhesins, capsules and toxins.

Adhesins

Pathogens use adhesins to attach to host tissues and to resist the flushing action of body fluids; thus, adhesins play a significant role throughout the course of an infection. The expression of adhesins is subject to phase variation in response to local conditions. Many pathogens express several different adhesins, each of which may recognize host cell receptors on different cell types or on the same cell type in different locations. For instance, type 1 fimbriae of uropathogenic *E. coli* attach to the epithelial cells of the urinary bladder, while P fimbriae of the

same bacteria attach to epithelial cells in the kidney. Colonization of a preferred niche may require several adhesins rather than a single adhesin. This is typical of colonization of the intestine by enteropathogenic bacteria, including *Salmonella* species. Interaction of adhesins with cell receptors activates signal transduction pathways, cell signalling cascades that can alter cell behaviour through changes in gene transcription, in cell metabolism, or in the cytoskeleton of the host cell. Adhesin–receptor interaction of enteropathogens with intestinal epithelium results in activation of signalling pathways that generate the release of nuclear factor-kappaB (NF-κB), a transcription factor that moves to the cell nucleus where it up-regulates the expression of a number of pro-inflammatory genes. For some invasive pathogens, such as *Listeria monocytogenes*, high-affinity interaction of their adhesins with host cell receptors activates signal transduction pathways that mediate the uptake of the bacteria by non-phagocytic epithelial cells.

Capsules

Encapsulated bacteria are often resistant to phagocytosis. Capsules interfere with opsonization, the binding of complement and antibody to the bacterial surface, thus protecting the bacterium from engulfment by phagocytes and from attack by antimicrobial agents. During growth, encapsulated bacteria may stick together, forming a microcolony or a biofilm that is resistant to phagocytic cells because of its large size. Some bacteria have capsules that may attach to host tissues. Many capsules are composed of polysaccharides and are hydrophilic; thus, these capsules may help the bacterium to resist desiccation. The capsule of *Bacillus anthracis* is composed of polyglutamic acid; it is anti-phagocytic and is regarded as an essential virulence factor.

Toxins

Traditionally, bacterial toxins have been considered to be of two types: exotoxins, produced and secreted by viable bacteria, and endotoxins, integral constituents of the bacterial cell wall not released until the microorganisms are lysed. Bacterial exotoxins and endotoxins differ in their structures and modes of action (Table 13.1).

Endotoxin is the lipopolysaccharide (LPS) of the outer leaflet of the outer membrane of Gram-negative bacteria. It is composed of three parts: a hydrophobic glycolipid (lipid A) and a hydrophilic polysaccharide composed of

a core oligosaccharide and an O-polysaccharide (O antigen). Toxicity resides in the lipid A portion. Lipopolysaccharide is released when bacterial cell walls are damaged by the complement system, phagocytes or antimicrobial drugs. The *in vivo* effects of endotoxin (BOX 13.1) depend on the amount present in the circulation. Small quantities of LPS enter the bloodstream every day, largely as a consequence of the death of commensal Gram-negative bacteria in the intestinal tract. LPS interacts with cells that have the toll-like receptor 4 (TLR-4): mononuclear phagocytes, neutrophils, platelets, dendritic cells and B lymphocytes. In this way, the innate immune system continues to be primed by the commensal microflora. High concentrations of circulating endotoxin greatly increase the release of cytokines, notably interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) from a range of mononuclear phagocytes, including Kupffer cells, monocytes, and macrophages in the spleen, bone marrow and pulmonary alveoli. Released cytokines induce fever, activate macrophages and clotting factor XII, and stimulate B cells to divide and produce antibodies. Cytokines stimulate the production of prostaglandins and leukotrienes which are mediators of inflammation. When present in toxic concentrations, LPS is responsible for the deposition of thrombi in small vessels (disseminated intravascular coagulation) and for a dramatic drop in blood pressure, giving rise to life-threatening endotoxic shock.

Table 13.1 Comparison of exotoxins and endotoxins.

Exotoxins	**Endotoxins**
Produced by live bacteria, both Gram-positive and Gram-negative	Component of the cell wall of Gram-negative bacteria released following cell death
Proteins, usually of high molecular weight	Lipopolysaccharide complex containing lipid A, the toxic component
Typically heat labile	Heat stable
Potent toxins, usually with specific activity; not pyrogenic. Highly antigenic; readily converted into toxoids which induce neutralizing antibodies	Toxins with moderate, non-specific generalized activity; potent pyrogens, weakly antigenic; not amenable to toxoid production. Neutralizing antibodies not associated with natural exposure
Synthesis determined extrachromosomally	Encoded in chromosome

BOX 13.1 Effects of endotoxins.

- Interaction with polymorphonuclear and mononuclear phagocytes, platelets and B lymphocytes
- Release of interleukin-1, leading to fever
- Activation of complement, promoting inflammatory changes

Exotoxins can be produced by either Gram-positive or Gram-negative bacteria. The toxins are highly antigenic proteins that can induce the production of

protective antitoxins. Occasionally, a potent exotoxin, such as *Clostridium botulinum* toxin, is ingested in contaminated food and produces systemic effects. More commonly, exotoxins are produced within the host and they may exert their effects either locally or systemically (BOX 13.2).

BOX 13.2 Effects of exotoxins.

- Cell membrane damage
  – Enzymatic digestion
  – Formation of pores
- Interference with protein synthesis
- Elevation of cAMP levels
- Disruption of functions relating to nervous tissue
- Digestion of components of interstitial tissue: collagen, elastin, hyaluronic acid

Many exotoxins have a two-subunit structure comprising an A subunit that possesses toxic enzymatic activity and a B subunit that is responsible for binding the exotoxin to specific receptors on the host cell membrane and may help in the transfer of the B subunit across the cell membrane. The enzymatic component, which acts on intracellular targets, does not become active until it is released in the cell. Thus, the B subunit determines the host cell specificity of the toxin: if the cell does not have receptors for the B subunit, it is not vulnerable to the toxin. Both tetanus toxin and botulinum toxin have the A–B subunit structure.

The exotoxins can be grouped into four major categories: (i) toxins that act on the extracellular matrix; (ii) toxins that act on the plasma membrane of their target cells, where they interfere with transmembrane signalling pathways or alter membrane permeability; (iii) toxins that act inside the cells, where they modify signalling pathways or the activities of the cytoskeleton; and (iv) toxins that cause dysfunction of the immune system, so-called superantigens. Some exotoxins damage the mucosal surface by enzymatic degradation of the epithelial cells, the intercellular junctions and the underlying tissues, thus removing physical barriers and facilitating the spread of bacteria through the tissues. Bacterial hyaluronidases, collagenases, lecithinases, elastases and phospholipases have the ability to degrade cell membranes and the tissue matrix.

At sublethal concentrations many of the membrane-acting toxins induce elevated concentrations of intracellular messengers, such as cyclic AMP, cyclic GMP and free cytosolic calcium ions. Because these messengers are involved in a wide variety of cellular processes, even sublytic amounts of the exotoxins are

capable of interfering with many essential signalling pathways, up-regulating some and down-regulating others. Heat-stable enterotoxin (ST) of *E. coli* activates membrane-bound guanylate cyclase in enterocytes, generating a signal that induces a significant rise in cyclic GMP within the cell. The resultant disturbance in the transport of ions causes diarrhoea. By triggering calcium signals, an exotoxin can up-regulate the expression of inducible genes that are responsible for the secretion of pro-inflammatory mediators such as IL-6 and IL-8. Some physiologically important cellular activities can be disturbed or turned off by an exotoxin that blocks the synthesis of intracellular proteins; this may result in the death of the target cells. Thus, both the cytolytic and non-cytolytic activities of an exotoxin can contribute to the local and systemic manifestations of a bacterial infection.

A number of toxins induce pore formation in cell membranes that disrupt the selective influx and efflux of ions across the membranes. For instance, uropathogenic *E. coli* release α-haemolysin which is cytotoxic to a variety of cells including erythrocytes, leukocytes, endothelial cells, fibroblasts and uroepithelial cells. It forms transmembrane pores that allow the loss of the normal ionic gradients without the loss of intracellular proteins; as a result, the internal macromolecules draw water into the cell, which is killed by osmotic lysis. At sublethal levels, the α-haemolysin of uropathogenic *E. coli* is a potent stimulus for the release of interleukin- 1β (IL-1β), which induces fever and triggers the release of acute phase proteins. The membrane-damaging group of toxins also includes the pore-forming haemolysins of *Streptococcus pyogenes, Listeria monocytogenes* and *Staphylococcus aureus*. At sublytic concentrations, pore-forming toxins can impair the defensive activities of the host: they inhibit or abolish phagocytosis by neutrophils and macrophages, and they induce apoptosis of T lymphocytes. Pore-forming toxins are synthesized and released when bacteria require iron; lysis of erythrocytes by the toxins releases haemoglobin, which becomes a source of iron for the pathogens.

A toxin may be released by a pathogen into the extracellular fluid (ECF), or it may be delivered directly into the cytosol of the target cell. If a toxin enters the ECF, the host can respond by producing neutralizing antibodies. Many bacterial toxins that act in the intracellular compartment are released into the ECF, attach to the plasma membrane and gain entry to the cell in a membrane-bound vesicle. In contrast, several Gram-negative pathogens, such as *Salmonella* species, *Shigella* species, enteropathogenic *E. coli* (EPEC), enterohaem- orrhagic *E. coli* (EHEC), and *Pseudomonas aeruginosa*, deliver toxins directly into the cytosol

of the target cell. The pathogens use a specialized system, a molecular syringe called a type III secretory system, to export the toxins in a hollow conduit across the inner bacterial membrane, the outer bacterial membrane, and the plasma membrane of the host cell. Because the exported toxins do not enter the extracellular spaces, the host does not develop neutralizing antibodies against them. Within the target cells, the exported proteins, known as ‘effectors’, interfere with signal trans- duction or act on the cytoskeleton. Effectors exported by *Salmonella* species and by *Shigella* species act on the cytoskeleton and are responsible for the uptake of the pathogens by non-phagocytic host epithelial cells. EPEC are also responsible for rearrangements of the cytoskeleton but, in contrast, the bacterium remains outside the cell. Moreover, EPEC supply both the ligand and the receptor that bring the bacterium into intimate contact with the host cell. The bacterial adhesin is an outer membrane protein, intimin. The receptor is a bacterial protein, Tir (translated intimin receptor), that is incorporated into the plasma membrane of the host epithelial cell after it has been transferred by an EPEC type III secretion apparatus. Binding of intimin to Tir leads to rearrangement of cytoskeletal components forming actin pedestals to which the EPEC attach.

Another family of microbial exotoxins, the superantigens (Fig. 13.2), subvert the adaptive immune response by cross-linking two of the most important antigen-recognition molecules, the T cell receptor (TCR) and the major histocompatibility complex (MHC) class II molecule. The superantigens bind first to MHC class II molecules on the surface of antigen- presenting cells (APC) but not in the antigen-binding groove. Then, the bimolecular superantigen–MHC protein complex interacts with subsets of the variable region of the β chain of the T cell receptor (TCR Vβ). The cross-linking of the two cell types results in a massive proliferation of T cells bearing particular segments of TCR Vβ and an associated release of large quantities of cytokines that can cause inflammation, fever, shock and multiple-organ system failure. A given superantigen can activate virtually all T cells that carry the subsets of the β chain with which it engages when it cross-links MHC with TCR. In contrast, a conventional antigen elicits a cellular response from only those T cells that have the appropriate receptor, a relatively small proportion of the T cell population. Hence, the T cell response to a superantigen exceeds the response to a conventional antigen by several orders of magnitude. However, because activation depends on the variable region of the β chain rather than on the antigenic specificity of the TCR, it does not provide the host with immune protection against the pathogen that

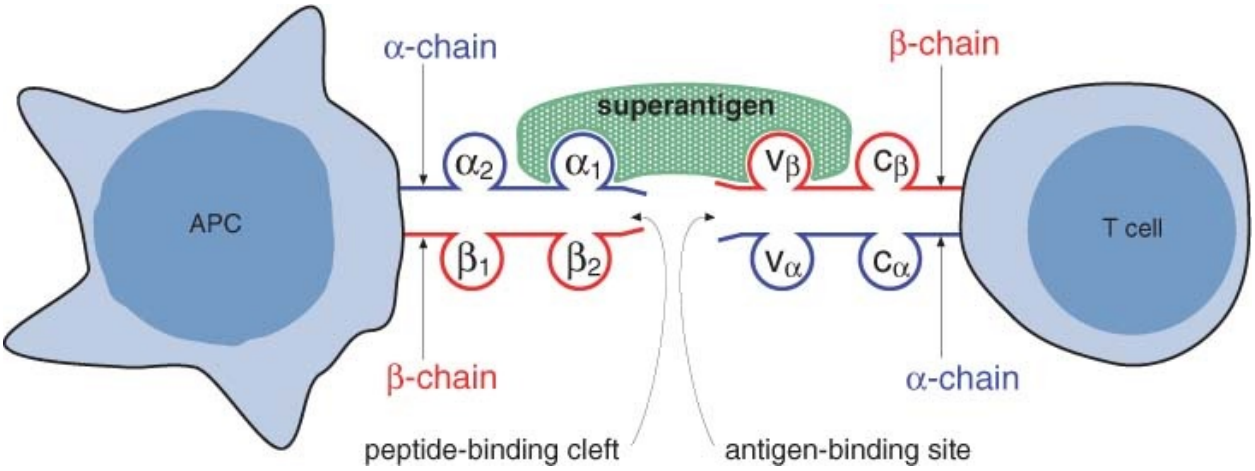
released the superantigen. In the course of some diseases, the initial proliferation is followed by failure of T cells to respond to the superantigen (T cell anergy) or by apoptosis and specific deletion of the particular TCR Vβ-bearing cells. This may increase the susceptibility of the host to infection. When a superantigen establishes a nonspecific linkage of TCR Vβ to MHC class II, the activated cells release large quantities of cytokines: principally, IL-2, TNF-α and IFN-γ from the expanding subset of T cells and IL-1 and TNF-α from the APCs. The excessive and uncoordinated release of the pro-inflammatory cytokines is responsible for the pathological features of the diseases attributed to superantigens released by staphylococci and streptococci.

Responses of the host to bacterial pathogens

The principal challenge for the host is to detect the pathogen and mount a rapid defensive response before there is significant tissue injury or interference with normal function. It takes several days for the adaptive immune system to mount a full response, an interval during which the host may experience severe injury or even death. Therefore, the ability of the innate immune system to promptly detect and engage the pathogen is crucial. The cells of the innate immune system express various pattern-recognition receptors (PRRs) capable of detecting conserved molecular ‘patterns’ that are unique to microorganisms and are not expressed by the hosts. Although these microbial ‘patterns’ are present in commensals and pathogens alike, they are known as pathogen- associated molecular patterns (PAMPs). In fact, it is molecules – specific, individual molecules – rather than ‘molecular patterns’ that are recognized in most instances (Beutler, 2004). Recognized ligands include LPS, lipoprotein, peptidoglycan, lipoteichoic acid and DNA from bacteria, double-stranded RNA from viruses, and glucans from fungi. These ligands are indispensable components of the microorganisms and, for that reason, they are not readily altered by mutation or selection (Beutler, 2004).

Figure 13.2 Superantigen binding to the Vβ domain of the T cell receptor and to the α-chain of a class II MHC molecule. Unlike conventional antigens, superantigens bind to class II MHC molecules without being processed and outside the peptide-binding cleft. Because superantigens bind to the Vβ regions of T cell receptors, regardless of their antigenic specificity, rather than to the antigen-binding site, activation of T cells is polyclonal with up to 15% of the

total T cell population becoming activated. APC: antigen-presenting cell.



Mammals possess a family of transmembrane PRRs, called toll-like receptors (TLRs), that are expressed constitutively in a variety of host immune cells, such as macrophages, dendritic cells, neutrophils, mast cells, B cells, and specific types of T cells. They are expressed also in some non-immune cells, such as epithelial cells, endothelial cells and fibroblasts. To date, 12 members of the family have been identified in mammals. Many of the TLRs are located in cell membranes but some are found almost exclusively within the cell. In addition, two intracellular proteins with nucleotide oligomerization domains (NOD) are known to act as sensors of pathogens in the cytoplasm of infected cells. Thus, the innate immune system is equipped to respond immediately to invading pathogens, whether extracellular or intracellular. Each TLR confers the ability to recognize distinct structural components, whether expressed by bacteria, viruses, protozoa, or fungi. For instance, TRL-4 in the host cell membrane recognizes LPS of Gram-negative bacteria; TRL-2 recognizes a discrete set of ligands, including lipoproteins and peptidoglycan of Gram-positive bacteria; while TLR-9, located within the host cell, detects nucleic acids derived from viruses and bacteria.

Recognition of PAMPs by TLRs activates intracellular signalling pathways that culminate in translocation of the transcription factor NF-κB to the nucleus where it binds to DNA and mediates the expression of the genes responsible for various pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-6, IL-1ß, and IL-12. The effector molecules and cells involved in the resultant inflammatory response include complement, monocytes, macrophages, dendritic cells, neutrophils, and natural killer cells. The neutrophils and macro-phages are efficient ‘professional phagocytes’ capable of killing most pathogens.

Macrophages secrete chemokines that attract neutrophils to the site of infection and they present microbial antigens to T cells. Signals from the TLRs also help to initiate pathogen-specific adaptive immune responses through the activation of dendritic cells. Some activated dendritic cells migrate from the site of infection to draining lymph nodes; there, in co-operation with MHC class II molecules, they present the microbial antigens to naïve CD 4^+ T cells and promote differentiation into T helper cells. In many instances, the process leads to the elimination of the pathogen without any clinical evidence of inflammation. However, when the outcome is less satisfactory, the inflammatory response can become an integral part of a pathological process.

Because the pathogen-recognition receptors are expressed constitutively and the pathogen-associated molecular patterns are conserved, the innate immune system is competent to detect microorganisms at all stages of their life histories. TRLs detect the molecular signatures of microbial pathogens, they orchestrate the innate immune responses, and they help to initiate the adaptive immune response. The efficacy of the adaptive immune response is built on the antigen-presenting function of the innate immune cells and on the actions of cytokines produced by cells of the innate immune system (Beutler, 2004).

The contest between host and pathogen does not always result in a clear victory for one or the other. Sometimes, pathogenic bacteria, endowed with an extensive set of virulence genes, reside within a host for an extended period without giving rise to any signs of disease. If an enteric pathogen, such as *Salmonella* Dublin in cattle or S. Typhi in humans, is sequestered in the gall bladder or in lymph nodes and is not excreted in the faeces, the host is described as a latent carrier; if the pathogen is shed in faeces, either continuously or intermittently, the host is considered an active carrier. In stressful situations, latent carriers can become active carriers, asymptomatic hosts that contaminate the environment and infect other susceptible animals. The most notorious active carrier recorded in history has been the healthy cook, ‘Typhoid Mary’, who worked in New York State in the early years of the twentieth century and infected over 200 people with *Salmonella* Typhi as she moved from one employer to another. In cattle herds, *Salmonella* Dublin can establish long-term residence in both latent carriers and active carriers. Although the tendency towards ‘restrained virulence’ is common amongst pathogens, there are exceptions. Living hosts are not essential for the dissemination of spore-forming anaerobes such as *Clostridium* species. These bacteria produce highly lethal toxins that kill the host, which then becomes an anaerobic substrate in which the

bacteria multiply and from which they can be disseminated as spores.

Dissemination of pathogens in the host

Some pathogens are not invasive and do not spread systemically, yet they release toxins and other signals that pose challenges to homeostasis, local or systemic. For instance, when enterotoxigenic strains of *Escherichia coli* (ETEC) adhere to the epithelial cells of the small intestine in newborn farm animals, they are not invasive but they induce profuse quantities of watery diarrhoea in the absence of evident histological damage to the mucosa. Likewise, *Clostridium tetani* can contaminate a wound in any region of the body, producing a neurotoxin. The pathogen remains *in situ* but the toxin causes systemic effects after it is taken up by peripheral nerve endings and transported intra-axonally to the central nervous system where it blocks the actions of inhibitory motor neurons. In contrast, other pathogenic bacteria penetrate epithelial barriers, gain access to the underlying tissue and induce inflammatory changes. For instance, *Pseudomonas aeruginosa* secretes a number of toxins that degrade the epithelial barrier and allow the opportunistic pathogen to enter the subepithelial tissues.

There are two routes by which invasive pathogens can breach the epithelial barrier: by passage through the intercellular spaces (the paracellular route), or by passage through the epithelial cells (the transcellular route). Some pathogens are capable of using either route. The paracellular route can be taken by bacteria that are able to disrupt the intercellular tight junctions: for example, enteropathogenic *E. coli, Listeria monocytogenes, Helicobacter pylori*, some *Clostridium* species and some *Salmonella* serovars. Other bacteria, such as *Shigella* species, may pass through the paracellular route when tight junctions are opened by emigrant neutrophils responding to chemotactic stimuli from pathogens present on the epithelial surface.

If it is to negotiate the transcellular route, an invasive bacterium must be able to enter intact epithelial cells. Epithelial cells are not phagocytic, so invasive bacteria have had to evolve molecular strategies that enable them to regulate their own entry into these host cells. The pathogens enter the epithelial cells through membrane-bound vacuoles formed by the host cell membrane in response to signals generated by the pathogens. There are two major types of induced uptake: a ‘zipper’ mechanism and a ‘trigger’ mechanism. The zipper mechanism is induced by specific ligand–receptor interactions at the cell membrane, whereas the trigger mechanism is induced by effector molecules

delivered into the cell by a type III secretory system. In each instance, the pathogens utilize existing signal transduction pathways within the cells to promote rearrangements of the actin cytoskeleton beneath the host cell membrane. The actin is responsible for the cell membrane forming pseudopodia or larger membrane ruffles that progress to envelop the pathogens in vacuoles, which are then taken into the host cell. The actin cytoskeleton provides the energy necessary for the formation of the vacuoles and for the entry of the vacuoles into the cell.

For pathogens that use the zipper mechanism, activation of the signalling pathways begins when bacterial ligands bind with high affinity to specific receptors on the epithelial cell membrane, as when internalins on the surface of *Listeria monocytogenes* bind to E-cadherin on the apical surface of the host cell, or when invasins on the surface of *Yersinia pseudotuberculosis* bind to β_1-integrins on the basolateral side of the host cell. For both pathogens, the actin cytoskeleton accumulates in the vicinity of the point of host–pathogen contact and the formation of the membrane- bound vacuole is achieved by incremental interactions between bacterial ligands and cell adhesins, resulting in a zipper-like process which tightly envelops the bacterium (Fig. 13.3).

Bacteria that use the trigger mechanism, including *Salmonella* and *Shigella*, deliver effector proteins that induce a profuse rearrangement of the actin cytoskeleton under the host plasma membrane, which results in the membrane extending into large ruffles that fold over, fuse and trap the pathogens and extracellular material in a membrane-bound, fluid-filled compartment that is incorporated into the cell. Thus, *Salmonella* and *Shigella* enter epithelial cells in spacious macropinocytotic vacuoles (Fig. 13.4).

Figure 13.3 Schematic diagram of the zipper mechanism of bacterial uptake by non-phagocytic cells. Bacterium contacts cell membrane and forms first ligand–receptor complexes. This leads to clustering of receptors and local changes in actin cytoskeleton that propagate incremental (zipper-like) ligand–receptor binding around the bacterium until the invading pathogen is enclosed within a vacuole that appears to sink into the cell. *Listeria* species are taken in through the apical surface of the epithelial cell and *Yersinia* species enter through the basolateral surface of the host cell.

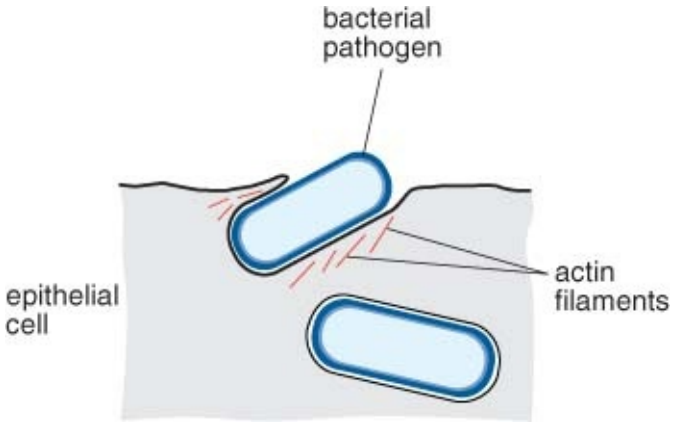
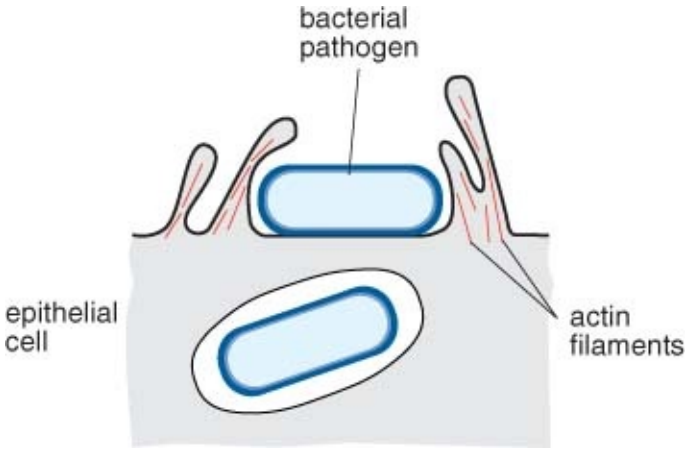


Figure 13.4 Schematic diagram of the trigger mechanism of bacterial uptake by non-phagocytic cells. Rather than bind to a receptor on a host cell, the pathogen injects bacterial effectors directly into the cell. The injected effectors promote massive actin polymerization and formation of large ruffles that incorporate the pathogen into the cell. Salmonellae are enveloped by ruffles on the apical surface of the epithelial cell and shigellae enter through the basolateral surface of the host cell.



The site of entry is subject to the polarity of the host cells. Tight junctions divide the epithelial plasma membrane into two surfaces: the apical surface and the basolateral surface. These two surfaces have different functions and distinctly different biochemical components, including receptors, pumps, channels, transporters, lipids, proteins and enzymes. Some invasive bacteria, such as *Listeria monocytogenes* and *Salmonella* species, attach to epitopes present in protein and glycolipid constituents of the apical surface before they are engulfed by the polarized epithelial cells. However, *Yersinia* species and *Shigella* species, also invasive bacteria, are not able to bind to the apical surfaces of the epithelial cells of the intestinal mucosa but they enter these cells through

the basolateral membranes. The pathogens are taken up by M cells, specialized epithelial cells that overlie Peyer's patches. When they reach the cell pockets beneath the M cells, they are engulfed by the resident macrophages in which they multiply and induce rapid cell death. On release from the dead macrophages, the bacteria induce ruffling of the basolateral membranes of adjoining columnar epithelial cells, which leads to those cells engulfing the bacteria in membrane-bound vacuoles.

Once inside epithelial cells, *Listeria* and *Shigella* are able to lyse the vacuolar membrane and escape into the cytoplasm. *Listeria monocytogenes* escapes because it secretes a pore-forming enzyme, listeriolysin O. While free in the cytoplasm, both *Listeria* and *Shigella* polymerize actin filaments forming a comet-like tail at one bacterial pole which propels them towards adjacent cells. The energy for the movement of the bacteria is provided by the host cell. When the motile bacteria reach the lateral cell membrane, they induce pseudopod-like projections. These projections and the bacteria they contain are engulfed by the adjacent cells. In the newly infected cells, the bacteria lyse the membranes surrounding them and the cycle is repeated allowing extensive cell-to-cell transmission and causing significant damage within the epithelial layer. In contrast, *Salmonella* are able to remain within the spacious vacuoles in which they enter the host cells. The persistence of the microorganisms in these novel intracellular compartments is attributed to a number of effector proteins they deliver into the host cell cytoplasm by the type III secretory system (Coburn *et al.*, 2007).

Bacteria that gain access to the bloodstream can be disseminated throughout the body either free in the plasma or in phagocytes. During bacteraemia, bacteria are present transiently in the bloodstream without replicating, whereas, during septicaemia, pathogenic organisms multiply and persist in the bloodstream, producing systemic disease. Dissemination of *Mycobacterium bovis* throughout the body can occur following phagocytosis by macrophages.

The clinical spectrum of bacterial disease

Disease is not an inevitable consequence of infection. Some individual pathogens tend to produce a predictable clinical picture following infection of a susceptible host. Anthrax in ruminants is invariably peracute and fatal. In contrast, infections with bacteria such as *Salmonella* Dublin in cattle may produce many different forms of disease.

Bacterial infections can be conveniently categorized as acute, subacute, chronic or persistent. Acute infections usually have a short severe clinical course, often a matter of days, and the invading bacteria are usually cleared from the body by the host's immune response. The host may shed the agent in large numbers for a short period. Subacute infections produce clinical effects of less intensity.

Chronic infections tend to occur when the host fails to eliminate the pathogen. Frequently, the infectious agent replicates initially to a high level and is subsequently cleared from most sites in the body by the host's immune response. Persistence occurs in certain sites such as the uriniferous tubules and the CNS in which the effects of cell-mediated and humoral immunity are minimal. Persistent shedding may occur from some of these sites as in bovine leptospirosis, in which leptospires may be shed in urine for more than a year. Some other chronic infections may be characterized by persistence with or without shedding of the aetiological agent. Cattle that have mounted an effective cell-mediated immune response to *Mycobacterium bovis* infection may remain chronically infected, with the organism persisting in localized foci without shedding.

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Section III
Pathogenic Bacteria

Chapter 14

Staphylococcus species

Staphylococci are Gram-positive cocci, approximately 1 μm in diameter, that tend to occur in irregular clusters resembling bunches of grapes (Fig. 14.1). The name derives from the Greek words *staphyle* and *kokkos* for a 'bunch of grapes' and a 'berry', respectively. *Staphylococcus* species occur as commensals on skin and mucous membranes; some may act as opportunistic pathogens causing pyogenic infections.

Most staphylococci are facultative anaerobes and catalase-positive. They are non-motile and oxidase-negative and do not form spores. Two species, *S. aureus* subsp. *anaerobius* and *S. saccharolyticus*, are anaerobic and catalase-negative.

A total of 43 species of *Staphylococcus* have been described to date, seven of which are coagulase-positive or coagulase-variable species (Table 14.1). The coagulase-positive *S. aureus* subsp. *aureus* (referred to as *S. aureus*), *S. pseudintermedius*, and the coagulase- variable *S. hyicus* are important pathogens of domestic animals (Table 14.1). *Staphylococcus intermedius* was previously thought to be the major staphylococcal pathogen of dogs and cats but it is now considered that *S. intermedius* strains isolated from these hosts belong to the species *S. pseudintermedius* (Sasaki *et al*., 2007; Devriese *et al*., 2009). Coagulase production correlates with pathogenicity. Although coagulase-negative staphylococci are usually of low virulence, some occasionally cause disease in animals and humans (Table 14.2).

Usual habitat

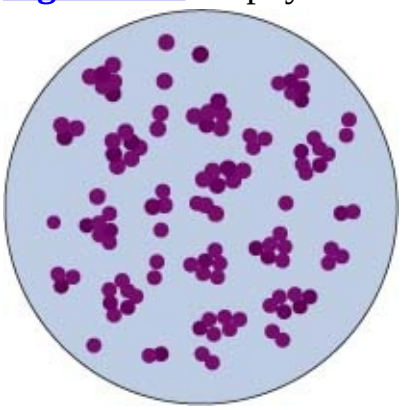
Staphylococcal species occur worldwide as commensals on the skin of animals and humans. They are also found on mucous membranes of the upper respiratory tract and lower urogenital tract and as transients in the digestive tract. The carriage sites of the pathogenic staphylococci are usually the mucous membranes and moist areas of the skin such as the axillae or perineal area. The nares are a major site of carriage of *S. aureus* in animals and humans, and approximately

20% of humans are permanent nasal carriers of this organism. *Staphylococcus sciuri* and *S. xylosus* are the most common coagulase-negative staphylococci isolated from healthy domestic animals. Staphylococci are comparatively stable in the environment, and staphylococcal strains display a selective affinity for particular animal species. Transfer of staphylococcal strains between animal species and between animals and humans is limited but of importance. Transfer of methicillin-resistant *S. aureus* strains from humans to animals or animals to humans is of particular significance.

Key points

- Gram-positive cocci in clusters resembling bunches of grapes
- Grow on non-enriched media
- Moderately-sized white or golden colonies
- Colonies of *S. aureus* and *S. pseudintermedius* produce double haemolysis
- Facultative anaerobes, non-motile, catalase-positive
- Commensals on mucous membranes and skin
- Coagulase production correlates with pathogenicity
- Comparatively stable in the environment
- Cause pyogenic infections

Figure 14.1 Staphylococci in characteristic ‘bunches of grapes’ formations.



Differentiation of *Staphylococcus* species

In clinical specimens, *Staphylococcus* species must be differentiated from *Streptococcus* species and from *Micrococcus* species (Table 14.3). Staphylococci are generally catalase-positive and streptococci catalase- negative. *Staphylococcus* species are usually categorized by their colonial appearance, haemolytic pattern, biochemical profiles and ribosomal RNA gene restriction

patterns (Thomson-Carter *et al*., 1989I. Some of the principal reactions of the coagulase-positive staphylococci are indicated in Table 14.4. It may be particularly important to distinguish *S. aureus* from *S. pseudintermedius* in certain clinical conditions, especially in dogs and cats.

Table 14.1 Coagulase-positive staphylococci and their clinical importance.

Species	Hosts	Clinical conditions
Staphylococcus aureus[a]	Cattle	Mastitis, udder impetigo
	Sheep	Mastitis
		Tick pyaemia (lambs)
		Benign folliculitis (lambs)
		Dermatitis
	Goats	Mastitis
		Dermatitis
	Pigs	Botryomycosis of mammary glands
		Impetigo on mammary glands
	Horses	Scirrhous cord (botryomycosis of the spermatic cord), mastitis
	Dogs, cats	Suppurative conditions similar to those caused by *S. pseudintermedius*
	Poultry	Arthritis and septicaemia in turkeys
		Bumblefoot
		Omphalitis in chicks
S. pseudintermedius	Dogs	Pyoderma, endometritis, cystitis, otitis externa, and other suppurative conditions
	Cats	Various pyogenic conditions
	Horses	Rarely isolated
	Cows	Rarely isolated
S. hyicus[b]	Pigs	Exudative epidermitis (greasy-pig disease)
		Arthritis
	Cattle	Mastitis (rare)
S. intermedius	Horses	Isolated from nares
	Pigeons	Isolated from upper respiratory tract
S. aureus subsp. *anaerobius*	Sheep	Lymphadenitis
S. delphini	Dolphins	Suppurative skin lesions
	Horses	Isolated from nares
	Pigeons	Isolated from upper respiratory tract
S. lutrae	Otters	Pathogenic significance uncertain
S. schleiferi subsp. *coagulans*	Dogs	Otitis externa

a, *S. aureus* can cause neonatal septicaemia and wound infections in many species.

b, 25–50% of *S. hyicus* strains are coagulase-positive.

In veterinary diagnostic laboratories, specific identification of the coagulase-negative staphylococci is ordinarily reserved for those organisms that are isolated in almost pure culture, or are recovered from sites that are normally

sterile such as joints or cerebrospinal fluid.

Table 14.2 Coagulase-negative staphylococci isolated from animals.

Species	Host/Source
S. arlettae	Goats/Nares
	Poultry/Skin
S. capitis	Cattle/Milk
S. caprae	Goats/Skin
S. chromogenes	Cattle/Milk[a]
	Pigs, poultry/Skin
S. cohnii	Cattle/Milk[a]
S. epidermidis	Cattle/Milk[a]
	Dogs, horses/Wound infections
S. equorum	Horses/Skin
S. felis[b]	Cats/Otitis externa, skin infections
S. gallinarum	Poultry/Skin infections
S. haemolyticus	Cattle/Milk[a]
S. hominis	Cattle/Milk
S. lentus	Pigs, sheep, goats/Skin infections
S. nepalensis	Goats/Respiratory tract
S. saprophyticus	Cats/Skin
	Cattle/Nostrils
S. sciuri	Cats and other animals/Skin infections
S. simiae	Squirrel monkeys/Gastrointestinal tract
S. simulans	Cattle/Milk[a]
	Dogs, cats, pigs/Skin
S. vitulinus	Cattle, sheep, pigs/Skin
S. warneri	Cattle/Milk[a]
S. xylosus	Cattle, sheep/Milk[a]
	Cats, poultry, pigs, horses/Skin

a, occasionally isolated from cases of subclinical or clinical mastitis.

b, described by Igimi *et al*. (1989).

- Colonial characteristics: Staphylococcal colonies are usually white, opaque and up to 4 mm in diameter. The colonies of bovine and human strains of *S. aureus* are golden yellow. Colonies of some coagulase-negative staphylococci are also pigmented.
- Haemolysis in sheep or ox blood agar: Four staphylococcal haemolysins are recognized, alpha, beta, gamma and delta. Individual haemolysins differ antigenically, biochemically and in their effects on the red blood cells of different animal species. Strains vary in their haemolysin-producing ability, and animal strains of *S. aureus* and *S. pseudintermedius* usually produce

both alpha-haemolysin and beta-haemolysin. On ruminant blood agar, the alpha-haemolysin causes a narrow zone of complete haemolysis immediately around the colony, and the beta-haemolysin produces a wider zone of partial or incomplete haemolysis. This is referred to as double haemolysis (Fig. 14.2). These haemolysins act as toxins *in vivo*. Coagulase-negative staphylococci exhibit variation in their ability to produce haemolysis which usually develops slowly. Isolates of *S. hyicus* are non-haemolytic.

- Slide and tube coagulase tests: In these tests, a suspension of staphylococci is mixed with rabbit plasma either on a slide or in a small tube. The fibrinogen in rabbit plasma is converted to fibrin by coagulase:

  – The slide test detects the presence of a bound coagulase or clumping factor on the bacterial surface. A positive reaction is indicated by clumping of bacteria within 1 to 2 minutes.

  – The tube test detects both free coagulase (staphylocoagulase), which is secreted by the bacteria into the plasma, and bound coagulase. It is the definitive test for coagulase production and a positive reaction is indicated by clot formation in the tube following incubation at 37°C for 24 hours.

  – Commercially available kits, which detect capsular polysaccharides and cell wall components including clumping factor and Protein A, are useful for the presumptive identification of *S. aureus*.

- Biochemical tests for differentiating *S. aureus* and *S. pseudintermedius* (Table 14.4):

  – A rapid test for detection of acetoin has been developed (Davis and Hoyling, 1973).

  – Purple agar, containing bromocresol purple as a pH indicator and 1% maltose, can be used to differentiate *S. aureus* from *S. pseudintermedius* and *S. intermedius* (Quinn *et al*., 1994). *Staphylococcus aureus* utilizes maltose and the acid produced changes the medium and colonies from purple to yellow. *Staphylococcus pseudintermedius* and *S. intermedius* are poor maltose fermenters and do not change the colour of the medium. Differentiation of *S. intermedius* and *S. pseudintermedius* can be carried out by bio-chemical methods including the detection of mannitol fermentation under anaerobic conditions. However, definitive differentiation of these species is most reliable using molecular methods. As recent molecular studies have shown that all *S. intermedius* isolates

from dogs belonged to a single major cluster containing the newly described *S. pseudintermedius* (Fitzgerald, 2009), it is likely that all isolates from dogs that were phenotypically identified as *S. intermedius* in the past are in fact *S. pseudintermedius*.

– Biochemical tests, which are commercially available, can be used to confirm the staphylococcal species, although those currently available do not differentiate *S. intermedius* from *S. pseudintermedius*.

- Molecular procedures such as the polymerase chain reaction are increasingly used by diagnostic laboratories as well as research laboratories for definitive identification of staphylococcal species.

Table 14.3 Differentiation of Gram-positive cocci.

Organism	Appearance in stained smears	Coagulase production	Catalase production	Oxidase production	O-F test[a]	Bacitracin disc (0.04 units)
Staphylococcus spp.	Irregular clusters	±	+	–	F	Resistant
Micrococcus spp.	Packets of four	–	+	+	O	Susceptible
Streptococcus and *Enterococcus* spp.	Chains	–	–	–	F	Resistant

a, oxidation-fermentation test, O oxidative, F fermentative.

Table 14.4 Distinguishing features of coagulase-positive staphylococci.

Species	Colony colour	Haemolysis on sheep blood agar	Coagulase production		Acetoin production	Maltose utilization[a]
			Tube test	Slide test		
S. aureus	Golden yellow[b]	+	+	+	+	+
S. pseudintermedius	White	+	+	-	+	±
S. intermedius	White	+	+	v	–	±
S. hyicus	White	–	v	–	–	–
S. aureus subsp. *anaerobius*[c]	White	+	+	–	–	na
S. delphini	White	+	+	–	–	na
S. schleiferi subsp. *coagulans*	White	+	+	–	+	na

a, 1% maltose in purple agar base.

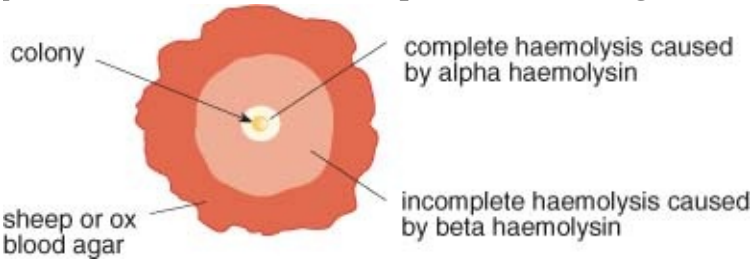
b, bovine and human strains only.

c, anaerobic.

+, over 90% strains positive. −, over 90% strains negative. ±, poor utilization. na, not available. v, variable reactions.

Figure 14.2 The characteristic double haemolysis of *S. aureus* and *S.*

pseudintermedius on sheep or ox blood agar.



Pathogenesis and pathogenicity

Because staphylococci are pyogenic bacteria, they often cause suppurative lesions. Minor trauma or immunosuppression may predispose to the development of infection. As with many other bacterial pathogens, virulence attributes can be broadly classified into those that promote tissue colonization, immune evasion and tissue destruction. Surface proteins such as staphylococcal cell wall proteins, which bind to fibronectin and fibrinogen, facilitate bacterial attachment to tissues. Structural features including capsular polysaccharide, teichoic acids and protein A interfere with opsonization and subsequent phagocytosis. Catalase production aids in survival within phagocytes, and coagulase has a role in shielding the organism from phagocytic cells. An array of virulence factors are involved in spread within tissues and the production of pathological changes within the host. Pathogenic effects can range from relatively minor local infections to life-threatening septicaemia. Although the major virulence factors of *S. aureus* have been well characterized, those of *S. pseudintermedius* are less defined. Nevertheless, many virulence attributes of *S. pseudintermedius* appear similar to those identified in *S. aureus* (Fitzgerald, 2009). Exoenzymes such as kinases and hyaluronidase promote tissue invasion, and exotoxins including haemolysins and leukocidin lyse host cell membranes. Exfoliative toxins have been identified in all three major staphylococcal pathogens of domestic animals (*S. aureus*, *S. hyicus* and *S. pseudintermedius*). These toxins are proteases that act on desmoglein, a cell-to-cell adhesion molecule in the skin (Nishifuji *et al*., 2008). They appear to act mainly in a species- specific manner as the predominant toxin produced in human staphylococcal scalded skin syndrome failed to produce lesions in pigs or dogs. Virulence factors of *S. aureus* and their pathogenic effects are indicated in Table 14.5. Genes encoding virulence factors in *S. aureus* are usually found on mobile genetic elements such as *S. aureus* pathogenicity islands and bacteriophages.

Regulatory genes such as *agr* and *sarA* control the expression of virulence genes as infection proceeds (O'Neill *et al*., 2007).

Production of coagulase by staphylococci is an important indicator of pathogenicity. Additional markers for pathogenicity are DNase activity and protein A production.

Biofilm formation by *S. aureus, S epidermidis* and *S. pseudintermedius* is a significant virulence determinant and these species are capable of causing chronic prosthetic-device-related infections.

Enterotoxins produced by *S. aureus* are important causes of food poisoning in humans. *S. pseudintermedius* is also known to produce some similar toxins but the role of these toxins in food poisoning is not well defined.

Diagnostic procedures

- Exudative epidermitis in piglets and tick pyaemia of lambs are the only clinical conditions of domestic animals with clinical signs sufficiently distinctive to indicate the involvement of pathogenic staphylococci. In suppurative conditions, the likelihood of staphylococcal infection must be considered and appropriate specimens such as exudates and mastitic milk collected for laboratory procedures.
- Gram-stained smears of pus or other suitable specimens may reveal typical staphylococcal clusters.
- Specimens are cultured on blood agar, selective blood agar and MacConkey agar and incubated aerobically at 37°C for 24 to 48 hours. Selective blood agar, which contains nalidixic acid and colistin, is used to inhibit *Proteus* species and other Gram-negative contaminants.
- Identification criteria for isolates:
  – Colonial characteristics
  – Presence or absence of haemolysis
  – Absence of growth on MacConkey agar
  – Catalase production
  – Coagulase production
  – Biochemical profile
  – Molecular typing usually based on PCR procedures. Bannoehr *et al*. (2009) reported a procedure for differentiation of the *S. intermedius* group (*S. intermedius, S. pseudintermedius* and *S. delphini*) involving PCR

amplification of a fragment of the *pta* gene, followed by the use of MboI in an RFLP procedure. Sasaki *et al*. (2010) developed a multiplex PCR based on the *nuc* gene which identified all coagulase-positive staphylococci.

- Phage typing, which was used for many years in epidemiological investigations such as those relating to outbreaks of staphylococcal food poisoning in humans, has been largely replaced by molecular methods. Pulsed-field gel electrophoresis following digestion of chromosomal DNA using a restriction endonuclease is one of the most important methods used in epidemiological investigations. However, efforts to harmonize PFGE protocols internationally have proved difficult, and common databases have been established only at national levels (Deurenberg *et al*., 2007). Apart from outbreaks of food poisoning, epidemiological investigation is frequently required in outbreaks of methicillin-resistant *S. aureus* (MRSA) in humans and animals. PFGE-based techniques are useful for such investigations but are limited by the clonal nature of some MRSA strains. Other methods used to characterize MRSA strains include multilocus sequence typing, PCR-based typing of the staphylococcal chromosomal cassette *mec* (SCC *mec*) region and *spa* typing (Frenay *et al*., 1996; Enright *et al*., 2002). The SCC *mec* region contains the *mec* gene which encodes methicillin resistance and the *spa* gene encodes staphylococcal protein A production.

Table 14.5 Virulence factors, including toxins, of *Staphylococcus aureus* and their pathogenic effects.

Virulence factor	**Pathogenic effects**
Coagulase	Conversion of fibrinogen to fibrin. Fibrin deposition may shield staphylococci from phagocytic cells
Lipase, esterases, elastase, staphylokinase, deoxyribonuclease, hyaluronidase, phospholipase	Enzymes which contribute to tissue destruction and virulence
Protein A	Surface component which binds Fc portion of IgG and inhibits opsonization
Leukocidin	Cytolytic destruction of phagocytes of some animal species
Alpha-toxin (alpha-haemolysin)	The major toxin in gangrenous mastitis. It causes spasm of smooth muscle and is necrotizing and potentially lethal
Beta-toxin (beta-haemolysin)	A sphingomyelinase which damages cell membranes
Exfoliative toxins	Proteases which contribute to skin lesion development in humans, dogs and pigs
Enterotoxins	Heat-stable toxins associated with staphylococcal food poisoning in humans
Toxic shock syndrome toxins (TSST)	Induce excessive lymphokine production, resulting in tissue damage. Bovine and human strains of *S. aureus* produce TSST-1. Sheep and goat strains produce a variant of this toxin. TSST-1 has superantigen activity

Clinical infections

Because staphylococci occur both as commensals on skin and mucous membranes and as environmental contaminants, infections can be either endogenous or exogenous in origin. Many infections are opportunistic and associated with trauma, immunosuppression, intercurrent parasitic or fungal infections, allergic conditions or endocrine and metabolic disturbances. Coagulase-positive staphylococci are responsible for the majority of these infections (Table 14.1). Some strains of low virulence which are coagulase negative are also capable of causing disease in animals (Table 14.2). Currently available vaccines are ineffective for preventing staphylococcal infections. Antibiotic susceptibility testing should precede treatment as antimicrobial resistance is a significant problem with staphylococcal species. Staphylococcal diseases of importance in domestic animals include mastitis, tick pyaemia, exudative epidermitis, botryomycosis and pyoderma.

Bovine staphylococcal mastitis

Staphylococcal mastitis, usually caused by *S. aureus*, is a common form of bovine mastitis worldwide. It may be subclinical, acute or chronic. The majority of infections are subclinical. Peracute and gangrenous forms are associated with severe systemic reactions and can be life-threatening. In gangrenous mastitis, the affected quarter, which becomes cold and blue– black, eventually sloughs. Tissue necrosis is attributed to the alpha-toxin which causes contraction and necrosis of smooth muscle in blood vessel walls, impeding blood flow in the affected quarter. In addition, this toxin causes release of lysosomal enzymes from leukocytes. Bovine staphylococcal mastitis is discussed in more detail in Chapter 93.

Tick pyaemia

Tick pyaemia, an infection of lambs that is caused by *S. aureus*, is confined to hill-grazing regions of Britain and Ireland where there are suitable habitats for the tick *Ixodes ricinus*. Lambs can carry *S. aureus* on their skin and nasal mucosa and infection occurs through minor skin trauma including tick bites. *Ixodes ricinus* is a vector for the rickettsial agent of tick-borne fever, *Anaplasma phagocytophilum*, which can cause immuno- suppression in lambs and may predispose to staphylococcal infection.

Tick pyaemia is characterized either by septicaemia and rapid death or by localized abscess formation in many organs. Clinical manifestations include arthritis, posterior paresis and ill-thrift. The condition can be of considerable economic importance on some farms where up to 30% of lambs between 2 and 10 weeks of age can be affected in spring and early summer.

Diagnosis

- In young lambs grazing rough pasture in Britain or Ireland, clinical signs may be indicative of the disease.
- Microscopic demonstration of the bacteria in pus, followed by isolation and identification of *S. aureus* from lesions, is confirmatory.

Treatment and control

Treatment is of limited value in severely affected lambs. Efforts should be directed at control within the flock.

- Prophylactic treatment of lambs with antibiotics, such as long-acting tetracycline, can be initiated at 1 week of age. Tetracyclines also protect lambs against *A. phagocytophilum*.
- Tick-control measures such as dipping should be introduced.

Exudative epidermitis (greasy-pig disease)

This disease, caused by *S. hyicus*, occurs worldwide in sucklers and weaned pigs up to 3 months of age. It is highly contagious and characterized by widespread excessive sebaceous secretion, exfoliation and exudation on the skin surface. Affected pigs, which are anorexic, depressed and febrile, have an extensive, non-pruritic dermatitis with a greasy exudate. Piglets under 3 weeks of age may die within 24 to 48 hours. Morbidity rates range from 20 to 100%, and mortality rates can reach 90% in severely affected litters. *Staphylococcus hyicus* can be isolated from the vaginal mucosa and skin of healthy sows. The organisms probably enter the skin of young pigs through minor abrasions such as bite wounds.

Predisposing stress factors include agalactia in the sow, intercurrent infections and weaning. Exfoliative toxin produced by *S. hyicus* appears to be the major virulence factor as injection of this toxin into the skin of young pigs produces exfoliation (Amtsberg, 1979).

Diagnosis

- A high mortality rate in young pigs with exudative, non-pruritic skin lesions is typical of the disease.
- Isolation and identification of *S. hyicus* from the dermal lesions is confirmatory.

Treatment and control

- Early systemic antibiotic therapy combined with topical treatment with antiseptic or antibiotic suspensions may be effective.
- Strict isolation of affected pigs is essential.
- Cleaning and disinfection of contaminated buildings should be carried out.
- Sows should be washed with a suitable antiseptic soap before farrowing.
- Prior colonization of the skin with an avirulent strain of *S. hyicus* prevented experimental infection with virulent *S. hyicus* (Allaker *et al*., 1988).

Botryomycosis

Botryomycosis is a chronic, suppurative granulomatous condition, often caused by *S. aureus*. It can occur within a few weeks of castration in the horse due to infection of the stump of the spermatic cord (scirrhous cord). Botryomycosis can also occur in mammary tissues of sows. The lesion is composed of a mass of fibrous tissue containing foci of pus and sinus tracts.

Staphylococcal infections in dogs and cats

Staphylococcus pseudintermedius is commonly isolated from pyoderma, otitis externa and other suppurative conditions including mastitis, endometritis, cystitis, osteomyelitis and wound infections. Occasionally, similar suppurative conditions are caused by *S. aureus*.

Methicillin-resistant staphylococcal infections in animals

Infection with MRSA has been a major problem in human hospitals for many years but methicillin- resistant staphylococcal infections have become a major problem in veterinary medicine and animal production in the last decade.

Infection with MRSA in small animals, principally in dogs, has been recorded in many countries, with wound infections, surgical site infections, pyoderma, otitis and urinary tract infections most commonly reported (Weese and van Duijkeren, 2010). Similar conditions have been reported in horses, including outbreaks of nosocomial infection in veterinary hospitals (Hartmann *et al*., 1997 ; O ' Mahony *et al*., 2005 ; Weese *et al*., 2005). Transmission of infection between pets and humans, including veterinary personnel, and between horses and humans has been reported (Leonard *et al*., 2006; Moodley *et al*., 2006). Colonization rates in veterinary personnel are generally higher than in the general population and exposure to MRSA may become an occupational hazard for this particular group (Weese and van Duijkeren, 2010).

Infection with MRSA in humans which is not associated with hospitalization, designated community- acquired MRSA, is a significant problem worldwide. Strains of *S. aureus* causing these infections are frequently positive for a leucocidin known as Panton- Valentine leucocidin (PVL toxin), which is implicated in haemolytic pneumonia and severe soft tissue and skin infections. Although *S. aureus* strains positive for PVL toxin have been isolated from animals infrequently, most MRSA strains in animals are PVL negative.

High colonization rates with MRSA in pig farmers (23%) and others in contact with pigs first emerged as a problem in The Netherlands in 2004 (Voss *et al*., 2005). Unlike MRSA in small animals and horses, the predominant strain in pigs, designated ST398, appears to be a pig-adapted strain and was not acquired from humans. However, human strains of MRSA have been isolated from pigs also, suggesting that human-to-pig transmission can occur in addition to transmission from pigs to humans (Khanna *et al*., 2008). Although the high colonization rates with MRSA in pig farmers are of public health concern, infection with MRSA is rare in pigs. In spite of high isolation rates of MRSA from nasal swabs in pigs on infected farms, there are only occasional reports of clinical conditions such as exudative epidermitis caused by MRSA.

Methicillin resistance can occur in coagulase- negative staphylococci also but is not of major significance worldwide. However, methicillin resistance in *S. pseudintermedius* is emerging as an important clinical problem in veterinary medicine in many countries, including the United States and parts of Europe (Weese and van Duijkeren, 2010). As these organisms are usually resistant to a wide range of antimicrobial agents including the β-lactam antibiotics, clinical management of animals infected with methicillin-resistant *S. pseudintermedius* represents a major challenge to the veterinary profession.

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Chapter 15

Streptococci

The Streptococci are a group of bacteria that can infect many animal species, causing suppurative conditions such as mastitis, metritis, polyarthritis and meningitis. The enterococci are a related group of organisms, previously classified in the genus *Streptococcus*. Strictly anaerobic Gram-positive cocci, previously placed in the genus *Peptostreptococcus*, are now classified as members of the genus *Peptoniphilus* (Ezaki *et al*., 2001). Most pathogenic species are in the genus Streptococcus. These Gram-positive cocci are approximately 1.0 μm in diameter and form chains of different lengths (Fig. 15.1).

Streptococcus species are catalase-negative, facultative anaerobes, which are non-motile. They are fastidious bacteria and require the addition of blood or serum to culture media. *Streptococcus pneumoniae* (pneumococcus) occurs as slightly pear-shaped cocci in pairs. Pathogenic strains have thick capsules and produce mucoid colonies. These bacteria cause pneumonia in humans, guinea-pigs and rats.

Peptoniphilus indolicus is an anaerobic Gram-positive coccus, which is aetiologically implicated in bovine 'summer mastitis' in association with *Arcanobacterium pyogenes* and *S. dysgalactiae*.

Usual habitat

The streptococci are distributed worldwide. Most species live as commensals on the mucosae of the upper respiratory tract and lower urogenital tract. These fragile bacteria are susceptible to desiccation and survive for only short periods off the host.

Key points

- Gram-positive cocci in chains
- Fastidious, requiring enriched media

- Small, usually haemolytic, translucent colonies
- Catalase-negative
- Facultative anaerobes, usually non-motile
- Commensals on mucous membranes
- Susceptible to desiccation
- Cause pyogenic infections

Differentiation of the streptococci

Three procedures are used for conventional laboratory differentiation of the streptococci, namely type of haemolysis, Lancefield grouping and biochemical testing.

- Type of haemolysis on sheep or ox blood agar:

  – Beta-haemolysis is complete haemolysis indicated by clear zones around colonies.

  – Alpha-haemolysis is partial or incomplete haemolysis indicated by greenish or hazy zones around colonies.

  – Gamma-haemolysis denotes no observable changes in the blood agar around colonies.

- Lancefield grouping is a serological method of classification based on the group-specific C-substance. Most pathogenic streptococci possess C-substance, a cell-wall polysaccharide antigen, which differs between species or groups of species and is widely used to classify clinical isolates of *Streptococcus*. Test methods include:

  – Ring precipitation test. The C-substance is extracted by acid or heat from the *Streptococcus* species under test. This antigen extract is layered over antisera of different specificities, in narrow tubes placed in plasticine on a slide. A positive reaction is indicated by the formation of a white ring of precipitate close to the interface of the two fluids within 30 minutes (Fig. 15.2).

  – Latex agglutination test. Specific C-substance antisera for groups A to G (with the exception of group E) are commercially available. Suspensions of latex particles are coated with each of the group-specific antibodies. The group antigen is extracted enzymatically from the *Streptococcus* under test. A drop of antigen is mixed on a plate with a drop of each latex-antibody suspension and rocked gently. A positive

reaction, which usually occurs within 1 minute, is indicated by agglutination (Fig. 15.3).

- Biochemical testing:

  – A number of commercial test systems are available for rapid biochemical identification of streptococci.

  – A short range of biochemical tests is used for differentiating equine group C streptococci.

- Differentiation of streptococcal species can be carried out using molecular tests such as PCR- based techniques. Multiplex PCR methods for the detection and differentiation of pathogens causing bovine mastitis, including streptococci, have been developed (Gillespie and Oliver, 2005). Molecular typing techniques have been applied to streptococci pathogenic for animals and include a MLST scheme for *S. zooepidemicus* (Webb *et al*., 2008), AFLP typing for *S. suis* (Rehm *et al*., 2007), and PFGE and MLST typing for *S. uberis* (Rato *et al*., 2008).

Figure 15.1 Streptococcal chains.

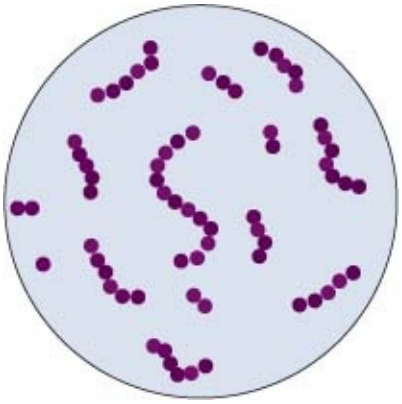


Figure 15.2 Ring precipitation test for streptococci.

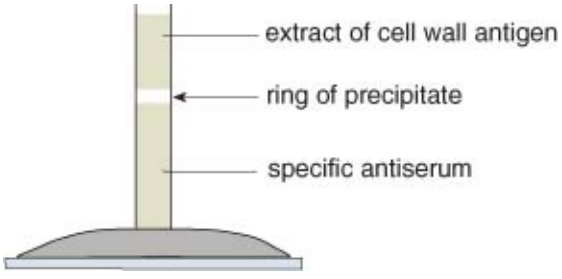
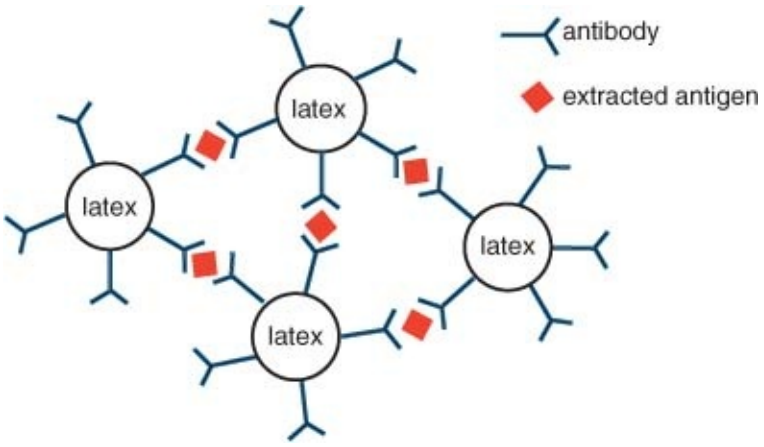


Figure 15.3 Diagrammatic representation of the latex agglutination test for streptococcal identification.



Pathogenesis and pathogenicity

Most of the streptococci that are pathogenic for animals are pyogenic and are associated with abscess formation, other suppurative conditions and septicaemia. *Streptococcus suis*, which is non-pyogenic, is a major pathogen of pigs, causing septicaemia, meningitis and pneumonia among other conditions. Beta-haemolytic streptococci are generally more pathogenic than those producing alpha-haemolysis. Virulence factors include enzymes and exotoxins such as streptolysins (haemolysins), hyaluronidase, DNase, NADase, streptokinase and proteases. The specific action and significance of some of these factors are poorly understood. Polysaccharide capsules, which are major virulence factors of *S. pyogenes, S. pneumoniae* and most strains of *S. equi*, are antiphagocytic. The cell-wall M proteins of *S. pyogenes, S. equi* and *S. porcinus* are also antiphagocytic. In the absence of antiphagocytic factors, these bacteria are rapidly killed by phagocytes.

Diagnostic procedures

History, clinical signs and pathology may be indicative of certain streptococcal infections such as strangles.

- Streptococci are highly susceptible to desiccation and specimens should be cultured promptly. Pus or exudate collected on swabs should be placed in transport medium if specimens cannot be processed immediately.
- A sensitive technique using the polymerase chain reaction has been developed for detecting both viable and non-viable *S. equi* in nasal swabs (Timoney and Artiushin, 1997).

- Chains of Gram-positive cocci may be demonstrable in smears from specimens.
- Specimens should be cultured on blood agar, selective blood agar and MacConkey agar. Plates are incubated aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates:
  – Small, translucent colonies, some of which may be mucoid
  – Type of haemolysis on blood agar
  – Chains of Gram-positive cocci
  – No growth on MacConkey agar with the exception of *Enterococcus* species
  – Negative catalase test
    - Lancefield grouping
    - Biochemical test profile.

Clinical infections

Streptococci are often commensals on mucous membranes and, consequently, many streptococcal infections are opportunistic. Infections may be primary as in strangles or secondary as in streptococcal pneumonia following a viral infection. Lymph nodes, genital tract or mammary glands may become infected. Neonatal septicaemia is often related to maternal genital tract infection. *Streptococcus pyogenes*, a human pathogen, occasionally causes bovine mastitis, tonsillitis in dogs and lymphangitis in foals. A strain of *S. pneumoniae*, capsular serotype 3, has been associated with respiratory disease in young horses during training, in combination with other pathogens (Chapman *et al*., 2000).

Streptococci of animal origin are of limited public health significance with the major exception of *S. suis*, which can cause severe, sometimes fatal, infections in individuals working with pigs. *Streptococcus zooepidemicus* subsp. *zooepidemicus* and *Streptococcus canis* are rare zoonoses also and can cause serious disease in humans. The group B streptococci, which cause disease in human infants, appear to be distinct from animal strains in this group.

Streptococcus canis, a significant pathogen of dogs, is associated with neonatal septicaemia, many suppurative conditions and toxic shock syndrome (Miller *et al*., 1996). *Streptococcus equi* subsp. *zooepidemicus* is a commensal on mucous

membranes and produces opportunistic infections in a number of animal species. In addition, outbreaks of acute haemorrhagic pneumonia in greyhounds and kenneled dogs have been reported (Pesavento *et al*., 2008). Strangles, porcine streptococcal meningitis and bovine streptococcal mastitis are important specific infections. Vaccines for the control of streptococcal infections are usually ineffective. The clinical consequences of streptococcal infections are listed in Table 15.1.

Strangles

Strangles is a highly contagious disease of horses caused by *Streptococcus equi* (*S. equi* subsp. *equi*). It is a febrile disease involving the upper respiratory tract with abscessation of regional lymph nodes.

Epidemiology

Although non-immune *Equidae* of all ages are susceptible, outbreaks of the disease occur most commonly in young horses. Assembling horses at sales, shows and racecourses increases the risk of acquiring infection. Transmission is via purulent exudates from the upper respiratory tract or from discharging abscesses. Unlike many of the streptococci, the organism is not a commensal and transmission of infection is frequently attributed to the introduction of animals incubating the disease and not yet showing clinical signs, or recovering carrier animals. Many horses shed the organism for up to 6 weeks after developing clinical signs. A chronic, convalescent carrier state can develop with bacteria present in the guttural pouch and such animals may remain carriers with intermittent shedding for many months. An atypical mild form, in which *S. equi* is present in small purulent foci, has been described.

Pathogenesis and pathogenicity

Infection appears to occur following entry of the organism into the tonsils with extension to the regional lymph nodes (Timoney and Kumar, 2008). The bacteria multiply in the lymph nodes, eliciting an inflammatory response, outpouring of neutrophils and abscess formation. Large numbers of neutrophils are attracted to the sites of invasion and replication of *S. equi*, because the peptidoglycan in its cell wall reacts with $C\bar{1}$ and stimulates the generation of chemotactic factors. The organism has many virulence factors which protect it from the host immune response and allow it to continue to multiply despite the presence of many

neutrophils. It possesses a hyaluronic capsule and M proteins which protect it against ingestion and killing and it may also produce a leukocidal toxin. The M proteins which project from the surface of the cell wall prevent activation of the alternative and classical complement pathways. However, once antibodies against these proteins are produced by the host, these effects are neutralized and organisms are effectively phagocytosed. Streptokinase may facilitate the spread of bacteria in tissue, and streptolysin S causes lysis of erythrocytes and appears to damage keratinocytes also (Timoney, 2004). *Streptococcus equi* produces a number of phage-encoded superantigens. These superantigens non-specifically stimulate T cells and are in part responsible for the clinical findings of high fever, neutrophilia and fibrinogenaemia observed in horses with strangles.

Table 15.1 Pathogenic streptococci, their habitats, hosts and consequences of infection.

Species	Lancefield groups	Haemolysis on blood agar[a]	Hosts	Consequences of infection	Usual habitat
S. pyogenes	A	β	Humans	Scarlet fever, septic sore throat, rheumatic fever	Mainly upper respiratory tract
S. agalactiae	B	β (α,γ)	Cattle, sheep, goats	Chronic mastitis	Milk ducts
			Humans, dogs	Neonatal septicaemia	Vagina
S. dysgalactiae	C	α (β,γ)	Cattle	Acute mastitis	Buccal cavity, vagina, environment
			Lambs	Polyarthritis	
S. equisimilis (*S. dysgalactiae* subsp. *equisimilis*)	A, C, G, L[b]	β	Horses	Abscesses, endometritis, mastitis	Skin, vagina
			Pigs, cattle, dogs, birds	Suppurative conditions	
S. equi (*S. equi* subsp. *equi*)	C	β	Horses	Strangles, suppurative conditions, purpura haemorrhagica	Upper respiratory tract, guttural pouch
S. zooepidemicus (*S. equi* subsp. *zooepidemicus*)	C	β	Horses	Mastitis, pneumonia, navel infections	Mucous membranes
			Cattle, lambs and pigs	Suppurative conditions, septicaemia	Skin, mucous membranes
			Dogs	Haemorrhagic pneumonia	
Enterococcus faecalis	D	α (β,γ)	Many species	Suppurative conditions following opportunistic invasion	Intestinal tract
S. suis	D	α (β)	Pigs	Septicaemia, meningitis, arthritis, bronchopneumonia	Tonsils, nasal cavity
			Cattle, sheep, horses, cats	Suppurative conditions	
			Humans	Septicaemia, meningitis	
S. porcinus	E, P, U, V[b]	β	Pigs	Submandibular lymphadenitis	Mucous membranes
S. canis	G	β	Carnivores	Neonatal septicaemia, suppurative conditions, toxic shock syndrome	Vagina, anal mucosa
S. uberis	Not assigned	α (γ)	Cattle	Mastitis	Skin, vagina, tonsils
S. pneumoniae	Not assigned	α	Humans, primates	Septicaemia, pneumonia, meningitis	Upper respiratory tract
			Guinea-pigs, rats	Pneumonia	
			Horses in training	Pneumonia (capsular type 3)	

a, types of haemolysis occurring less frequently are shown in brackets.

b, individual isolates of these species may belong to one of four groups.

Clinical signs

The incubation period is 3 to 6 days and the course of the uncomplicated disease

is 5 to 10 days. There is a high fever, depression and anorexia followed by an oculonasal discharge which becomes purulent. The lymph nodes of the head and neck are swollen and painful. Characteristically, the submandibular nodes are affected and they eventually rupture discharging purulent, highly infectious material. Guttural pouch empyema is a common finding. The morbidity may be up to 100% and mortality rate is usually less than 5%. Reinfection may occur in some recovered horses but immunity appears to be solid in approximately 75% of infected animals (Timoney, 2004).

Death may result from complications such as pneumonia, neurological involvement, asphyxia due to pressure on the pharynx from enlarged lymph nodes, or purpura haemorrhagica. Purpura haemorrhagica, considered to be an immune-mediated disease, may occur in some affected horses 1 to 3 weeks after initial illness.

Streptococcus zooepidemicus and *S. equisimilis*, which produce mild upper respiratory tract infections, must be differentiated from *S. equi*.

Bastard strangles, in which abscessation develops in many organs, is a serious complication in about 1% of affected animals.

Diagnosis

- Clinical signs and a history of recent exposure to suspect animals may allow a presumptive diagnosis of strangles.
- Colonies are usually mucoid, up to 4 mm in diameter, and surrounded by a wide zone of beta- haemolysis.
- *Streptococcus equi* must be distinguished from other Lancefield group C streptococci, particularly *S. equisimilis* and *S. zooepidemicus*, by sugar fermentation in peptone water containing serum (Table 15.2) and by other confirmatory biochemical tests. Alternatively, rapid differentiation of the species may be carried out using PCR-based tests, including real-time PCR (Båverud *et al*., 2007).
- Asymptomatic carriers can be detected using the polymerase chain reaction test. However, as PCR-based techniques can detect both live and dead organisms and DNA may persist in the absence of viable organisms, the culture of lavage samples from the guttural pouch is probably the ideal method for detection of carrier animals.
- Subtyping of *S. equi* strains based on analysis of the gene encoding the *S. equi* M protein has been reported recently and suggests that certain strains

may be associated with particular geographical areas (Waller and Jolley, 2007). This procedure may be useful for tracing the origin of some strangles outbreaks.

Table 15.2 Differentiation of equine group C streptococci by sugar fermentation.

	Trehalose	Sorbitol	Lactose	Maltose
S. equi	–	–	–	+
S. zooepidemicus	–	+	+	+(–)
S. equisimilis	+	–	v	+

v, variable reactions.

(−), a few strains are negative.

Treatment and control

- Administration of penicillin to in-contact and infected horses before development of lymphadenopathy is recommended by some practitioners. Treated horses are often susceptible to reinfection as treatment prevents the development of an effective immune response (Sweeney *et al*., 2005). Antibiotic therapy is of limited benefit if abscesses have formed.
- Clinically suspect animals should be isolated.
- Horses should be isolated for 2 weeks when first introduced or when returning to a property.
- A live attenuated intranasal vaccine is available in North America but it is not licensed for use in Europe because of concerns about reversion to virulence and other undesirable side effects. Another live vaccine, attenuated by deletion of the *aroA* gene and administered by the submucosal route, is licensed in Europe. The vaccine reduces clinical signs but immunity is relatively short-lived. Research on vaccines is now targeting the development of multicomponent subunit vaccines (Waller and Jolley, 2007).
- Predisposing factors such as overcrowding and mixing of different age groups should be avoided.
- Following outbreaks of the disease, buildings and equipment should be cleaned and disinfected.

Streptococcus suis infections

Streptococcus suis is recognized worldwide as a cause of significant losses in the pig industry. It is associated with meningitis, arthritis, septicaemia and bronchopneumonia in pigs of all ages, and with sporadic cases of endocarditis, neonatal deaths and abortion.

Serological and biochemical characteristics of isolates

Streptococcus suis properly belongs to Lancefield group D, although strains were previously assigned to groups R, S (RS) and T. Serological testing is based on antigenic differences in capsular material, largely carbohydrate in nature. At least 35 serotypes of varying virulence have been recognized. About 70% of *S. suis* isolates belong to serotypes 1 to 9 and to serotype 1/2, which has both type 1 and type 2 antigens. Of these, serotype 2 is the most prevalent serotype with carrier rates of up to 90%. This serotype is associated with meningitis in both pigs and humans. Two biotypes, *S. suis* I and *S. suis* II, are identifiable using commercial test systems.

Clinical signs and epidemiology

Asymptomatic carrier pigs harbour *S. suis* in tonsillar tissue. Disease outbreaks are most common in intensively reared pigs when they are subjected to overcrowding, poor ventilation and other stress factors. Sows carrying the organisms can infect their litters, leading either to neonatal deaths or to carrier animals in which characteristic signs develop later in life. Meningitis, which is often fatal, is characterized by fever, tremors, incoordination, opisthotonos and convulsions.

In North America, *S. suis* is often isolated from cases of respiratory disease in conjunction with *Mycoplasma* and *Pasteurella* species. Serious infections occur periodically in humans directly involved in pig husbandry or processing. Infections with *S. suis* have also been recorded in cattle, small ruminants, horses and cats.

Pathogenesis

Although a number of virulence factors have been described for *S. suis*, their exact role in the pathogenesis of infection has not been fully elucidated. The capsule and an opacity factor are both essential pathogenic factors as isogenic

mutants lacking either factor are markedly less virulent. In addition, strains positive for suilysin, extracellular factor (EF) and muramidase- released protein (MRP) are considerably more virulent than strains lacking these factors and are positively associated with systemic infection (Beineke *et al*., 2008; Wei *et al*., 2009).

Suilysin is a haemolysin and many *S. suis* isolates are haemolytic on blood agar. Adhesins such as glyceraldehyde-3-phosphate dehydrogenase and fibronectin-binding proteins are virulence factors that facilitate binding of *S. suis* to host cells and it is thought that hyaluronidase may function in local spread of the organism. Genes encoding for adhesins are up-regulated at the early stages of infection whereas genes encoding factors such as EF are up- regulated as the disease progresses (Tan *et al*., 2008).

Control

These bacteria tend to become endemic in a herd and eradication is not feasible. Improved husbandry may decrease the prevalence of clinical disease. Research is ongoing on the development of subunit vaccines containing capsular antigens, EF and MRP as immunogens.

Most strains of *S. suis* are susceptible to penicillin or ampicillin. Prophylactic long- acting penicillin, given by injection to sows 1 week prior to farrowing and to piglets during the first 2 weeks of life, has proved worthwhile in herds experiencing neonatal deaths or meningitis at weaning. *Streptococcus suis* strains remain susceptible to penicillin and β-lactam antibiotics in many countries but levels of resistance to these drugs of between 4.0% and 22% have been reported from China (Zhang *et al*., 2008).

Bovine streptococcal mastitis

Streptococcus agalactiae, S. dysgalactiae and *S. uberis* are the principal pathogens involved in streptococcal mastitis. *Enterococcus faecalis, S. pyogenes* and *S. zooepidemicus* are less commonly isolated from cases of mastitis.

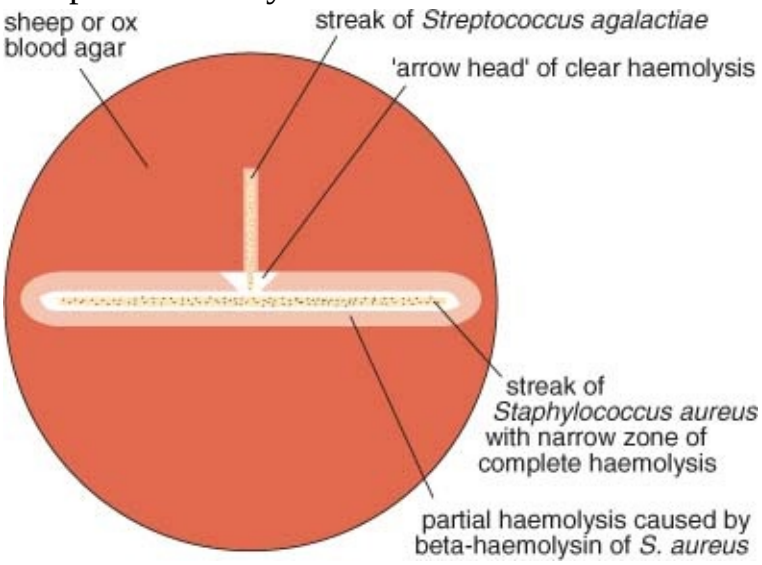
- *Streptococcus agalactiae* colonizes the milk ducts and produces persistent infection with intermittent bouts of acute mastitis.
- *Streptococcus dysgalactiae*, which is found in the buccal cavity and genitalia and on the skin of the mammary gland, causes acute mastitis.
- *Streptococcus uberis*, a normal inhabitant of skin, tonsils and vaginal mucosa, is a major cause of clinical mastitis, usually without systemic

signs.

Table 15.3 Differentiation of streptococci which cause bovine mastitis.

	Haemolysis on blood test agar	CAMP test	Aesculin hydrolysis (Edwards medium)	Growth on MacConkey agar	Lancefield group
Streptococcus agalactiae	β (α,γ)	+	–	–	B
S. dysgalactiae	α	–	–	–	C
S. uberis	α	–	+	–	Not assigned
Enterococcus faecalis	α	–	+	+	D

Figure 15.4 CAMP test. *Streptococcus agalactiae* elaborates a factor which completely lyses the red cells already damaged by the β-haemolysin of *Staphylococcus aureus*, producing a characteristic clear 'arrow head' pattern of complete haemolysis.



Diagnosis

- Clinical signs include inflammation of mammary tissue and clots in the milk.
- Milk samples should be collected carefully to avoid contamination.
- Samples should be cultured on blood agar, Edwards medium and MacConkey agar and incubated aerobically at 37°C for 24 to 48 hours.
- Differentiation of the mastitis-producing streptococci is outlined in Table 15.3. A positive CAMP test (Christie, Atkins and Munch-Petersen) is illustrated in Fig. 15.4.
- Sugar fermentation tests.
- Molecular-based methods are increasingly used for the detection and

identification of mastitis pathogens, including streptococci. A real-time PCR reaction assay for the identification of 11 different organisms responsible for intramammary infections in cattle has been developed (Koskinen *et al*., 2009).

Treatment and control

A detailed description of bovine mastitis including streptococcal mastitis is presented in Chapter 93.

Enterococcus species

These enteric organisms are found in the intestinal tract of animals and humans. They are opportunistic pathogens and differ phenotypically from the *Streptococcus* species in two important respects:

- They tolerate bile salts and grow on MacConkey agar as red, pin-point colonies.
- Some isolates are motile.

There are a number of species in the genus *Enterococcus* but *E. faecalis* is the species most frequently isolated from domestic animals and poultry. *Enterococcus faecium* is also found in the intestinal tract of many species. *Enterococcus* species cause opportunistic infections such as wound infections in all species, mastitis in cattle, and urinary tract and ear infections in dogs. They are intrinsically resistant to many antimicrobial agents, which can cause problems relating to the selection of antimicrobial drugs. In addition, vancomycin-resistant enterococci are important nosocomial pathogens in human hospitals, and vancomycin resistance in animal isolates of *E. faecium* is a major public health concern (Aarestrup *et al*., 2001). Increasing levels of resistance to vancomycin in animal isolates was associated with the use of avoparcin in animals. However, levels of resistance have decreased markedly since the use of avoparcin was banned in 1997, although resistance in animal isolates has not disappeared completely (DANMAP, 2005).

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Chapter 16

Actinobacteria

The class *Actinobacteria* comprises a phylogeneti-cally diverse group of Gram-positive bacteria which includes a number of families containing pathogens of veterinary importance (Fig. 16.1). In addition, the class contains a large number of organisms that are not of pathogenic significance. This chapter presents information mainly on genera within the family *Actinomycetaceae* of the suborder *Actinomycineae*, *Actinomyces* species, *Arcanobacterium* species and *Actinobaculum* species. The genera *Nocardia, Derma-tophilus* and *Crossiella*, which are members of the class *Actinobacteria*, are included also. Because of filament formation and granulomatous responses to tissue invasion, these organisms were originally regarded as fungi. However, filaments of these bacteria rarely exceed 1μ m in width, whereas hyphae of the eukaryo-tic fungi are usually more than 5 μ m wide. The comparative features of these genera are summarized in Table 16.1.

Some thermophilic actinobacteria, such as *Saccha-ropolyspora rectivirgula*, found in poor-quality overheated hay, produce spores that can induce allergic pulmonary disease in cattle, horses and humans. *Streptomyces* species are saprophytic soil actinobacteria and are common contaminants on laboratory media. They elaborate a variety of antimicrobial substances, many with therapeutic activity.

Key points

- Gram-positive bacteria, many species with branching filaments
- Relatively slow growth on laboratory media
- Opportunistic pathogens producing diverse inflammatory responses
- ***Actinomyces, Arcanobacterium***and ***Actinobaculum species***
  –anaerobic or facultatively anaerobic
  –morphologically heterogeneous
  –non-spore-forming, non-motile
  –modified Ziehl-Neelsen-negative
  –colonize mucous membranes

- ***Nocardia* species**
  –aerobic, non-motile
  –spores from aerial filaments
  –growth on Sabouraud dextrose agar
  –modified Ziehl-Neelsen-positive
  –soil saprophytes
- ***Dermatophilus congolensis***
  –aerobic and capnophilic
  –motile zoospores
  –no growth on Sabouraud dextrose agar
  –found in scabs and in foci on skin of carrier animals

Figure 16.1 Pathogenic actinobacteria of veterinary importance.

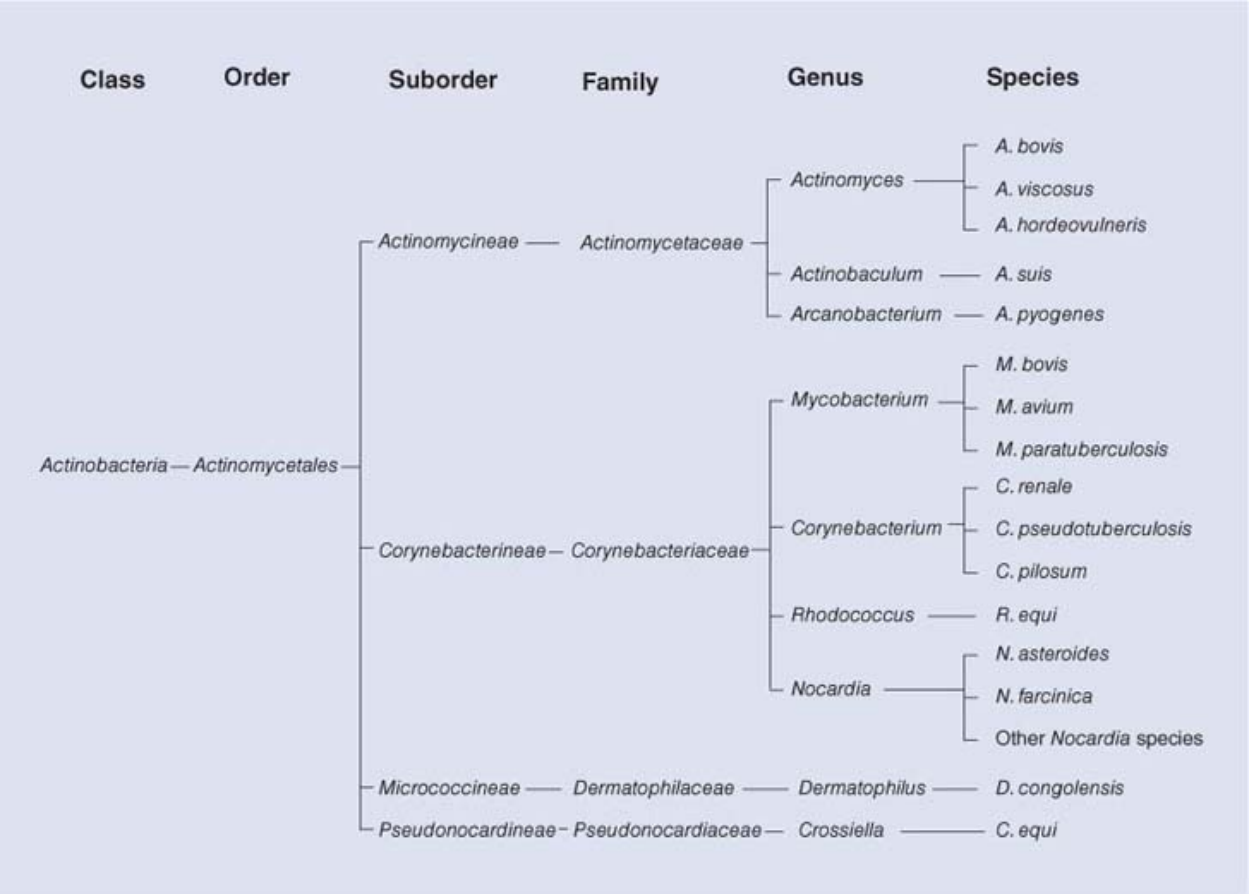


Table 16.1 Comparative phenotypic features of actinobacteria of veterinary importance.

Feature	*Actinomyces* species	*Arcanobacterium pyogenes*	*Actinobaculum suis*	*Nocardia* species	*Dermatophilus congolensis*
Atmospheric growth requirements	Anaerobic or facultatively anaerobic and capnophilic	Facultatively anaerobic and capnophilic	Anaerobic	Aerobic	Aerobic and capnophilic
Aerial filament production	–	–	–	+	–
Modified Ziehl-Neelsen staining	–	–	–	+	–
Growth on Sabouraud dextrose agar	–	–	–	+	–
Usual habitat	Nasopharyngeal and oral mucosae	Nasopharyngeal mucosa of cattle, sheep and pigs	Prepuce and preputial diverticulum of boars	Soil	Skin of carrier animals, scabs from lesions
Site of lesions	Many tissues including bone	Soft tissues	Urinary tract of sows	Thoracic cavity, skin and other tissues	Skin

Other genera of significance in veterinary medicine within the class *Actinobacteria* are presented in Chapters 17 (*Corynebacterium* species), 18 (*Rhodococcus equi*) and 23 (*Mycobacterium* species).

Actinomyces, Arcanobacterium and Actinobaculum species

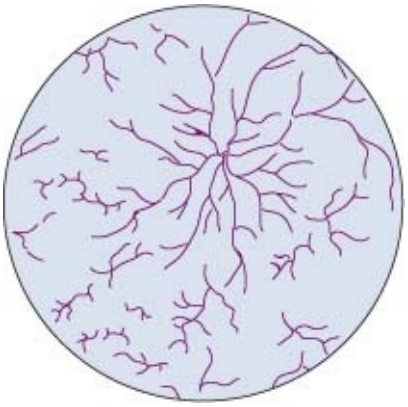
The species in these genera are non-motile, non-spore-forming, Gram-positive bacteria which require enriched media for growth. *Arcanobacterium pyogenes* was formerly called *Actinomyces pyogenes* and previously *Corynebacterium pyogenes. Actinobaculum suis* has also undergone a number of name changes and is closely related to the genus *Arcanobacterium* (Lawson *et al*., 1997). Both of these organisms have a coryne-form morphology whereas the *Actinomyces* species are usually long and filamentous although short V, Y and T configurations also occur (Fig. 16.2). The species of veterinary importance in the group are *Arcanobacterium pyogenes*, *Actinobaculum suis, Actinomyces bovis, Actinomyces viscosus* and *Actinomyces hordeovulneris*. The main diseases associated with these bacteria are summarized in Table 16.2.

Usual habitat

With the exception of *A. hordeovulneris*, pathogenic members of these genera colonize the mucous membranes of mammals. *Actinomyces bovis* is found in the oropharynx of cattle and other domestic animals, and *Actinomyces viscosus* is a

commensal in the oral cavity of dogs and humans. *Arcanobacterium pyogenes* is commonly present on the nasopharyngeal mucosa of cattle, sheep and pigs. The usual habitat of *Actinobaculum suis* is the preputial mucosa of boars. Although the habitat of *Actinomyces hordeovulneris* is uncertain, the organism appears to be closely associated with awns in the seed heads of grasses of the genus *Hordeum*. The seed heads of these grasses are often referred to as foxtails in North America.

Figure 16.2 Long branching filaments and shorter V, Y and T forms, typical of many *Actinomyces* species as they appear in smears from lesions.



Differentiation of the genera

Differentiating features of the genera are presented in Table 16.3.

- Morphology of individual species in stained smears aids differentiation. *Arcanobacterium pyogenes* and *Actinobaculum suis* have coryneform morphology.
- Each species has a defined atmospheric growth requirement.
- Colonial morphology and haemolytic activity:

  – *Arcanobacterium pyogenes* produces a characteristic hazy haemolysis along streak lines after aerobic incubation for 24 hours. Pin-point colonies become visible after 48 hours.

  – *Actinomyces bovis* and *A. hordeovulneris* colonies typically adhere to agar media and are usually non-haemolytic.

  – *Actinomyces viscosus* can produce two colony types, one large and smooth and the other small and rough. The large colony is composed of V, Y and T cell configurations and the smaller colonies are formed of short branching filaments.

  – Actinobaculum suis produces colonies which are up to 3 mm in

diameter, with shiny raised centres and dull edges. Poorly defined haemolysis is observed on ruminant blood agar.

- *Biochemical reactions*. Specialized techniques, usually conducted in reference laboratories, are required for definitive identification of most of these fastidious, slow-growing organisms. In routine diagnosis, a presumptive identification of *A. pyogenes* is based on colonial morphology and pitting of a Loeffler's serum slope within 24 hours, which indicates pro-teolytic activity. It also hydrolyses gelatin.
- *Granules in pus*. Granules can be detected when pus is diluted with distilled water in a Petri dish. In infections caused by *A. bovis*, pinhead-sized, yellowish 'sulphur granules' are found. Whitish, soft, grey granules are demonstrable in pus from animals infected with *A. viscosus*. Granules in lesions caused by *A. bovis* contain characteristic clubs (Fig. 16.3). Club colony formation is a feature of other chronic infections such as bovine actinobacillosis caused by *Actinobacillus lignieresii* and botryomycosis usually associated with *Staphylococcus aureus*.
- Urease is produced by *A. suis*.
- Analysis of 16s rRNA gene sequences can be used for identification and classification of organisms in this group (Lawson *et al*., 1997; Jost *et al*., 2002).

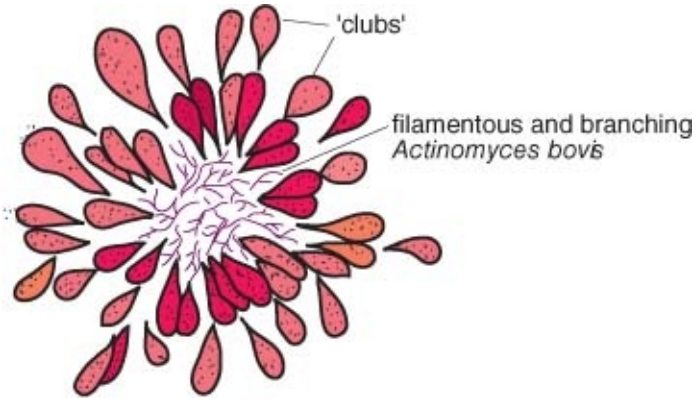
Table 16.2 Disease conditions produced by *Actinomyces*, *Arcanobacterium* and *Actinobaculum* species in domestic animals.

Species	Hosts	Disease conditions
Arcanobacterium pyogenes	Cattle, sheep, pigs	Abscessation, mastitis, suppurative pneumonia, endometritis, pyometra, arthritis, umbilical infections
Actinomyces hordeovulneris	Dogs	Cutaneous and visceral abscessation, pleuritis, peritonitis, arthritis
A. bovis	Cattle	Bovine actinomycosis (lumpy jaw)
A. viscosus	Dogs	Canine actinomycosis:
		– cutaneous pyogranulomas
		– pyothorax and proliferative pyogranulomatous pleural lesions
		– disseminated lesions (rare)
	Horses	Cutaneous pustules
	Cattle	Abortion
Actinomyces species (unclassified)	Pigs	Pyogranulomatous mastitis
	Horses	Poll evil and fistulous withers
Actinobaculum suis	Pigs	Cystitis, pyelonephritis

Table 16.3 Phenotypic differentiation of *Actinomyces*, *Arcanobacterium* and *Actinobaculum* species of veterinary importance.

Characteristic	*Actinomyces bovis*	*Actinomyces viscosus*	*Actinomyces hordeovulneris*	*Arcanobacterium pyogenes*	*Actinobaculum suis*
Morphology	Filamentous branching, some short forms	Filamentous branching, short forms	Filamentous branching, short forms	Coryneform	Coryneform
Atmospheric requirements	Anaerobic + CO_2	10% CO_2	10% CO_2	Aerobic	Anaerobic
Haemolysis on sheep blood agar	±	–	±	+	±
Catalase production	–	+	+	–	–
Pitting of Loeffler's serum slope	–	–	–	+	–
Granules in pus	'Sulphur granules'	White granules	–	–	–

Figure 16.3 A club colony with a core of branching filaments of *Actinomyces bovis* surrounded by club-shaped structures. These structures are part of the host response to this chronic infection.



Pathogenesis and pathogenicity

Arcanobacterium pyogenes produces a haemolytic exotoxin, pyolysin, which is cytolytic for several cell types including neutrophils and macrophages, and is dermonecrotic and lethal for laboratory animals. It is a major virulence factor of *A. pyogenes* as mutants lacking the gene for pyolysin production are cleared rapidly from experimentally infected mice (Jost and Billington, 2005). Neuraminidases produced by *A. pyogenes* are thought to assist in adherence of the organism to host tissues, probably by exposing hidden host cell receptors for other adhesins (Jost and Billington, 2005). *Arcanobacterium pyogenes* produces a number of other adhesins also, including extracellular matrix-binding proteins and fimbriae. This bacterium also produces proteases whose role as potential virulence factors is not yet determined. Virulence attributes of *Actinomyces* species important in veterinary medicine have not been established. Purulent reactions are typical of infections with *A. pyogenes*, whereas *A. bovis* and *A.*

viscosus provoke pyogranulomatous reactions.

Diagnostic procedures

- Species of animal affected, clinical presentation and type and location of lesions may suggest the species involved.
- Specimens suitable for laboratory procedures include exudates, aspirates and tissue samples for culture and histopathology.
- Gram-stained smears may reveal morphological forms typical of the aetiological agent (Fig. 16.2). Unlike *Nocardia* species, *Actinomyces* species are modified Ziehl-Neelsen (MZN) negative (Table 16.1).
- Histopathological examination of specimens from lesions caused by *A. bovis* reveals aggregates of filamentous organisms surrounded by eosinophilic club-shaped structures (Fig. 16.3).
- Blood and MacConkey agars are inoculated with the specimen and incubated at 37°C for up to 5 days. The atmospheric requirements for different species are indicated in Table 16.3. Species identification based on phenotypic characteristics is difficult except in the case of *A. pyogenes*.
- Identification criteria for isolates: – Colonial characteristics
  – Morphology in stained smears
  – Presence or absence of haemolysis on blood agar
  – Absence of growth on MacConkey agar
  – Presence or absence of growth when subcultured onto Sabouraud dextrose agar
  – Pitting of a Loeffler' s serum slope (*A. pyogenes*) – Urease production (*A. suis*).
- Because species identification is difficult for organisms within the group, PCR analysis of the 16S rRNA gene sequence is increasingly used for confirmation of identity.

Clinical infections

The disease conditions produced by the pathogenic *Actinomyces*, *Arcanobacterium* and *Actinobaculum* species are presented in Table 16.2. In some conditions, the identity of the actinomycete has not been clearly defined. An unclassified *Actinomyces* species resembling *A. bovis* has been isolated from pyogranu-lomatous mastitis in sows. Abortion in sows has been ascribed to *A.*

naeslundii, an organism usually associated with human dental caries (Palmer *et al*., 1979). An *Actinomyces species*, probably *A. bovis*, has been identified in the suppurative discharges from poll evil and fistulous withers in horses.

Infections with Arcanobacterium pyogenes

Arcanobacterium pyogenes is an opportunistic pathogen and is a common cause of suppurative lesions in many domestic species worldwide, especially cattle, pigs and sheep. Any organ system may be affected. Cases of lymphadenitis, osteomyelitis, peritonitis and neural abscessation are commonly encountered. The organism has also been associated with pyometra, metritis and acute mastitis in cows. In the acute bovine mastitis referred to as 'summer mastitis' in Britain and Ireland, the anaerobic bacterium *Peptoniphilus indoli-cus* is frequently associated with *A. pyogenes*. *Arcanobacterium pyogenes* also occurs in association with anaerobes in other mixed infections such as foot abscesses in cattle and sheep (see Chapter 91). Diagnosis is based on the typical pleomorphic cell morphology in Gram-stained smears from specimens, colonial characteristics and the ability of *A. pyogenes* to pit a Loeffler's serum slope. Although the organism was previously regarded as susceptible to many antimicrobial agents, there are reports of resistance to agents such as the macrolides and tetracyclines, which are commonly used for growth promotion and disease prevention in the USA (Trinh *et al*., 2002).

Canine actinomycosis

Actinomyces viscosus is the aetiological agent of canine actinomycosis. Infection can result in subcutaneous pyogranulomatous lesions and extensive fibrovascular proliferation on the peritoneal or pleural surfaces with sanguinopurulent exudate in the affected cavity. The thoracic lesions closely resemble those of canine nocardiosis. The main clinical finding is respiratory distress. *Actinomyces viscosus* has also been isolated from cutaneous lesions in a horse (Specht *et al*., 1991) and from a heifer which had aborted (Okewole *et al*., 1989). The lesions associated with *A. hordeovulneris* infection in the dog include cutaneous and visceral pyogranulomas, pleuritis, peritonitis and arthritis. In uncomplicated infections, *A. viscosus* is usually responsive to treatment with penicillin. A new species, *Actinomyces canis*, which was isolated from a number of different clinical conditions in dogs, has been described (Hoyles *et al*., 2000).

Bovine actinomycosis (lumpy jaw)

Invasion of the mandible and, less commonly, the maxilla of cattle by *A. bovis* causes a chronic rarefying osteomyelitis. The organism is presumed to invade the tissues following trauma to the mucosa from rough feed or through dental alveoli during tooth eruption. A painless swelling of the affected bone enlarges over a period of several weeks. The swelling becomes painful, and fistulous tracts discharging purulent exudate develop. Spread to contiguous soft tissues may occur but there is minimal involvement of regional lymph nodes.

Diagnosis

- Clinical signs are often distinctive in advanced cases.
- Radiography can be used to determine the degree of bone destruction.
- Other appropriate diagnostic techniques (see Diagnostic procedures).
- Lumpy jaw should be distinguished from other conditions which result in swelling of the bones of the jaw and from actinobacillosis which may involve the soft tissues of the head.

Treatment

- When lesions are small and circumscribed, surgery is the therapy of choice. In advanced cases, surgical treatment is frequently unrewarding.
- Prolonged therapy with penicillin, given parenterally to animals early in the disease, may be of value. Isoniazid *per os* for 30 days has also been recommended.

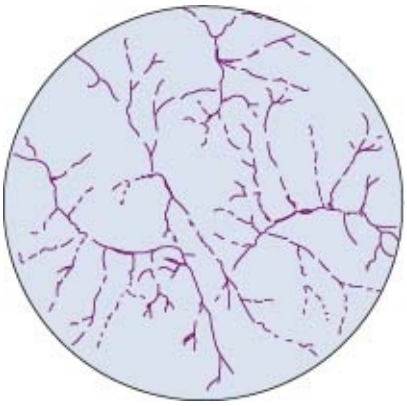
Porcine cystitis and pyelonephritis

This specific disease, which affects the urinary tract of pregnant sows, is transmitted at coitus and is potentially fatal. The pathogen, *Actinobaculum suis*, can be isolated from the prepuce and preputial diverticulum of healthy boars but not from the urogenital tract of healthy sows. Boars are rarely affected clinically and sows usually develop evidence of disease 3 to 4 weeks after mating. Anorexia, arching of the back, dysuria and haematuria are prominent signs. If both kidneys are extensively damaged, death may result. The diagnostic procedures and therapeutic measures appropriate for this disease are similar to those for bovine pyelonephritis (see Chapter 17).

Nocardia species

Members of the *Nocardia* species are Gram-positive, aerobic, saprophytic actinobacteria. In smears of exudate from infected tissue, they appear as long, slender, branching filaments with a tendency to fragment into rods and cocci (Fig. 16.4). When cultured, these organisms produce aerial filaments which may form spores. Components of the cell wall, especially mycolic acid, render *Nocardia* species partially acid fast (MZN-positive).

Figure 16.4 Branching filaments of *Nocardia asteroides* as they appear in smears from lesions. The filaments have a tendency to fragment.



Usual habitat

Nocardia species are saprophytes found in soil and decaying vegetation.

Differentiation of *Nocardia* species

Phenotypic identification of *Nocardia* species is difficult and is based on specialized biochemical tests and analysis of mycolic acid composition. Molecular methods based on sequencing of the 16S rRNA gene are now frequently employed for definitive identification. Up to 90 *Nocardia* species are recognized, including *N. asteroides, N. farcinica* and many other species which have been associated with disease in animals and humans.

Pathogenesis and pathogenicity

Infection, which is opportunistic, is usually associated with immunosuppression or, alternatively, may follow a heavy challenge. The usual mode of infection is by inhalation but it may also occur through skin wounds or via the teat canal. An

intestinal form of nocardiosis may result from ingestion of the organisms.

Virulent strains of *N. asteroides* survive intracellularly. The production of superoxide dismutase and catalase and the presence of a thick peptidoglycan layer in the cell wall confer resistance to microbiocidal activity of phagocytes. Cell-mediated immunity is essential for protection against infection by this facultative, intracellular bacterium (Deem *et al*.,1982).

Diagnostic procedures

A presumptive diagnosis of infection with *Nocardia* species is based on clinical findings and laboratory procedures.

- Specimens suitable for laboratory examination include exudates, aspirates, mastitic milk, tissue from granulomas and fixed tissue for histopathology.
- Smears of exudate should be stained by the Gram and MZN methods. *Nocardia* species are MZN-positive, unlike *Actinomyces* species which are MZN-negative.
- Histopathological examination of tissue specimens may reveal clusters of nocardial filaments.
- The organism can be cultured on blood agar or on selective growth-enhancing media such as charcoal– yeast extract medium. Plates are incubated aerobically at 37°C for up to 10 days.
- Identification criteria for isolates:

  –Colonies on blood agar are usually visible after incubation for about 5 days. They are white, powdery and firmly adherent to the agar. Colonies are variably haemolytic and odourless.

  – Subculture on to Sabouraud dextrose agar yields dry, wrinkled, orange-coloured colonies after incubation for up to 5 days.

  – Gram-stained smears from colonies show some filamentous forms with a preponderance of rod and coccal forms.
- *Nocardia* species require differentiation from *Streptomyces* species which may contaminate laboratory media. Features of *Streptomyces* species which distinguish them from *Nocardia* species include a strong, earthy odour, MZN-negative filaments and colonies on Sabouraud dextrose agar which are powdery-white in appearance.
- DNA sequencing of the 16S rRNA gene is a frequently used method for identification of nocardial species.

Clinical infections

Historically, *Nocardia asteroides* was thought to account for most nocardial infections in domestic animals. However, with the advent of new, molecular-based methods of identification, many organisms previously classified as *N. asteroides* are now known to belong to other nocardial species (Brown-Elliott *et al*., 2006). Thus, it is likely that nocardial disease in animals is due to a number of different species within the genus (Table 16.4). The most commonly encountered conditions are cutaneous and systemic infections in dogs and mastitis in cattle, with recent publications documenting a *Nocardia farcinica* mastitis epizootic in Canada (Brown *et al*., 2007) and an outbreak of *Nocardia neocaledoniensis* mastitis in Italy (Pisoni *et al*., 2008). Nocardial infection has been reported occasionally in horses, immunosuppression being an important predisposing factor (Biberstein *et al*., 1985). *Nocardia aster-oides* has also been associated with abortion in sows (Koehne, 1981). *Nocardia farcinica* is implicated in bovine farcy. *Nocardia brasiliensis* and *N. otitidiscavi-arum* (*N. caviae*) are pathogenic for humans and rarely cause disease in domestic animals.

Table 16.4 Disease conditions produced by *Nocardia* species in domestic animals.

Species	**Hosts**	**Disease conditions**
Nocardia species	Dogs	Canine nocardiosis:
		– cutaneous pyogranulomas
		– pyogranulomatous pleural lesions and pyothorax
		– disseminated lesions
	Cattle	Chronic mastitis, abortion Bovine farcy[a]
	Pigs	Abortion
	Sheep, goats, horses	Wound infections, mastitis, pneumonia, other pyogranulomatous conditions

a, some mycobacteria have also been implicated in bovine farcy.

Canine nocardiosis

Infections in dogs due to *Nocardia* species are acquired by inhalation, through skin wounds or by ingestion. Thoracic, cutaneous and disseminated forms of the disease are recognized. The thoracic form is characterized by fever, anorexia and respiratory distress. There is a fibrovascular proliferative reaction on the pleura and sanguinopurulent fluid accumulates in the thoracic cavity. The cutaneous form presents either as an indolent ulcer or as a granulomatous swelling with discharging fistulous tracts. In the disseminated form, which occurs typically in

dogs less than 12 months of age, clinical signs are non-specific and are referable to the organ system mainly affected.

Diagnosis

Although canine nocardiosis is clinically similar to canine actinomycosis, antibiotic therapy for nocardiosis is less effective. Consequently, it is essential to distinguish the main aetiological agents, *Nocardia* species and *A. viscosus*. The main differentiating features of these organisms are listed in Table 16.5.

Treatment

Nocardia species show a marked variation in their susceptibility to antibiotics. Susceptibility testing is carried out by specialized laboratories using the broth microdilution technique. Effective antibiotics, which include amikacin, imipenem-cilastatin and cotrimoxazole, should be administered systemically for at least 6 weeks.

Table 16.5 Differentiation of *Nocardia* species and *Actinomyces viscosus*.

Characteristic	***Nocardia* species**	***Actinomyces viscosus***
MZN staining of filaments	+	–
Atmospheric requirement	Aerobic	10% CO_2
Growth on Sabouraud dextrose agar	+	–
Susceptibility to penicillin G	–	+

MZN, modified Ziehl-Neelsen stain.

Bovine nocardial mastitis

A chronic form of bovine mastitis results from infection with *Nocardia* species. Fibrosis, either diffuse or multifocal, develops and white clots are evident intermittently in the milk. Multifocal fibrosis can be detected clinically as discrete hard masses, up to 5 cm in diameter, palpable in the affected gland after milking. Infection during early lactation may occasionally induce a systemic reaction with fever, depression and anorexia. Nocardial mastitis is usually sporadic, affecting only one or two cows in a herd, and is usually refractory to chemotherapy. However, outbreaks associated with the use of dry-cow therapy, particularly neomycin - containing products, have been reported (Brown *et al*., 2007).

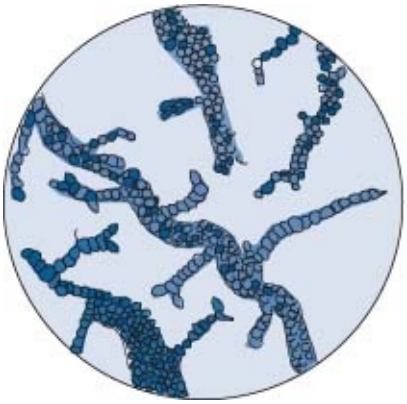
Bovine farcy

This disease, also known as bovine nocardiosis, is limited to the tropics. It is a chronic infection of super-ficial lymphatic vessels and lymph nodes. Early lesions consist of small cutaneous nodules, often on the medial aspect of the legs and on the neck. These nodules enlarge slowly and coalesce to form swellings, up to 10 cm in diameter, which rarely ulcerate. The lymphatic vessels may become thickened and cord - like. Internal organs may be affected occasionally and the condition is important because lesions resemble those of tuberculosis. Because *Nocardia farcinica, Mycobacterium farcinogenes* and *M. senegalense* have been isolated from such lesions, the aetiology of bovine farcy requires clarification.

Dermatophilus congolensis

Dermatophilus congolensis is a Gram-positive, filamen-tous, branching actinobacterium with distinctive morphology (Fig. 16.5). This organism is unusual because it produces motile coccal zoospores about 1.5µ m in diameter. Mature zoospores produce germ tubes which develop into filaments 0.5 to 1.5 µm in width. Within these filaments, transverse and longitudinal divisions form segments that ultimately develop into zoospores. Mature filaments may be more than 5µm in width and contain columns of zoospores which impart a ‘tram-track’ appearance to the filaments. Although skin infections caused by *D. congo-lensis* occur worldwide, dermatophilosis is most prevalent in tropical and subtropical regions.

Figure 16.5 *Dermatophilus congolensis* in a smear from scab material. Wide filaments contain coccal zoospores. Side branches divide into segments prior to the formation of zoospores.



Usual habitat

The organism seems to persist in foci in the skin of many clinically normal animals, particularly in endemic areas. Dormant zoospores may become activated when microenvironmental moisture and temperature levels are favourable. Duration of zoospore survival in the environment is usually limited but may be up to 3 years in dry scabs.

Pathogenesis and pathogenicity

Dermatophilus congolensis does not usually invade healthy skin. Trauma and persistent wetting predispose to skin invasion. Microenvironmental conditions which interfere with normal surface protective mechanisms, such as sebaceous secretions, also lead to activation of dormant zoospores. When activated, zoospores produce germ tubes and these develop into filaments which invade the epidermis. The ability of individual strains to invade the epidermis is related to their virulence. Strains vary in virulence, which may be related to the ability to produce enzymes such as phospholipases, proteolytic enzymes and an alkaline ceramidase (Norris *et al*., 2008).

Invasion leads to an acute inflammatory response characterized by large numbers of neutrophils which ultimately form microabscesses in the epidermis. A cyclical pattern of invasion of regenerating epithelial cells by the pathogen, together with serous exudation and microabscess formation, leads to the development of raised scab-like crusts containing numerous branching filaments. Factors that depress specific immune responses, including intercurrent diseases and pregnancy, may increase host susceptibility to dermatophilosis.

Diagnostic procedures

- The clinical picture is usually indicative of the infection, particularly in endemic areas.
- Specimens suitable for laboratory examination include scab material and samples of skin fixed in formalin.
- Smears from the undersurface of scabs or from softened scab material, stained by the Giemsa method, reveal the characteristic branching filaments containing zoospores (Fig. 16.5). When there is difficulty demonstrating the organism in smears, histopathological or immunofluorescent techniques may be employed.

- Scab material softened with water is cultured on blood agar at 37°C in an atmosphere of 2.5 to 10% CO_2 for up to 5 days.
- Zoospores, which exhibit chemotaxis for CO_2, can be recovered from heavily contaminated specimens by placing infected scab material in distilled water at room temperature for 3.5 hours, followed by exposure to an atmosphere of CO_2 for 15 minutes. A sample from the surface of the water contains motile zoospores, which can be cultured.
- Identification criteria:

  – After incubation for 48 hours, colonies are up to 1 mm in diameter, yellow and haemolytic. When incubated for 3 to 4 days, they become rough, golden-yellow and embedded in the agar. Older colonies may have a mucoid appearance.

  – Giemsa-stained smears from colonies reveal solidly staining filaments.

  – Growth does not occur on Sabouraud dextrose agar.

  – Biochemical tests are rarely required for identifi-cation. The organism liquefies Loeffler's serum medium, hydrolyses gelatin and casein, and produces acid from glucose and fructose.

  – As with other members of the actinobacteria, methods of identification based on sequencing of the 16s rRNA gene have been developed. Genotyping based on random amplified polymorphic DNA analysis has been reported, and suggested a correlation between genotypic variation and host species (Larrasa *et al*., 2002).

Clinical infections

Infections with *D. congolensis* are usually confined to the epidermis. However, invasion of subcutaneous tissue has been described in a cat (Jones, 1976). Commonly used designations for infection with this organism are dermatophilosis and cutaneous streptothricosis. Mycotic dermatitis (a misnomer) and lumpy wool are used to describe infection of woolled areas of the skin in sheep. When the skin of the lower limbs of sheep is involved, the condition is termed strawberry footrot.

Although the disease affects animals of all ages, it is more prevalent and often more severe in young animals. Damage to the skin predisposes to infection with *D. congolensis. Zoospores* are most often transmitted by direct contact with infected animals. In endemic tropical regions, the prevalence and severity of

dermatophilosis correlates with infestation with *Amblyomma variegatum* (Morrow *et al*., 1989). Anumber of blood-sucking insects may also be important in disease transmission in the tropics. Economic loss derives from damage to hides and fleeces. In addition, dermatophilosis creates a strong predisposition to fly strike in sheep (Norris *et al*., 2008). Human skin infections, occasionally acquired through close contact with infected animals, are rare (Stewart, 1972; Burd *et al*., 2007).

Clinical signs

Lesion distribution usually correlates with those areas of skin predisposed to infection. Heavy prolonged rainfall in association with warm environmental temperatures can result in lesions predominantly affecting the dorsum of farm animals. Trauma to the face and limbs of animals grazing in thorny scrub can predispose to lesions in these sites. Early lesions present as papules and are often detectable only by palpation. As lesions progress, serous exudate causes matting of hairs giving them a tufted appearance. Lesions may coalesce to form irregular elevated crusty scabs. Tufts of hair can be readily plucked from the lesion along with adherent scab material and underlying exudate. Scab formation tends to be more pronounced in cattle and sheep than in horses.

Localized infections are usually of little consequence. Lesions may resolve spontaneously within a few weeks, particularly in dry conditions. In severe infections, lesions may be extensive and deaths may occasionally occur, particularly in calves and lambs. Rarely, oral lesions result in depression, difficulty with eating and loss of condition.

Diagnosis

Diagnosis is based on the clinical appearance of lesions and demonstration of *D. congolensis* in scabs. Isolation of the organism is confirmatory.

Treatment

Parenterally administered antibiotics such as long-acting oxytetracycline are usually effective. Alternatively, high doses of penicillin–streptomycin combinations on three consecutive days may be used. For treatment to be effective, satisfactory epidermal concentrations of the antibiotics are required. The outcome of treatment is influenced by the severity and extent of lesions. Topical treatments are ineffective.

Control

Control measures vary with geographical location and climatic factors; they are based on minimizing the effects of predisposing factors and early treatment of clinical cases.

- Clinically affected animals should be isolated and treated promptly.
- Shelter should be provided during periods of prolonged rainfall.
- Grazing areas should be cleared of thorny scrub.
- Tick infestation must be reduced by dipping or spraying with acaricides at weekly intervals and by elimination of tick habitats.
- Prophylactic use of long-acting tetracyclines may be required in endemic regions.
- Control of intercurrent diseases reduces the severity of dermatophilosis.
- Research is continuing on increasing the resistance of cattle and sheep to infection by *D. congolensis* and on methods for modifying the microbial populations of the skin in order to reduce the potential of *D. congolensis* for invasion (Norris *et al*., 2008).

Crossiella equi

This organism is a member of the *Actinobacteria* in the suborder *Pseudonocardineae*. It is associated with equine nocardiform placentitis, leading to loss of the foal in approximately 50% of cases (Donahue *et al*., 2002).

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Chapter 17

Corynebacterium species

Corynebacterium species are small, pleomorphic, Gram-positive bacteria which occur in coccoid, club and rod forms (coryneform morphology). In stained smears they occur singly, in palisades of parallel cells and in angular clusters resembling Chinese characters (Fig. 17.1). The type species is *Corynebacterium diphtheriae*, the cause of diphtheria in children.

The genus *Corynebacterium* formerly contained a miscellaneous collection of bacteria. Recently, DNA and 16S rRNA studies have assigned several former members of the corynebacteria to other genera. The corynebacteria are considered to belong to a group of organisms within the *Corynebacteriaceae* known as the CMN group, which contains the genera *Corynebacterium, Mycobacterium, Nocardia* and *Rhodococcus*. Although the group is heterogeneous, the organisms all have a high G+C content and their cell walls contain mycolic acids. The mycolic acids of the corynebacteria are shorter than those of other genera within the group and the carbon chains are usually saturated.

Most corynebacteria are catalase-positive, oxidasenegative, non-spore-forming facultative anaerobes which require enriched media for growth. Pathogenic corynebacteria are non-motile. Tissue trauma usually precedes the establishment of pathogenic corynebac-teria and the resulting lesions are characterized by suppuration.

Usual habitat

Many *Corynebacterium* species are commensals on mucous membranes (Table 17.1). *Corynebacterium pseudotuberculosis* (formerly C. *ovis*) can survive for months in the environment.

Key points

- Gram-positive, pleomorphic bacteria
- Fastidious, requiring enriched media
- Majority are commensals on mucous membranes
- Cause pyogenic infections
- *Corynebacterium* species:
  –non-motile facultative anaerobes
  –catalase-positive, oxidase-negative

Differentiation of the corynebacteria

Most pathogenic corynebacteria are relatively host specific and produce identifiable clinical syndromes. The host species and the nature of the disease may suggest the causal agent. Identification criteria include bacterial cell morphology, colonial appearance and biochemical reactions. An enhancement of the haemolysis test is used for the identification of C. *pseudotuberculosis*

- Colonial characteristics:
  –*Corynebacterium bovis* is a lipophilic bacterium which produces small, white, dry, non-haemolytic colonies in the well of plates inoculated with a bovine milk sample.
  –*Corynebacterium kutscheri* produces whitish colonies. Occasional isolates are haemolytic.
  – *Corynebacterium pseudotuberculosis* has small, whitish colonies surrounded by a narrow zone of complete haemolysis, which may not be evident for up to 72 hours. After several days the colonies become dry, crumbly and cream-coloured.
  – Members of the *C. renale* group produce small non-haemolytic colonies after incubation for 24 hours. Pigment production after incubation for 48 hours is one of the differentiating features of the three species in the group (Table 17.2).
- Biochemical reactions:
  – Conventional or commercially available biochemical tests can be used to differentiate the corynebacteria.
  – Two biotypes of *C. pseudotuberculosis* are recognized. The ovine/caprine strains lack nitrate-reducing capacity, while the equine/bovine strains usually reduce nitrate. Cross-infection by biotypes is thought to be minimal although non-nitrate-reducing strains have been

isolated from clinical infections in cattle (Yeruham *et al*., 2004) and from horses (Connor *et al*., 2000).

–The biochemical reactions used to distinguish members of the *C. renale* group are indicated in Table 17.2.

– Urease is produced by all pathogenic corynebac-teria with the exception of *C. bovis*.

- Enhancement of haemolysis test:

  – The haemolysis produced by *C. pseudotuber-culosis* is enhanced when the organisms are inoculated across a streak of *Rhodococcus equi* (Fig. 17.2).

Table 17.1 The pathogenic corynebacteria, their hosts, usual habitats and the disease conditions which they produce.

Pathogen	Host	Disease condition	Usual habitat
Corynebacterium bovis	Cattle	Subclinical mastitis	Teat cistern
C. diphtheriae	Humans	Diphtheria	Human pharyngeal mucosa
	Horses	Rare cases of wound infection	
C. kutscheri	Laboratory rodents	Superficial abscesses, caseopurulent foci in liver, lungs and lymph nodes	Mucous membranes, environment
C. pseudotuberculosis			
Non-nitrate-reducing biotype	Sheep, goats	Caseous lymphadenitis	Skin, mucous membranes, environment
Nitrate-reducing biotype	Horses, cattle	Ulcerative lymphangitis, abscesses	Environment
C. renale group			
C. renale (type I)	Cattle	Cystitis, pyelonephritis	Lower urogenital tracts of cows and bulls
	Sheep and goats	Ulcerative (enzootic) balanoposthitis	Prepuce
C. pilosum (type II)	Cattle	Cystitis, pyelonephritis	Bovine urogenital tract
C. cystitidis (type III)	Cattle	Severe cystitis, rarely pyelonephritis	Bovine urogenital tract
C. ulcerans	Cattle	Mastitis	Human pharyngeal mucosa
	Cats	Rare cases of upper respiratory tract infection	
	Humans	Diphtheria (toxigenic strains)	

Figure 17.1 Characteristic pleomorphism of corynebacteria showing their typical arrangement in stained smears.

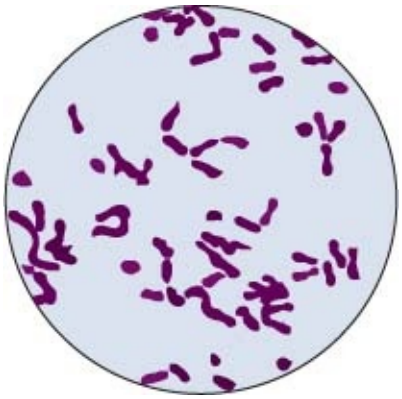
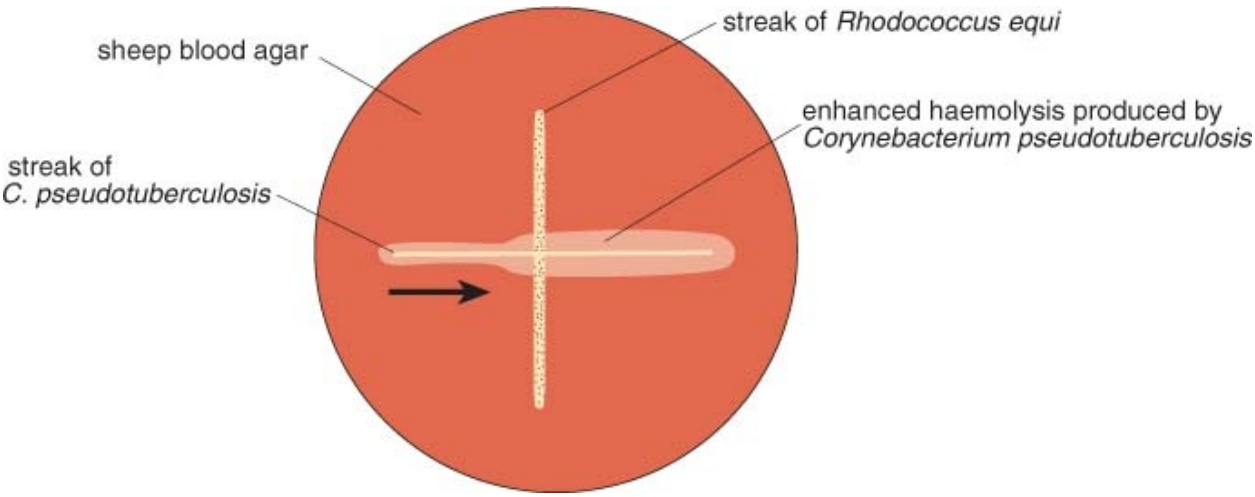


Table 17.2 Differentiation of bacteria in the *Corynebacterium renale* group.

Feature	*C. renale* (type I)	*C. pilosum* (type II)	*C. cystitidis* (type III)
Colour of colony	Pale yellow	Yellow	White
Growth in broth at pH 5.4	+	–	–
Nitrate reduction	–	+	–
Acid from xylose	–	–	+
Acid from starch	–	+	+
Casein digestion	+	–	–
Hydrolysis of Tween 80	–	–	+

Figure 17.2 Enhancement of haemolysis test for *Corynebacterium pseudotuberculosis*. When a streak of *C. pseudotuberculosis* is drawn at right angles (arrow) across a streak of *Rhodococcus equi*, enhancement of haemolysis occurs.



Pathogenesis and pathogenicity

Many corynebacteria are opportunistic pathogens. Corynebacteria, with the exception of *C. bovis*, are pyo-genic organisms that cause a variety of suppurative conditions in domestic animals. *Corynebacterium bovis*, which is found in the teat canal of up to 20% of apparently healthy dairy cows, provokes a mild neutrophil response. It has been suggested that this response may protect the mammary gland against invasion by more virulent pathogens (Pociecha, 1989). However, there are conflicting data on the significance of infection with *C. bovis* in the mammary gland and Huxley *et al*. (2004) suggest that this may be due in part to the incorrect identification of isolates in previous studies. They found 2.8% of a collection of 762 isolates to be species other than *C. bovis* when tested using molecular methods.

Corynebacterium pseudotuberculosis is a facultative intracellular pathogen capable of surviving and repli-cating in phagocytes. The virulence of this pathogen is linked to its cell wall lipid and to the production of an exotoxin, phospholipase D (PLD). This enzyme hydrolyses sphingomyelin in mammalian cell membranes, releasing choline. In the early stages of infection, PLD may enhance survival and multiplication of *C. pseudotuberculosis* in the host. Another protective antigen, corynebacterial secreted protease 40 (CP40), is also thought to have a significant role in virulence as immunization with this antigen provokes a strong immune response which provides considerable protection against infection (Baird and Fontaine, 2007). Both *C. ulcerans* and *C. pseudotuberculosis* can produce diphtheria toxin when lysogenized by corynephage beta which possesses the *tox* gene. Although the effect of this toxin in animals is unclear, its presence in raw milk from cows infected with *C. ulcerans* may have public health implications.

Bacteria in the *C. renale* group are urinary tract pathogens which cause cystitis and pyelonephritis in cattle. These organisms produce urease, which hydrolyses urea. Members of the *C. renale* group possess fimbriae which allow attachment to the urogenital mucosa.

Minor trauma to the skin may allow entry of *C. kutscheri* and *C. pseudotuberculosis*, whereas the urinary tract pathogens avail of diminished immunological defences or local tissue damage following parturition.

Diagnostic procedures

- The species of animal affected and the clinical signs may suggest a specific diagnosis.
- Suitable specimens for laboratory examination include pus, exudate, samples of affected tissue and mid-stream urine.
- Direct microscopic examination of Gram-stained smears from specimens may reveal coryneform organisms (Fig. 17.1).
- Culture media for routine use include blood agar, selective blood agar and MacConkey agar. Inoculated plates are incubated aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates: – Colonial characteristics
  – Presence or absence of haemolysis
  – Aerobic or anaerobic incubation requirements
  – Absence of growth on MacConkey agar
  – Typical coryneform pleomorphism in a Gram-stained smear from culture (Fig. 17.1)
  – Results of conventional or commercially available biochemical tests
  – Specific tests for distinguishing members of the *C. renale* group (Table 17.2)
  – Enhancement of haemolysis test for C. *pseudotuberculosis*
- Molecular techniques have been developed for the detection and identification of *C. pseudotubercu-losis*. Cetinkaya *et al*. (2002) reported a PCR-based method for the identification of isolates, and Pacheco *et al*. (2007) used a multiplex PCR technique both for identification of isolates and for direct detection of the organism in specimens of pus.

Clinical infections

The main diseases caused by infections with *Corynebacterium* species are summarized in Table 17.1. *Corynebacterium pseudotuberculosis* causes occasional human infections, some acquired from infected animals and others from environmental sources. Toxigenic strains of *C. diphtheriae* and *C. ulcerans* are occasionally found in animals and may represent a rare source of infection for humans (Henricson *et al*., 2000 ; de Zoysa *et al*., 2005).

Caseous lymphadenitis

Caseous lymphadenitis, caused by the non-nitrate-reducing biotype of *C. pseudotuberculosis*, is a chronic suppurative condition of sheep, goats and rarely cattle. Infection results in abscessation and enlargement of superficial or internal lymph nodes. The incubation period is about 3 months. The disease is prevalent in Australia, New Zealand, the Middle East, Asia, Africa and parts of North and South America. Caseous lymphadenitis is being reported more frequently in Britain and other European countries. Ill-thrift may be evident in affected animals, and the disease invariably results in condemnation of car-casses and devaluation of hides. Infection is spread by pus from ruptured abscesses and from nasal and oral secretions. The organism can survive in the environment for several months. *Corynebacterium pseudotuber*-culosis has been isolated from the milk of affected goats.

Sheep become infected through contamination of shearing wounds, by arthropod bites or from contaminated dips. Affected lymph nodes are enlarged and, when the infection becomes chronic, exhibit characteristic encapsulated abscesses which have an ‘onion ring’ appearance in cross-section. The abscess material is caseous, initially greenish and later putty-coloured. Haematogenous spread can lead to abscessation of internal lymph nodes without obvious superficial lesions. Ill-thrift and pneumonia may be present. The visceral form of the disease may not be detectable ante mortem. Goats usually develop the superficial form of the disease with subcutaneous abscesses in the head and neck regions. The different distribution of lesions observed in sheep and goats probably reflects differences in management practices and behavioural factors which facilitate transmission such as shearing of sheep and grooming and head-butting behaviour in goats.

Diagnosis

- The disease may be suspected on clinical grounds or at post-mortem examination.
- Smears from lesions may reveal Gram-positive coryneform bacteria.
- Isolation and identification of *C. pseudotuberculosis* from abscess material is confirmatory.
- Several ELISAs have been developed for the sero-logical detection of *C. pseudotuberculosis* infection. These tests detect antibodies directed against either cell wall antigens or the exotoxin (PLD). A double antibody sandwich ELISA based on PLD was reported to be sensitive (79 ± 5%) and

highly spe-cific (99 ± 1%) in sheep (Dercksen *et al*., 2000).

- Sunil *et al*. (2008) investigated the use of an interferon -γ test for detection of infection and eradication of caseous lymphadenitis in sheep flocks, and concluded that it showed considerable promise.

Treatment

Because of the chronic nature of lesions and the ability of the organisms to survive intracellularly, therapy is usually ineffective, although *C. pseudotuberculosis* strains are susceptible to most classes of antimicrobial agents *in vitro*.

Control

Appropriate control measures for individual countries are determined by the prevalence of the disease.

- Exclusion of caseous lymphadenitis from countries free of the disease:
  – Sheep and goats should be imported only from countries which are either free of the disease or have a low incidence of infection. Animals must be selected from flocks or herds officially certified to be free of infection for 3 years.
  – Animals should be subjected to pre-importation testing using ELISA.
  – Imported animals should be quarantined for several months and infected animals should be slaughtered.
- Eradication of caseous lymphadenitis from countries with a low prevalence of the disease:
  – Animals with obvious lesions should be segregated and culled.
  – Regular testing of flocks or herds using ELISA should be followed by culling of animals with positive or doubtful results.
  – Lambs should be removed from seropositive dams at birth and reared artificially.
  – Contaminated buildings and equipment should be thoroughly disinfected.
- Control measures in countries with a high prevalence of caseous lymphadenitis:
  – Strict hygienic measures should be applied in buildings such as shearing sheds. Shearing and docking equipment should be regularly and

thoroughly disinfected.

– Vaccines are available for use in some countries and may have a place in control programmes. Bacterins, toxoid and combinations of bacterin and toxoid vaccines have been developed. In addition, live attenuated vaccines and DNA vaccines have been investigated, some of which gave substantial protection against infection (reviewed by Baird and Fontaine, 2007).

Ulcerative lymphangitis

The nitrate-reducing biotype of *C. pseudotuberculosis* causes sporadic cases of ulcerative lymphangitis in horses and cattle. Ulcerative lymphangitis occurs in Africa, the Americas, the Middle East and India. In the USA, the disease is prevalent in autumn and early winter and is more common in horses than in cattle. Infection occurs through skin wounds or arthropod bites or by contact with contaminated harness. The condition presents either as lymphangitis of the lower limbs or abscessation in the pectoral region. The onset of lymphangitis is slow and the condition usually becomes chronic. Affected lymphatic vessels are swollen and firm and nodules form along their length. Oedema develops in affected limbs, and ulcerated nodules exude a thick, odourless, greenish, blood-tinged pus. Infection in cattle is reported most frequently in Israel and manifests as lymphadenitis and lymphangitis with abscess formation and ulceration. Houseflies are thought to play a role in dissemination of the organism. Less commonly, mastitis and a clinical syndrome involving lesions of the coronary band with resulting lameness in affected dairy cattle have been reported (Steinman *et al*., 1999; Yeruham *et al*., 2004).

Diagnosis is based on isolation and identification of *C. pseudotuberculosis* from lesions, since lymphangitis can also result from infection with other pyogenic bacteria. Systemic antibiotic therapy may be combined with topical treatment using an iodophor shampoo. Affected animals must be isolated and contaminated areas should be disinfected.

Bovine pyelonephritis

Organisms belonging to the *C. renale* group can be isolated from the vulva, vagina and prepuce of apparently normal cattle. The stress of parturition and the short urethra in the cow predispose to infection of the urinary tract. Although infection by any member of the group can cause cystitis, the most severe form is associated with *C. cystitidis*. Ascending infection from the bladder through the

ureters can result in pyelonephritis. Clinical signs of pyelonephritis include fever, anorexia and decreased milk production. Restlessness and kicking at the abdomen may indicate renal pain. Dysuria, an arched back and blood-tinged urine are invariably present. Long-standing infections lead to extensive renal damage.

Diagnosis

- Clinical signs may suggest urinary tract disease.
- Thickened ureters and enlarged kidneys may be detected by rectal palpation. The condition is often unilateral.
- Red blood cells and protein are present in the urine.
- Culture of *C. renale* from urinary deposits, in association with characteristic clinical signs, is confirmatory.

Treatment

Antibiotic therapy, based on susceptibility testing, must be instituted early in the disease and should be continued for at least 3 weeks. Because penicillin is excreted in the urine, treatment with this antibiotic is particularly effective for susceptible isolates. Following a review of 17 cases Braun *et al*. (2008) considered unilateral nephrectomy to be the treatment of choice if pyelonephritis is unilateral and severe.

Ulcerative balanoposthitis

Ulcerative (enzootic) balanoposthitis (‘pizzle rot’), particularly common in Merino sheep and Angora goats, is caused by *C. renale* and is characterized by ulceration around the preputial orifice, with a brownish crust developing over the lesion. Similar lesions sometimes occur on the vulva in ewes. *Corynebacterium renale* can hydrolyse urea to ammonia which may cause mucosal irritation and ulceration. A high urinary urea level, a consequence of high protein intake, may predispose to the development of disease. Animals grazing pastures containing high oestrogen levels are also prone to the condition. Castrated sheep are affected more frequently than rams. A heavy wool or mohair cover around the prepuce predisposes to infection. Untreated cases may progress to total occlusion of the preputial orifice.

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Chapter 18

Rhodococcus equi

Rhodococcus equi ormerly called *Corynebacterium equi*, is a Gram-positive, aerobic, soil saprophyte which occurs worldwide. It is an opportunistic pathogen of foals under 6 months of age. *Rhodococcus equi* grows on non-enriched media such as nutrient agar and produces characteristic mucoid salmon-pink colonies, features indicative of capsule formation and pigment production. Some strains of *R. equi* appear as cocci and others as rods up to 5 μ m in length (Fig. 18.1). The organism is non-motile, catalase-positive, oxidase-negative and weakly acid-fast. It is taxonomically related to *Nocardia* and *Mycobacterium* species; all three genera are classified in the order *Actinomycetales*. The acid-fast properties of *R. equi*, its ability to grow intracellularly and production of lung lesions are further evidence of its relationship with *Mycobacterium* species. The complete genome sequence of the organism is available online (Muscatello *et al*., 2007).

Usual habitat

Rhodococcus equi is an inhabitant of both soil and the intestinal tracts of animals. It can replicate in soils enriched with faeces of herbivores at warm temperatures and is present in the soil of most equine establishments.

Clinical infections

Suppurative bronchopneumonia of foals is the major disease caused by this pyogenic organism. Superficial abscesses due to *R. equi* have been recorded in horses over 6 months of age. Infected pigs develop a granulomatous lymphadenitis involving the cervical lymph nodes, and disease has been recorded on rare occasions in most other domestic species (Table 18.1). Associated with the increasing number of humans infected with human immunodeficiency virus worldwide, opportunistic infection with *R. equi* has become a disease of considerable importance in human medicine.

Key points

- Gram-positive rods or cocci
- Growth on non-enriched media
- Salmon-pink, mucoid, non-haemolytic colonies
- Aerobic, non-motile
- CAMP test-positive
- Soil saprophyte
- Respiratory pathogen of foals

Suppurative bronchopneumonia of foals

This important disease of foals, 1 to 4 months of age, is characterized by bronchopneumonia and lung abscessation.

Epidemiology

Infection is generally acquired by inhalation of dust contaminated with *R. equi*. The organism is often present in large numbers in the faeces of healthy foals under 3 months of age and in extremely high numbers of those with clinical disease. It can also be isolated from the faeces of older horses and many other mammals and birds. Some farms may have repeated problems with *R. equi* pneumonia each year with disease recorded only rarely on other premises. Environmental influences appear to be important in the occurrence of disease including factors such as dry weather and poor grass cover on paddocks leading to dusty conditions. High foal density and large numbers of horses on farms are risk factors for disease. In European countries with wet temperate climates, acquisition of infection in dusty stables appears to be important (Muscatello *et al*., 2006). Recent studies suggest that clinically and subclinically affected foals may excrete *R. equi* in aerosols (Muscatello *et al*., 2005). Thus, infection may occur by contagion as well as through the inhalation of organisms with dust. Granulomatous ulcerative enterocolitis and mesenteric lymphadenitis sometimes occur when affected foals swallow sputum containing large numbers of *R. equi*. Ingestion of low numbers of organisms does not result in disease. Because the immunological competence of foals progresses as they mature, foals over 6 months of age appear to be refractory to pulmonary infection.

Figure 18.1 Cocci and rods, the two distinct morphological forms of *Rhodococcus equi*.

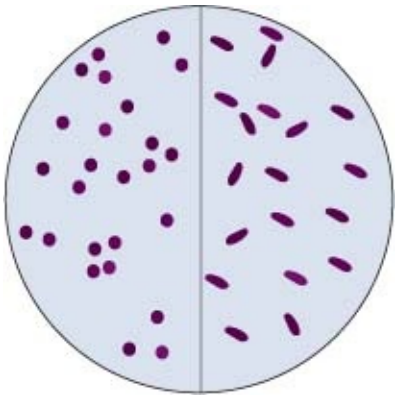


Table 18.1 Clinical conditions associated with *Rhodococcus equi*.

Host	Clinical condition
Foals 1 to 4 months of age	Suppurative bronchopneumonia and pulmonary abscessation
Horses	Superficial abscessation
Pigs, cattle	Mild cervical lymphadenopathy
Cats	Subcutaneous abscesses, mediastinal granulomas
Immunosuppressed humans	Pneumonia

Pathogenesis and pathogenicity

The virulence of *R. equi*, an intracellular pathogen, is principally associated with a large plasmid. This plasmid contains genes that encode a number of proteins associated with virulence, the most important of which is the vapA protein. Only virulent strains of *R. equi* are isolated from lesions of naturally infected foals and thus these virulence-associated proteins and plasmids can be used as epidemiological markers. Characterization of isolates from different animal species using a plasmid typing scheme has shown definite associations between particular plasmid types and individual animal species (Ocampo-Sosa *et al*., 2007). Only equine isolates appear to be positive for the *vapA* gene. Virulence is associated with an ability to survive and multiply within macrophages through interference with endosomal maturation and prevention of acidification of the vacuole in which the organism is found. Other factors enhancing virulence include capsular polysaccharides and mycolic acids in the cell wall which retard phagocytosis, and also a variety of exoenzymes. The particular susceptibility of foals under 4 months of age to bronchopneumonia caused by this pathogen is attributed to impaired cellular immunity in the lungs (Prescott *et al*., 2004).

Clinical signs

Clinical signs vary with the age at which the foal becomes infected. Acute

disease often occurs in 1-month-old foals, with sudden onset of fever, anorexia and signs of bronchopneumonia. The disease tends to be insidious in foals 2 to 4 months old, and lesions can be well advanced before the animal exhibits coughing, dyspnoea, weight loss, exercise intolerance and characteristic loud, moist rales on auscultation of the lungs. Affected foals may occasionally have diarrhoea.

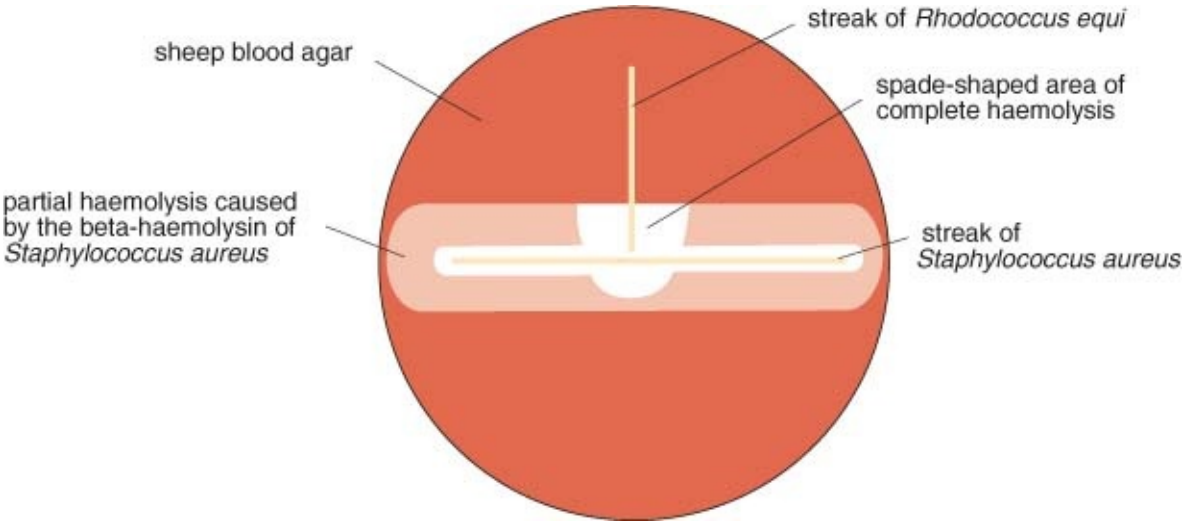
Diagnosis

Although clinical signs and findings on auscultation are highly suggestive of *R. equi* infection in foals affected by the classical form of the disease, it is important to diagnose the disease before clinical signs are evident as by that stage serious lung damage will have occurred. Thus, monitoring of foals on farms with a history of *R. equi* infection is frequently undertaken.

- A history of the disease on the farm, the age of the affected foal and clinical and laboratory findings such as leukocytosis and hyperfibrinogenaemia may suggest infection with *R. equi*.
- Ultrasonography of the thorax is frequently used to provide evidence of pulmonary involvement.
- Specimens for laboratory examination include tracheal aspirates and pus from lesions.
- Cytological examination of respiratory secretions is useful as visualization of pleomorphic organisms within cells strongly supports a diagnosis of *R. equi* infection.
- Blood agar and MacConkey agar plates inoculated with suspect material are incubated aerobically at 37°C for 24 to 48 hours. Sensitivity of culture is low and is attributed to the intracellular location of organisms (Muscatello *et al*., 2007).
- Identification criteria for isolates:
  – Colonies on blood agar are non-haemolytic, salmon-pink and mucoid
  – Absence of growth on MacConkey agar
  – CAMP-test positive (Fig. 18.2)
  – Unreactive in the oxidation-fermentation test and in sugar fermentation tests
  – Biochemical profile using commercially available kits.
- Molecular techniques for demonstration of the organism in clinical specimens are available but sensitivity and specificity are variable. PCR for

the *vapA* gene was reported to be 100% sensitive on tracheal wash fluid (Sellon *et al*., 2001) and a new real-time PCR for the cholesterol oxidase gene developed by Rodríguez-Lázaro *et al*. (2006) was highly sensitive also.

Figure 18.2 CAMP test. *Rhodococcus equi* produces a factor which completely lyses the red cells previously damaged by the betahaemolysin of *Staphylococcus aureus*, producing a spade-shaped pattern of complete haemolysis which extends across the streak of *S. aureus*.



Treatment

- A combination of oral rifampin and a macrolide antibiotic such as erythromycin, azithromycin or clarithromycin for 4 to 10 weeks, although expen-sive, is the preferred treatment. However, severely affected foals may fail to respond. The response to therapy can be evaluated radiographically and by ultrasonography.
- Supportive therapy includes rehydration and the use of bronchodilatory agents or expectorants.

Control

- Commercial vaccines are not available.
- On farms where the disease has occurred, foals should be kept under observation and examined clinically twice weekly until they are 4 months of age.
- Recent studies have shown that the level of virulent *R. equi* present in the

environment of foals is not directly related to the prevalence of disease and may not be important if the organisms remain bound to the soil and cannot be inhaled (Muscatello *et al*., 2007; Cohen *et al*., 2008). However, prevention of exposure of foals to aerosols of *R. equi* is desirable.

- Dusty conditions in paddocks and holding yards should be minimized by measures such as irrigation and maintenance of good grass cover.
- The numbers of foals kept together in paddocks or yards should be reduced in order to limit the generation of aerosols in dry weather. In addition, keeping smaller numbers of foals together limits foal-to-foal spread of infection although the relative importance of this route of infection has not been determined.
- If foals are stabled, time spent indoors should be reduced where possible and ventilation should be improved. The use of non-dusty bedding materials may help minimize airborne *R. equi*.
- Stables in which clinically affected foals were housed must be thoroughly cleaned and disinfected.
- It is claimed that hyperimmune serum from the dam, administered to the foal in the first month of life, reduces the prevalence of disease on some farms.

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Chapter 19

Listeria species

Most *Listeria* species are small, Gram-positive, coccobacillary rods, up to 2 μ m in length (Fig. 19.1). They are catalase-positive, oxidase-negative, motile, facultative anaerobes. The genus is composed of six species, two of which are pathogenic for animals. *Listeria mono-cytogenes*, the most important of these pathogens, has been implicated worldwide in diseases of many animal species and humans. It was first isolated from laboratory rabbits with septicaemia and monocytosis (Murray *et al*., 1926). The organism can grow over a wide temperature range from 4°C to 45°C and can tolerate pH values between 5.5 and 9.6. The clinical manifestations of infections with *Listeria* species are summarized in Table 19.1. *Listeria monocytogenes* is a pathogen mainly of ruminants as is *L. ivanovii*, although the latter is less frequently implicated in diseases of animals. *Listeria monocytogenes* is of major public health significance and infects humans via contaminated food and occasionally by direct contact. *Listeria innocua* is usually regarded as non - pathogenic but it has been isolated from sheep with meningoencephalitis on rare occasions.

Usual habitat

Listeria species can replicate in the environment. They are widely distributed and can be recovered from herbage, faeces of healthy animals, sewage effluent and bodies of fresh water.

Key points

- Small, Gram-positive rods
- Grow on non-enriched media
- Tolerate wide temperature and pH ranges
- Small haemolytic colonies on blood agar
- Facultative anaerobes, catalase-positive, oxidase-negative

- Tumbling motility at 25°C
- Aesculin hydrolysed
- Environmental saprophytes
- Outbreaks of listeriosis often related to silage feeding
- Pathogenicity associated with intracellular replication

Differentiation of *Listeria species*

The pattern of haemolysis on sheep blood agar, CAMP tests and acid production from a short range of sugars are useful differentiating laboratory methods for *Listeria* species (Table 19.2). After incubation for 24 hours, the colonies are small, smooth and transparent.

- Commercially available biochemical test kits can be used to distinguish *Listeria* species.
- Based on cell wall and flagellar antigens, 13 serotypes of *L. monocytogenes* are recognized (Murray *et al*., 2007).
- Phage typing is reproducible and discriminating but its diagnostic applications are limited as some strains are not typable.
- Molecular methods are commonly used for strain typing of *L. monocytogenes*. Digestion of nucleic acid using restriction endonucleases followed by PFGE is a widely recognized method of typing. The Center for Disease Control (CDC) PulseNet public health laboratories utilize a standardized protocol which permits inter-laboratory comparison of PFGE patterns via the internet. Other methods of typing include amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA analysis, and repetitive extragenic palindro-mic element - based PCR (REP–PCR). All of these techniques have good discriminatory ability (Fonnesbech Vogel *et al*., 2004 ; Chou and Wang, 2006). Many of the methods currently available for the isolation and differentiation of *Listeria* organisms have been reviewed (Gasanov *et al*., 2005).

Figure 19.1 The typical coccobacillary form of *Listeria monocytogenes* from an actively growing culture.

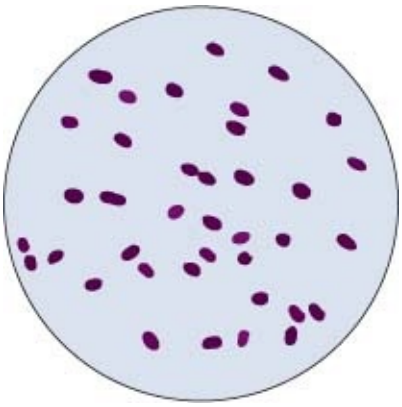

Table 19.1 Clinical manifestations of infections with *Listeria* species in domestic animals.

Species	Hosts	Forms of disease
Listeria	Sheep,	Encephalitis (neural form)
monocytogenes	cattle, goats	Abortion Septicaemia Endophthalmitis (ocular form)
	Cattle	Mastitis (rare)
	Dogs, cats, horses	Abortion, encephalitis (rare)
	Pig	Abortion, septicaemia, encephalitis
	Birds	Septicaemia
L ivanovii	Sheep, cattle	Abortion
L innocua	Sheep	Meningoencephalitis (*rare*)

Pathogenesis and pathogenicity

Infection with *L. monocytogenes* usually follows ingestion of contaminated feed and may result in septicaemia, encephalitis or abortion. Organisms penetrate the intestine by a mechanism that is not well defined. Spread occurs via lymph and blood to various tissues. In pregnant animals, infection results in transplacental transmission. There is evidence that the organism can invade through breaks in the oral or nasal mucosa. It is thought that the organism may penetrate via the dental pulp when sheep are cutting or losing teeth (Barlow and McGorum, 1985). From this site, migration in cranial nerves is thought to be the main route of infection in neural listeriosis. Lesions in the brain stem, often unilateral, are composed of microabscesses and perivascular lymphocytic cuffs.

Listeria monocytogenes has the ability to invade both phagocytic and non-phagocytic cells, to survive and replicate intracellularly and to transfer from cell to cell without exposure to humoral defence mechanisms. Specific surface proteins, internalins, facilitate both the adherence of organisms to epithelial cells of the host and their subsequent uptake by these cells. Phagocytosis by

macrophages is mediated by receptors that recognize the lipoteichoic acid of the Gram-positive cell wall of the *Listeria* organism (Flannagan *et al*., 2009). Survival of virulent strains within cells is by means of escape from the phagosome before its maturation to a phagolysosome. The action of a cytolytic toxin, listeriolysin, and a phospholipase enzyme destroys the membranes of phagocytic vacuoles allowing *Listeria* to escape into the cytoplasm. In the cytoplasm, an actin-polymerizing protein, ActA, produced by the organism, directs the formation of tail-like structures from host cellular microfilaments which aid the motility of the invading pathogens. The motile *Listeria* contact the internal surface of the cytoplasmic membrane and induce pseudopod-like projections. These projections, containing the bacteria, are taken up by adjacent cells. The entire process is then repeated following replication of *Listeria* in newly infected cells (Chakraborty and Wehland, 1997). Replication within cells is facilitated by expression of genes which allow the organism to utilize nutrients present within the host cell. *Listeria monocytogenes* survives within cells in part because of its ability to escape the phagosome but also because of its ability to inhibit autophagy, by mechanisms which have not yet been fully elucidated (Ray *et al*., 2009). Autophagy occurs as a normal part of the growth and development of a cell and involves the catabolism of normal cell components through the lysosomal machinery. The role of autophagy is to maintain cellular homeostasis through the degradation and recycling of cellular components. However, it may also play a role in protection against infection by intracellular organisms such as *Listeria monocytogenes*.

Table 19.2 Laboratory methods for differentiating *Listeria* species.

Listeria species	Haemolysis on sheep blood agar	CAMP test		Acid production from sugars		
		S. aureus	*R. equi*	D-mannitol	L-rhamnose	D-xylose
L. monocytogenes	+	+	–	–	+	–
L. ivanovii	++	–	+	–	–	+
L. innocua	–	–	–	–	v	–
L. seeligeri	+	+	–	–	–	+
L. welshimeri	–	–	–	–	v	+
L. grayi	–	–	–	+	v	–

v, variable reaction.

Clinical infections

Infections with *L. monocytogenes* have been recorded in more than 40 species of domestic and wild animals. Sporadic abortions in sheep and cattle have been attributed to infection with *L. ivanovii. Listeria innocua* has been implicated in a case of ovine meningoencephalitis (Walker *et al*., 1994). Forms of listeriosis that occur in domestic animals are listed in Table 19.1.

Listeriosis in ruminants

Listeriosis in ruminants may present as encephalitis, abortion, septicaemia or endophthalmitis. Usually only one form of the disease occurs in a group of affected animals. Septicaemia, often encountered in newborn piglets, foals, cage birds and poultry, can also occur in adult sheep.

Although *L. monocytogenes* is widely distributed in the environment, outbreaks of listeriosis tend to be seasonal in European countries and to affect silage-fed animals in late pregnancy. *Listeria monocytogenes* can replicate in the surface layers of poor-quality silage with pH values above 5.5. In such circumstances, liste-rial numbers may reach 10^7colony-forming units kg^{-1} of silage. In good quality silage, multiplication of the organisms is inhibited by the acid produced by fermentation. Susceptibility to infection with *L. monocy-togenes* has been attributed to decreased cell - mediated immunity associated with advanced pregnancy.

Clinical signs

The incubation period of neural listeriosis (circling disease) ranges from 14 to 40 days. Dullness, circling and tilting of the head are common clinical signs. Unilateral facial paralysis results in drooling of saliva and drooping of the eyelid and ear. Exposure keratitis may occur in some cases. Body temperature may be elevated in the early stages of the disease. In sheep and goats, recumbency and death may follow within a few days of the emergence of clinical signs. The duration of illness is usually longer in cattle. Abortion without evidence of systemic illness may occur up to 12 days after infection. Septicaemic listeriosis, with a short incubation period of 2 to 3 days, is most commonly encountered in lambs although it may occur occasionally in pregnant sheep. In cattle and sheep, keratoconjunctivitis and iritis (ocular listeriosis) are localized and often unilateral, and have been attributed to direct ocular contact with contaminated silage.

Diagnosis

- Characteristic neurological signs or abortion in association with silage feeding may suggest listeriosis.
- Appropriate specimens for laboratory examination depend on the form of the disease:

  – Cerebrospinal fluid (CSF) and tissue from the medulla and pons of animals with neurological signs should be sampled. Fresh tissue is required for isolation of organisms and fixed tissue for histopathological examination.

  – Specimens from cases of abortion should include cotyledons, foetal abomasal contents and uterine discharges.

  – Suitable samples from septicaemic cases include fresh liver or spleen and blood.
- Smears from cotyledons or from liver lesions may reveal Gram-positive coccobacillary bacteria.
- Immunofluorescence, using monoclonal antibodies, may facilitate a rapid diagnosis.
- Histological examination of brain tissue reveals microabscesses and heavy perivascular mononuclear cuffing in the medulla and elsewhere in the brain stem.
- White cell numbers exceeding 1.2×10^{7} litre^{-1} and a protein concentration of greater than 0.4 g litre$^{™1}$ in CSF are found in neural listeriosis.
- Isolation methods:

  – Specimens from cases of abortion and septicaemia can be inoculated directly on to blood agar, selective blood agar and MacConkey agar. The plates are incubated aerobically at 37°C for 24 to 48 hours.

  – A cold-enrichment procedure is necessary for isolating the organism from brain tissue. Small pieces of medulla are homogenized and a 10% suspension is made in nutrient broth. The suspension is held at 4°C in a refrigerator and subcultured weekly on to blood agar for up to 12 weeks.
- Identification criteria for *L. monocytogenes* isolates:

  – Colonies are small, smooth and flat with a bluegreen colour when illuminated obliquely. Rough variants occur infrequently. Individual colonies are usually surrounded by a narrow zone of complete haemolysis.

  – Catalase test is positive, distinguishing this organism from streptococci

and *Arcanobacterium pyogenes* which have similar colonies but are catalase-negative.

– CAMP test is positive with *Staphylococcus aureus* but not with *Rhododoccus equi* (Table 19.2).

– Aesculin is hydrolysed.

– Isolates incubated in broth at 25°C for 2 to 4 hours exhibit a characteristic tumbling motility.

– Most isolates of animal origin are virulent, a characteristic that can be confirmed by animal inoculation. Instillation of a drop of broth culture into the eye of a rabbit induces keratoconjunctivitis (Anton test).

- Molecular methods have been developed for the detection of *Listeria* species in clinical specimens and in food. Such methods are more rapid than cultural procedures and are frequently more sensitive. The methods used are usually based on PCR and include commercially available PCR systems.

Treatment

Ruminants in the early stages of septicaemic listeriosis respond to systemic therapy with ampicillin or amoxicillin. Response to antibiotic therapy may be poor in neural listeriosis although prolonged high doses of ampicillin or amoxicillin combined with an aminoglycoside may be effective. Reports differ as to the effectiveness of different treatments. Braun *et al*. (2002) reported that a gentamicin/ampicillin combination was most effective for the treatment of sheep and goats whereas Schweizer *et al*. (2006) found no differences between penicillin G, oxytetracycline, amoxicil-lin or amoxicillin combined with gentamicin when treating a number of cases of listeriosis in cattle. Ocular listeriosis requires treatment with antibiotics and corticosteroids injected sub-conjunctivally. Cepha-losporins are not effective in the treatment of *Listeria* infections in animals or humans.

Control

- Poor-quality silage should not be fed to pregnant ruminants. Silage feeding should be discontinued if an outbreak of listeriosis is confirmed.
- Feeding methods which minimize direct ocular contact with silage should be implemented.
- Vaccination with killed vaccines, which do not induce an effective cell-

mediated response, is not protective because *L. monocytogenes* is an intracellular pathogen. Although antibodies may increase the ability of phagocytes to ingest *L. monocytogenes*, they do not confer immunity to infection. Neutrophils are important in protection against listerial infection but cell-mediated immunity involving activation of macrophages by IFN -γ released from T-helper cells has long been considered the principal method of protection. However, as multiplication of *L. monocytogenes* occurs in hepatocytes and other cells, the achievement of complete immunity must require additional defence mechanisms which have not yet been fully elucidated. Live, attenuated vaccines, which are available in some countries, are reported to reduce the prevalence of listeriosis in sheep (Gudding *et al*., 1989). Subunit vaccines are under investigation and may induce protection if combined with effective adjuvants.

Human listeriosis

If normal healthy adults acquire infection, the disease usually presents as a mild febrile illness resembling influenza. Papular lesions on the hands and arms, principally in veterinarians and farmers, can result from contact with infective material. Infection with *L. monocytogenes* can lead to abortion in pregnant women and can be life-threatening in neonates, the elderly and immunosuppressed individuals.

Human infections usually result from consumption of contaminated food such as raw milk, soft cheeses, coleslaw and uncooked vegetables. Because of its intracellular localization and tolerance to heat, *Listeria monocytogenes* may survive pasteurization. Direct transfer from infected animals to humans is uncommon and is of little consequence in healthy, non-pregnant individuals.

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Chapter 20

Erysipelothrix rhusiopathiae

Erysipelothrix rhusiopathiae is a non-motile, Gram-positive, facultative anaerobe. It is catalase-negative, oxidase-negative, resistant to high salt concentrations and grows in the temperature range 5°C to 42°C and in the pH range of 6.7 to 9.2. Isolates from animals with acute infections form smooth colonies while isolates from chronically infected animals form rough colonies. Smears from smooth colonies yield slender rods (0.2 to 0.4 × 0.8 to 2.5μ m) whereas rough colonies are usually composed of short filaments which decolorize readily (Fig. 20.1). The bacterium grows on nutrient agar but growth is improved in media containing blood or serum.

Erysipelothrix rhusiopathiae causes erysipelas in pigs and turkeys worldwide. Sheep and other domestic animals are occasionally infected. The bacterium also causes erysipeloid, a localized cellulitis, in humans. The disease conditions associated with infection in domestic species are listed in Box 20.1.

Several serotypes of *E. rhusiopathiae* have been reclassified as a new species, *E. tonsillarum*, using DNA–DNA hybridization studies (Takahashi *et al*., 1992). This species appears to be non-pathogenic for pigs but causes endocarditis in dogs (Eriksen *et al*., 1987).

Usual habitat

It is claimed that up to 50% of healthy pigs harbour *E. rhusiopathiae* in tonsillar tissues. Carrier pigs excrete the organism in faeces and in oronasal secretions. The bacterium has also been isolated from sheep, cattle, horses, dogs, cats and poultry and from 50 species of wild mammals and over 30 species of wild birds. Although soil and surface water can become contaminated with *E. rhusiopathiae*, survival time in soil probably does not exceed 35 days under optimal conditions. The bacterium is often present in the slime layer of fish, a potential source of human infection.

Key points

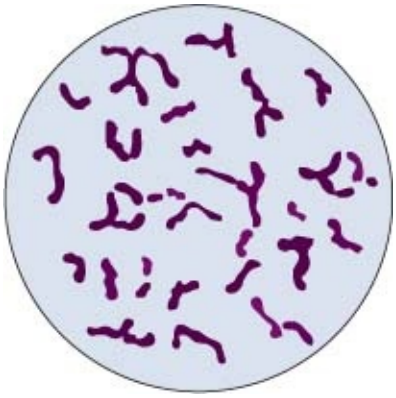
- Gram-positive, small rods (smooth form) or filaments (rough form)
- Growth on non-enriched media
- Small colonies, with incomplete haemolysis in 48 hours
- Growth over wide temperature and pH ranges
- Catalase-negative
- Coagulase-positive
- Non-motile, oxidase-negative, facultative anaerobe
- H_2S formed along stab line in triple sugar iron agar
- Found in porcine tonsils
- Causes swine erysipelas, turkey erysipelas and polyarthritis in lambs

Definitive identification of *Erysipelothrix rhusiopathiae*

- Colonial morphology and haemolytic activity:

  –Non-haemolytic, pin-point colonies appear after incubation for 24 hours, and after 48 hours a narrow zone of greenish, incomplete haemolysis develops around the colonies. At this stage, differences in colony morphology are evident. Smooth colonies are up to 1.5 mm in diameter, convex and circular with even edges, while rough colonies are slightly larger, flat and opaque with irregular edges. A “bottle-brush” type of growth is characteristic of rough isolates when they are stab-inoculated into nutrient gelatin and incubated at room temperature for up to 5 days.

Figure 20.1 Filaments of *Erysipelothrix rhusiopathiae* from a chronic lesion, showing morphological variation.



Box 20.1Clinical manifestations of Erysipelothrix rhusiopathiae infection in domestic animals.

- Pigs (swine erysipelas)
  - – septicaemia
  - “diamond” skin lesions
  - – chronic arthritis
  - – chronic valvular endocarditis
  - – abortion
- Sheep
  - – polyarthritis in lambs
  - –post-dipping lameness
  - – pneumonia
  - – valvular endocarditis
- Turkeys (turkey erysipelas)
  - – septicaemia –arthritis
  - – valvular endocarditis

- Biochemical reactions:

  – Commercially available biochemical test kits can be used for definitive identification. Reactions for presumptive identification include:

  – Catalase-negative.

  – Coagulase-positive (Tesh and Wood, 1988). Few pathogens produce this enzyme apart from some staphylococci (see Chapter 14).

  – H_2S production is detected by a thin, black, central line in triple sugar iron (TSI) agar when this medium is stab-inoculated.
- Serotyping for epidemiological studies:

  – A heat-stable peptidoglycan extracted from the cell wall is used for serotyping in precipitation reactions. Twenty-six serotypes have been identified. Some isolates are non-typable. The serotypes most commonly involved in porcine disease are 1a, 1b and 2.
- Virulence testing in laboratory animals:

  – Isolates of *E. rhusiopathiae* vary considerably in virulence. If necessary, the virulence can be confirmed by the intraperitoneal inoculation of mice or pigeons.

- PCR-based methods for the detection and identification of *E. rhusiopathiae* have been described. Yamazaki (2006) developed a multiplex PCR method which is capable of differentiating *E. rhusiopathiae* and *E. tonsillarum*. Methods using pulsed -field gel electrophoresis (Eriksson *et al*., 2009) and randomly amplified polymorphic DNA (RAPD) analysis have been used for typing and epidemiological investigation of *E. rhusiopathiae* strains.

Pathogenesis and pathogenicity

Infection is usually acquired by ingestion of material contaminated by pig faeces. Entry may occur through the tonsils, skin or mucous membranes. Virulence factors include a capsule which protects the organism against phagocytosis by macrophages and neutrophils and appears to protect the organism within macrophages, allowing intracellular replication. The ability to produce neuraminidase is correlated to virulence; this enzyme plays a major role in adherence of the organism to endothelial cells and subsequent invasion of these cells. Neuraminidase acts by cleaving α-glycosidic linkages in neuraminic (sialic) acid which is found on body cell surfaces. Other factors that contribute to the pathogenicity of *E. rhusiopathiae* include surface proteins which aid adherence to tissues and biofilm formation. A hyaluronidase enzyme is important in dissemination of the bacteria within tissues. The spaA protein, found on the cell surface, is a major protective antigen used in vaccine development. Antibodies against recombinant spaA protein were shown to greatly enhance the opsonic activity of porcine neutrophils against the organism (Imada *et al*., 1999). In the septicaemic form of the disease, vascular damage is characterized by swelling of endothelial cells, adherence of monocytes to vascular walls and widespread hyaline microthrombus formation. Localization of the bacteria in joint synovia and on heart valves during haematogenous spread accounts for the development of chronic lesions at these sites. Long-term articular damage may result from an immune response to persistent bacterial antigens. Viable *E. rhusiopathiae* are rarely isolated from chronically affected joints.

Clinical infections

Infections with *E. rhusiopathiae* are encountered in pigs, turkeys and sheep (Box

20.1). In addition, several major outbreaks of disease have been reported in poultry (Mazaheri *et al.*, 2005). Other domestic animals are occasionally affected.

Swine erysipelas

Subclinically infected carrier pigs are the main reservoir of infection. Pigs with acute disease excrete large numbers of organisms in faeces. Organisms are also excreted in urine, saliva and nasal discharges. Infection is usually acquired through ingestion of contaminated food or water and less commonly through minor skin abrasions. In pigs kept outdoors, repeated faecal contamination of the soil occurs and may represent a source of infection.

The susceptibility of individual pigs and the virulence of the *E. rhusiopathiae* strain, both of which are highly variable, determine the course and outcome of infection. Pigs under 3 months of age are normally protected by maternally-derived antibodies while animals over 3 years of age usually have acquired a protective active immunity through exposure to strains of low virulence. Stress factors appear to be important in the occurrence of disease, with acute cases frequently observed following sudden changes in weather, transport or weaning.

Clinical signs

Swine erysipelas can occur in four forms. The septicaemic and cutaneous (‘diamond’) forms are acute while arthritis and vegetative endocarditis are chronic forms of the disease. Chronic arthritis has the most significant negative impact on productivity.

Septicaemia occurs after an incubation period of 2 to 3 days. During an outbreak of acute disease, some pigs may be found dead and others are febrile, depressed and walk with a stiff, stilted gait or remain recumbent. Mortality may be high in some outbreaks. Pregnant sows with the septicaemic form of disease may abort.

In the diamond-skin form, systemic signs are less severe and mortality rates are much lower than in animals with septicaemia. Pigs are febrile, and cutaneous lesions progress from small, light pink or purple, raised areas to more extensive and characteristic diamond-shaped erythematous plaques. Some of these lesions resolve within a week; others become necrotic and may slough.

Arthritis, which is commonly encountered in older pigs, can present as stiffness, lameness or reluctance to bear weight on affected limbs. Joint lesions,

which may be initially mild, can progress to erosion of articular cartilage with eventual fibrosis and ankylosis. *Erysipelothrix rhusiopathiae* is one of the most frequently detected causes of non-suppurative arthritis in pigs at slaughter (Hariharan *et al*., 1992; Buttenschon *et al*., 1995). In vegetative endocarditis, the least common form, wart-like thrombotic masses are present, usually on the mitral valves. Many affected animals are asymptomatic but some may develop congestive heart failure or die suddenly if stressed by physical exertion or by pregnancy.

Diagnosis

- Diamond-shaped skin lesions are pathognomonic.
- Specimens for laboratory examination include blood for haemoculture and post-mortem specimens of liver, spleen, heart valves or synovial tissues. Organisms are rarely recovered from skin lesions or chronically affected joints.
- Microscopic examination of specimens from acutely affected animals may reveal slender Gram-positive rods. Filamentous forms may be demonstrable in smears from chronic valvular lesions (Fig. 20.1).
- Blood agar and MacConkey agar plates inoculated with specimen material are incubated aerobically at 37°C for 24 to 48 hours. Selective media, containing either sodium azide (0.1%) or crystal violet (0.001%), may be used for contaminated samples.
- Identification criteria for isolates:
  - Colonial morphology after incubation for 48 hours
  - Absence of growth on MacConkey agar
  - Appearance in Gram-stained smears from colonies
  - Negative catalase test
  - Coagulase production
  - H_2 S production in TSI agar slants
  - Biochemical test profile.
- Serological tests are not applicable for diagnosis.
- Several PCR-based methods for the detection of *E. rhusiopathiae* in clinical specimens have been described. Shimoji *et al*. (1998) reported a PCR-based method for the detection of virulent *E. rhusiopathiae* isolates. This method utilized a selective enrichment broth for incubation of samples prior to the

PCR procedure, in order to improve sensitivity of detection. In addition, PCR methods have been used to investigate the occurrence of *Erysipelothrix* species in abattoir and meat samples (Wang *et al*., 2002).

Treatment

Both penicillin and tetracyclines are effective for treatment although resistance to tetracyclines has been reported in some countries (Yamamoto *et al*., 2001). When chronic lesions have developed, antibiotic therapy is ineffective.

Control

- Hygiene and management practices should be evaluated and, where necessary, brought to a satisfactory standard.
- Chronically affected animals should be culled.
- Affected pigs should be isolated.
- Both live attenuated and inactivated vaccines are available. Attenuated vaccines can be given orally, systemically or by aerosol. They should not be administered to animals receiving antibiotic therapy. However, use of attenuated vaccines may be associated with an increase in the chronic arthritic form of erysipelas. Imada *et al*. (2004) typed 800 *E. rhusiopathiae* strains isolated in Japan using randomly amplified polymorphic DNA (RAPD) analysis and concluded that 37% of the chronic cases of erysipelas detected over an 11-year period had occurred as a side effect of vaccine use.

Erysipelas in fowl

Erysipelas in turkeys is an important disease worldwide and birds of all ages are susceptible. Toms may excrete the organisms in their semen and turkey hens may die suddenly within 4 to 5 days of artificial insemination. The disease usually presents as a septicaemia and mortality rates may be high. Dark-coloured, swollen snoods are characteristic of the disease. Post-mortem findings include enlarged friable livers and spleens. Chronically affected birds may exhibit arthritis and vegetative endocarditis and they gradually lose weight and become emaciated. Vaccination with an inactivated vaccine stimulates protective immunity.

Erysipelas in chickens can cause outbreaks of severe disease occasionally, with mortality rates of up to 50% (Mazaheri *et al*., 2005). It is suggested that the red

poultry mite (*Dermanyssus gallinae*) may serve as a reservoir and vector for the organism as PFGE banding patterns of isolates recovered from poultry and mites were indistinguishable (Chirico *et al*., 2003; Eriksson *et al*., 2009).

Infections in sheep

Non-suppurative polyarthritis of lambs may result from entry of organisms through the navel or, more commonly, through docking or castration wounds. Post-dipping lameness, which affects older lambs and adult sheep, is due to cellulitis and laminitis. The organism enters through skin abrasions in the region of the hoof from heavily contaminated dipping solutions. Valvular endocarditis and pneumonia in ewes, associated with *E. rhusiopathiae*, have also been reported (Griffiths *et al*., 1991).

Human erysipeloid

Many human infections with *E. rhusiopathiae* are occupational in origin. Workers engaged in the fish and poultry industries and other agriculturally-based occupations may be at risk of acquiring infection. Organisms enter through minor skin abrasions causing a localized cellulitis referred to as erysipeloid (Mutalib *et al*., 1993). Rarely, extension by haematogenous spread in untreated patients can lead to joint and heart involvement.

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Chapter 21

Bacillus species

Most *Bacillus* species are large, Gram-positive, endospore-producing rods up to 10.0 µm in length. A few non-pathogenic species are Gram-negative, and organisms in smears prepared from old cultures decolorize readily. In smears from tissues or cultures, cells occur singly, in pairs or in long chains (Fig. 21.1). The genus is comprised of more than 200 species with diverse characteristics. *Bacillus* species are catalase-positive, aerobic or facultatively anaerobic and most species are motile although the major pathogen, *Bacillus anthracis*, is non-motile. Most species are saprophytes with no pathogenic potential. However, they often contaminate clinical specimens and laboratory media. *Bacillus anthracis* is the most important pathogen in the group. The name *Clostridium piliforme* has been proposed for *Bacillus piliformis*, the agent of Tyzzer's disease (Duncan *et al*., 1993). *Bacillus larvae*, a major pathogen of bees, has been reclassified as *Paenibacillus larvae* subsp. *larvae* (Genersch *et al*., 2006).

Usual habitat

Bacillus species are widely distributed in the environment, mainly because they produce highly resistant endospores. In soil, endospores of *B. anthracis* can survive for more than 50 years. Some *Bacillus* species can tolerate extremely adverse conditions such as desiccation, high temperatures and chemical disinfectants.

Key points

- Large, Gram-positive rods
- Endospores produced
- Aerobes or facultative anaerobes
- Growth on non-enriched media
- Most species motile, catalase-positive and oxidase-negative
- Majority are non-pathogenic environmental organisms
- *Bacillus anthracis* causes anthrax

- *Bacillus licheniformis* is implicated in sporadic abortions in cattle and sheep

Differentiation of *Bacillus* species

The ability to grow aerobically and to produce catalase distinguishes *Bacillus* species from the clostridia, which are also Gram-positive, endospore-forming rods. Phenotypic differentiation of *Bacillus* species is largely based on colonial characteristics and biochemical tests. Many species, including *B. anthracis*, do not produce capsules when grown on laboratory media. Taxonomic studies based on genome sequencing data show that *B. anthracis*, *B. cereus* and *B. thuringiensis* are closely related, and these organisms, in addition to three other species, make up what is informally referred to as the '*B. cereus group*' (Arnesen *et al*., 2008). The presence of specific virulence genes on plasmids and the mechanisms by which these virulence genes are regulated are the principal differentiating features of these species (Arnesen *et al*., 2008; Kolstø *et al*., 2009).

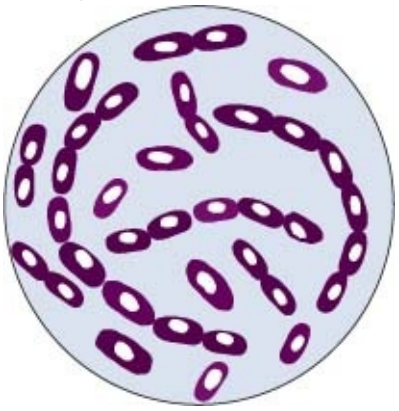


Figure 21.1 Rods of *Bacillus* species in chain formation. Endospores appear as unstained areas within the cells.

- Colonial characteristics of *Bacillus* species which are pathogenic for animals and humans:

  – *Bacillus cereus* colonies are similar to those of *B. anthracis* but are slightly larger with a greenish tinge. The majority of strains produce a wide zone of complete haemolysis around the colonies. Because they have some similar characteristics, *B. anthracis* and *B. cereus* require careful differentiation (Table 21.1).

  – *Bacillus anthracis* colonies are up to 5 mm in diameter, flat, dry, greyish

and with a ‘ground glass’ appearance after incubation for 48 hours. At low magnification, curled outgrowths from the edge of the colony impart a characteristic, ‘medusa head’ appearance. Rarely, isolates are weakly haemolytic.

– *Bacillus licheniformis* colonies are dull, rough, wrinkled and strongly adherent to the agar. Characteristic hair-like outgrowths are produced from streaks of the organisms on agar media. Colonies become brown with age. The name of this species derives from the similarity of its colonies to lichen.

- Commercial biochemical test kits for confirming the identity of *Bacillus* species are available.
- Confirmation of identity and virulence of *B. anthracis* can be made by detection of its two virulence plasmids using PCR-based techniques (Anon., 2008).
- All *B. anthracis* isolates fall into a single clonal group but high resolution molecular typing techniques such as multilocus variable number of tandem repeats analysis (MLVA) can differentiate three phylogenetic branches A, B and C (Kolstø *et al*., 2009). These lineages differ in geographical distribution, although lineage A is widely dispersed throughout the world (Simonson *et al*., 2009).

Table 21.1 Differentiating features of *Bacillus anthracis* and *B. cereus*.

Feature	***B. anthracis***	***B. cereus***
Motility	Non-motile	Motile
Appearance on sheep blood agar	Non-haemolytic	Haemolytic
Susceptibility to penicillin (10-unit disc)	Susceptible	Resistant
Lecithinase activity on egg yolk agar	Weak and slow	Strong and rapid
Effect of gamma phage	Lysis	Lysis rare
Pathogenicity for laboratory animals (application to scarified area at tail base of mouse)	Death in 24 to 48 hours	No effect

Clinical infections

The major disease conditions caused by bacteria in this group are listed in Table 21.2. Anthrax is the most important of these diseases. *Bacillus licheniformis* is an emerging pathogen as a cause of abortion in cattle and sheep. *Bacillus cereus* is important in human food poisoning and may cause an emetic or diarrhoeal syndrome. It is occasionally associated with cases of mastitis in cows.

Infections with *Bacillus licheniformis*

Bacillus licheniformis, an organism widespread in the environment and associated with food spoilage, has recently been recognized as a cause of abortion in cattle and sheep. On some farms in Britain, multiple bovine abortions have been attributed to infection with *B. licheniformis*. In 2006 in the UK, a diagnosis of *B. licheniformis* infection was made in 21% of abortion cases in which a diagnosis was reached (Cabell, 2007). An association with the feeding of silage or mouldy hay has been suggested. Because this organism is ubiquitous, it is only of diagnostic significance when isolated in heavy, pure culture from foetal abomasal contents.

Table 21.2 Clinical manifestations of diseases caused by *Bacillus anthracis* and other *Bacillus* species.

***Bacillus* species**	**Susceptible animals**	**Clinical manifestations**
B. anthracis	Cattle, sheep	Fatal peracute or acute septicaemic anthrax
	Pigs	Subacute anthrax with oedematous swelling in pharyngeal region; an intestinal form with higher mortality is less common
	Horses	Subacute anthrax with localized oedema; septicaemia with colic and enteritis sometimes occurs
	Humans	Skin, pulmonary and intestinal forms of anthrax are recorded in humans periodically
B. cereus	Cattle	Mastitis (rare)
	Humans	Food poisoning, eye infections
B. licheniformis	Cattle, sheep	Sporadic abortion

Anthrax

Anthrax is a severe disease that affects virtually all mammalian species including humans. The disease, which occurs worldwide, is endemic in some countries and in defined regions of other countries. Ruminants are highly susceptible, often developing a rapidly fatal septicaemic form of the disease. Pigs and horses are moderately susceptible to infection, while carnivores are comparatively resistant. Birds are almost totally resistant to infection, a characteristic attributed to their relatively high body temperatures.

Epidemiology

Endospore formation is the most important factor in the persistence and spread of anthrax and is a response to nutrient depletion and other adverse environmental conditions. The endospores of *B. anthracis* can survive for

decades in soil. Spores may become concentrated in some geographically defined regions where soil conditions favour spore survival. Soils in such regions are alkaline and rich in calcium and nitrogen, and have a high moisture content. In addition, repeated cycles of flooding and evaporation may concentrate spores in particular low-lying locations (Dragon and Rennie, 1995). The increased survival of spores in alkaline calcium-rich soils is because of the important role of calcium in the core of the spore. Calcium, in combination with dipicolinic acid, forms a lattice that stabilizes the DNA and enzymes in the core and ensures spore survival. Leaching of calcium from the spore, which may occur in calcium-poor environments, impairs spore survival. Outbreaks of anthrax in herbivores can occur when pastures are contaminated by spores originating from buried carcasses. Spores may be brought to the surface by flooding, excavation, subsidence, or the activity of earthworms.

Sporadic outbreaks of the disease have been associated with the importation of contaminated meat-and-bone meal, fertilizers of animal origin, and hides. Infection is usually acquired by ingestion of spores and, less commonly, by inhalation or through skin abrasions. Although carnivores are comparatively resistant to infection, the ingestion of large numbers of *B. anthracis* from an anthrax carcass can produce disease.

Pathogenesis and pathogenicity

The virulence of *B. anthracis* derives from the presence of a capsule and the ability to produce a complex toxin. Both virulence factors are encoded by plasmids and are required for disease production. The plasmid PXO1 encodes the three components which form two exotoxins; the genes that regulate their expression are also found on the plasmid. The genes encoding capsule production and their regulators are found on plasmid PXO2. The expression of virulence factors is regulated by a number of variables including host temperature and carbon dioxide concentration. The capsule, composed of poly-γ-D-glutamic acid, inhibits phagocytosis. The complex toxin consists of three antigenic components: protective antigen, oedema factor and lethal factor. Individually each factor lacks toxic activity in experimental animals, although protective antigen induces antibodies which confer partial immunity. Protective antigen acts as the binding moiety for both oedema factor and lethal factor. Oedema factor is a calmodulin-dependent adenylate cyclase, and once it has entered cells following binding to protective antigen, it causes increased levels of cyclic AMP. The resultant disturbance of water homeostasis causes the fluid

accumulation seen in clinical disease. Neutrophils are the principal target of oedema factor which severely inhibits their function. Lethal toxin consists of lethal factor, a zinc metalloprotease and protective antigen which acts as the binding domain as for oedema factor. It causes the death of macrophages and other cells including dendritic cells, neutrophils and some epithelial and endothelial cells. In naturally occurring disease, local effects of the complex toxin include swelling and darkening of tissues due to oedema and necrosis. When septicaemia occurs, increased vascular permeability and extensive haemorrhage lead to shock and rapid death.

Clinical signs and pathology

The incubation period of anthrax ranges from hours to days. The clinical presentation and pathological changes vary with the species affected, the challenge dose and the route of infection.

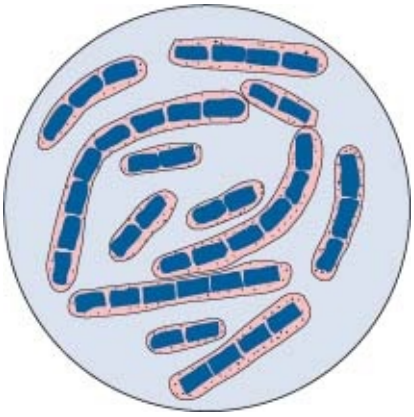
In cattle and sheep, the disease is usually septicaemic and rapidly fatal. Although most animals are found dead without premonitory signs, pyrexia with temperatures up to 42°C (108°F), depression, congested mucosae and petechiae may be observed ante mortem. Animals that survive for more than a day may abort or display subcutaneous oedema and dysentery. In cattle, post-mortem findings include rapid bloating, incomplete rigor mortis, widespread ecchymotic haemorrhages and oedema, dark, unclotted blood and blood-stained fluid in body cavities. An extremely large soft spleen is characteristic of the disease in cattle. Splenomegaly and oedema are less prominent post-mortem features in affected sheep, which are reported to be more susceptible than cattle and succumb more rapidly.

In pigs, infection generally results in oedematous swelling of the throat and head along with regional lymphadenitis. If oedema in the laryngeal region does not interfere with breathing, affected pigs may survive. Intestinal involvement manifests clinically as dysentery due to multifocal, haemorrhagic enteric lesions. Mortality rates may be high.

The clinical course of anthrax in horses is often prolonged for several days. Following introduction of spores into abrasions, extensive subcutaneous oedema of the thorax, abdomen or legs may develop. Swelling of the pharynx, similar to that in pigs, has been described. Less commonly, colic and dysentery due to severe haemorrhagic enteritis may result from ingestion of spores. If septicaemia occurs, extensive ecchymoses and splenomegaly are found post mortem.

In dogs, which are rarely affected, the course of the disease and pathological changes resemble those observed in affected pigs.

Figure 21.2 Numerous chains of *Bacillus anthracis* as they appear in a thin blood smear. When stained with polychrome methylene blue, the blue-staining organisms are surrounded by pink capsules (M'Fadyean reaction).



Diagnosis

- Carcasses of animals that have died from anthrax are bloated, putrefy rapidly and do not exhibit rigor mortis. Dark, unclotted blood may issue from the mouth, nostrils and anus. The carcasses of such animals should not be opened because this will facilitate sporulation, with the risk of long-term environmental contamination.
- Peripheral blood from the tail vein of ruminants or peritoneal fluid from pigs should be collected into a sterile syringe. Cotton wool soaked in 70% alcohol should be applied to the site after collection to minimize leakage of contaminated blood or fluid. Thin smears of blood or fluid, stained with polychrome methylene blue, reveal chains of square-ended, blue-staining rods surrounded by pink capsules (Fig. 21.2). The amount of capsular material diminishes with time after the death of the animal.
- Culture and isolation are regarded as the gold standard for diagnosis of disease. Blood agar and MacConkey agar are inoculated with the suspect specimens and incubated aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates:
  – Colonial morphology
  – Microscopic appearance in a Gram-stained smear
  – Absence of growth on MacConkey agar
  – Cultural features and, if necessary, pathogenicity tests in laboratory

animals (Table 21.1)

– PCR-based tests can be used to confirm the virulence of isolates by demonstration of the two virulence-associated plasmids, PXO1 and PXO2 (Anon., 2008)

– Biochemical test profile.

- PCR may also be used for the direct detection of the organism in clinical samples and is particularly useful for detection of *B. anthracis* DNA in deteriorating or stored samples (Berg *et al*., 2006).
- The Ascoli test is a thermoprecipitation test designed to detect antigens of *B. anthracis* in biological materials such as hides. Homogenized material is boiled and clarified by filtration. The filtrate is used as the source of antigen in ring precipitation or gel diffusion tests with *B. anthracis* antiserum. This test lacks specificity because B. *anthracis* shares thermostable antigens with other *Bacillus* species.
- Agar gel immunodiffusion, complement fixation, ELISA and immunofluorescence tests have been evaluated for the diagnosis of anthrax, but they are either too insensitive or lack the required specificity for routine use.

Treatment

If administered early in the course of the disease, high doses of penicillin G or oxytetracycline may prove effective. Although inducible β-lactamase production has been demonstrated in *B. anthracis* isolates, naturally occurring penicillin resistance in clinical isolates appears to be rare (Shadomy and Smith, 2008).

Control

Suspected cases of anthrax must be reported immediately to appropriate regulatory authorities. Control measures should be designed to take account of the prevalence of disease in a particular country or geographical region.

- In endemic regions:

  – Annual vaccination, particularly of cattle and sheep, is advisable. The Sterne strain spore vaccine should be given about 1 month before anticipated outbreaks. The spores in this live vaccine convert to non-encapsulated avirulent vegetative organisms.

  – Chemoprophylaxis, employing long-acting penicillin, should be considered when outbreaks threaten valuable livestock.

– Killed vaccines, which contain protective antigen as the major protective component, are available for humans who may be exposed to infection in the course of their work.

- In non-endemic regions following a disease outbreak:

  – Movement of animals, their waste products, feed and bedding from affected and adjacent premises must be prohibited.

  – Personnel implementing control measures should wear protective clothing and footwear which must be disinfected before leaving the affected farm.

  – Foot-baths containing sporicidal disinfectant (5% formalin, or 3% peracetic acid) should be placed at entrances to affected farms.

  – Contaminated buildings should be sealed and fumigated with formaldehyde before bedding is removed. Following removal of bedding and loose fittings, all drains should be blocked and the building should be sprayed with 5% formalin which should be left to act for at least 10 hours before final washing.

  – Immediate disposal of carcasses, bedding, manure, fodder and other contaminated material is mandatory. Carcasses should be incinerated or buried deeply away from water courses. Contaminated material and equipment must be disinfected with 10% formalin or, if appropriate, incinerated.

  – Scavenger animals should not be allowed access to suspect carcasses and insect activity should be minimized by application of insecticides on and around carcasses.

  – In-contact animals should be isolated and kept under close observation for at least 2 weeks.

Anthrax in humans

Anthrax in humans has been known from ancient times and was thought to be one of the plagues of Egypt. Public awareness of the disease has increased in recent years since its use in bioterrorist attacks in the USA in 2001 (Jernigan *et al*., 2001). Three main forms of the disease occur in humans. Cutaneous anthrax (malignant pustule) is the result of endospores entering abraded skin. This localized lesion can progress to septicaemia if not treated. Pulmonary anthrax (woolsorters' disease) follows inhalation of spores, and intestinal anthrax results from ingestion of infective material. The disease may prove fatal in the absence

of early treatment. The use of *B. anthracis* as an agent of bioterrorism has greatly increased the amount of research conducted on this pathogen, in particular, research into diagnostic techniques and vaccination. Approaches for improving currently available vaccines include investigation of the use of adjuvants and alternative delivery systems. Recombinant vaccines using bacterial and viral vectors have been developed and some products showed considerable promise (Friedlander and Little, 2009).

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Chapter 22

Clostridium species

The clostridia are large, Gram-positive bacteria which are fermentative, catalase-negative and oxidase-negative, and require enriched media for growth. They are straight or slightly curved rods and the majority are motile by flagella which are peritrichous. *Clostridium* species produce endospores which usually cause bulging of mother cells (Fig. 22.1). The size, shape and location of endospores can be used for species differentiation. Although most pathogenic clostridial species are strict anaerobes, some are comparatively aerotolerant. Clostridia occur worldwide and particular species may be associated with defined geographical regions.

Although more than 100 clostridial species are recognized, less than 20 are pathogenic. These pathogenic species can be grouped in three categories, based on toxin activity and tissues affected (Fig. 22.2). *Clostridium tetani* and *C. botulinum*, the neurotoxic clostridia, affect neuromuscular function without inducing observable tissue damage. In contrast, histotoxic clostridia produce relatively localized lesions in tissues such as muscle and liver and may subsequently cause toxaemia. *Clostridium perfringens* types A to E, important members of the third category, produce inflammatory lesions in the gastrointestinal tract along with enterotoxaemia. *Clostridium difficile* is an emerging enteric pathogen of animals and is a significant nosocomial pathogen of humans. *Clostridium spiro-forme* causes diarrhoea in rabbits and *C. colinum* is an intestinal pathogen of fowl. *Clostridium piliforme*, which causes hepatic necrosis in foals, is an atypical member of the clostridia and does not have characteristics that permit its inclusion in any of the three categories described.

Key points

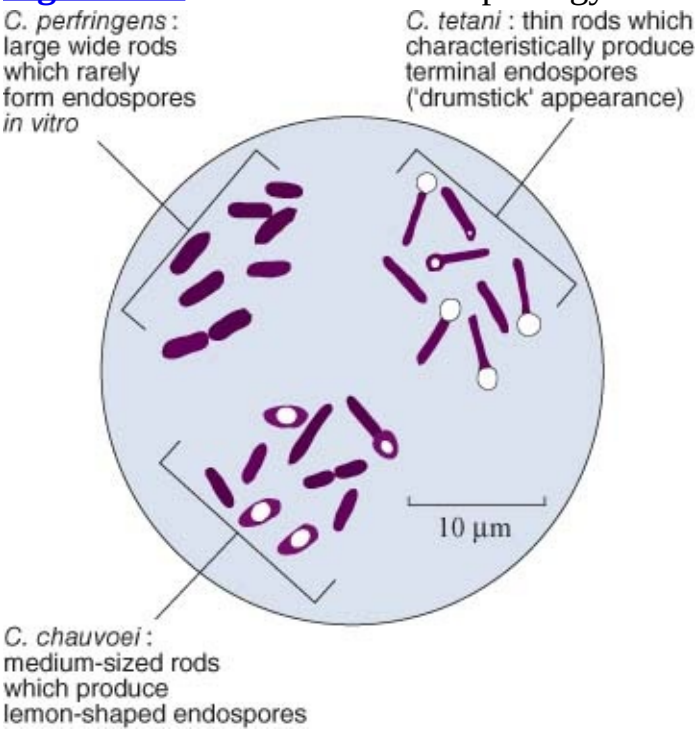
- Large, Gram-positive rods
- Endospores produced
- Anaerobic, catalase-negative and oxidase-negative
- Motile (except *C. perfringens*)
- Enriched media required for growth

- Colonies of *C. perfringens* surrounded by zones of double haemolysis
- Present in soil, in alimentary tracts of animals and in faeces
- Pathogens can be grouped according to the mode and sites of action of their potent exotoxins:
  - – neurotoxic clostridia
  - – histotoxic clostridia
  - – enteropathogenic and enterotoxaemia-producing clostridia
- Produce diverse forms of disease in many animal species

Usual habitat

Clostridia are saprophytes which are found in soil, freshwater or marine sediments with suitably low redox potentials. They constitute part of the normal intestinal flora and some may be sequestered as endospores in muscle or liver. Sequestered endospores, if activated, may produce disease.

Figure 22.1 Characteristic morphology of some clostridial species.



Specimen collection and cultural requirements

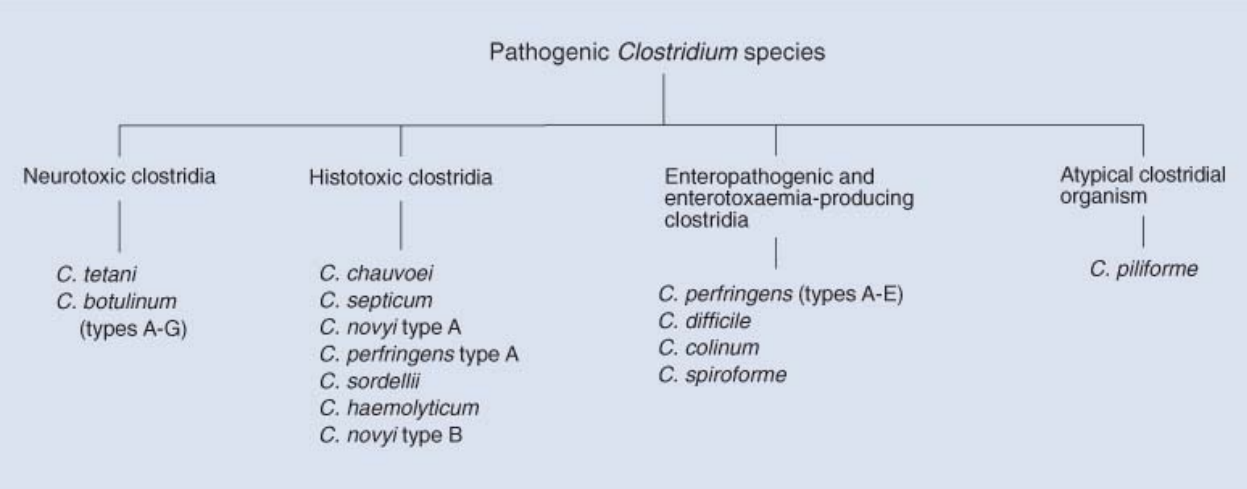
To ensure survival of these fastidious anaerobes, special methods are required for collection and processing of specimens.

- Unless specimens are taken from live or recently dead animals, post-mortem clostridial invaders may spread rapidly from the intestine into tissues, leading to difficulty with the interpretation of laboratory results.
- Blocks of tissue or fluids from affected animals should be placed in anaerobic transport media for transfer to the laboratory. Specimens must be cultured promptly after collection.
- Blood agar enriched with yeast extract, vitamin K and haemin is suitable for the culture of clostridia. Media should be freshly prepared or pre-reduced to ensure absence of oxygen.
- Suitable atmospheric requirements are provided by culturing in anaerobic jars containing hydrogen supplemented with 5 to 10% carbon dioxide to enhance growth. Some vegetative clostridia may not survive exposure to the air for more than 15 minutes.

Detection and differentiation of clostridia

Apart from the use of cultural techniques, clostridia can be demonstrated in clinical specimens by molecular and other methods. PCR-based techniques have been developed for demonstration of histotoxic clostridia in tissues (Sasaki *et al*., 2001; Uzal *et al*., 2003) and fluorescent antibody techniques are also widely used for the rapid identification of these organisms in tissues.

Figure 22.2 Pathogenic *Clostridium* species of veterinary importance.



Laboratory procedures for differentiating most clostridia include colonial

morphology, biochemical tests, toxin neutralization methods and gas–liquid chromatography for identification of organic acids.

- Colonial morphology is of limited value for differentiating most clostridial species. However, colonies of *C. perfringens* are surrounded by a characteristic double-zone of haemolysis.
- Miniaturized commercial kits are available for biochemical identification.
- PCR techniques can be used for identification of clostridial isolates and multiplex PCR tests have been developed for differentiation of a number of different species. Sasaki *et al*. (2002) found a multiplex PCR system, based on the flagellin gene, useful for the differentiation of *C. chauvoei, C. haemolyticum, C. novyi* types A and B, and *C. septicum*.
- Specific toxins can be identified in body fluids or in intestinal contents by toxin neutralization or protection tests in laboratory rodents, usually mice.
- Immunoassay methods such as ELISA can be used for toxin detection. The polymerase chain reaction can be employed for the detection of genes coding for toxin production in isolates of *C. botulinum*. These tests have now replaced many of the mouse bioassay tests but are not yet sufficiently sensitive and specific to replace animal tests in all circumstances.

Clinical conditions caused by neurotoxic clostridia

The neurotoxic clostridia, *C. tetani* and *C. botulinum*, produce their effects by elaborating potent neurotoxins. The neurotoxin of *C. tetani* is produced by organisms replicating locally in damaged tissues. Absorbed toxin exerts its effect on synaptic junctions remote from the site of toxin production. The neurotoxin of *C. botulinum* is usually produced by organisms replicating in decaying organic matter or in the anaerobic conditions in contaminated cans of meat or vegetables. When absorbed from the gastrointestinal tract into the bloodstream, the toxin affects the functioning of neuromuscular junctions. Some features of the neurotoxins of *C. tetani* and *C. botulinum* are presented in Table 22.1. The toxins of both *C. tetani* and *C. botulinum* are similar in structure and function. The differing clinical signs observed in each disease can be explained by the differing sites of action of the toxins produced by each organism.

Tetanus

Tetanus is an acute, potentially fatal intoxication which affects many species including humans. However, species susceptibility to toxin varies considerably. Horses and humans are highly susceptible, ruminants and pigs moderately so and carnivores are comparatively resistant. Poultry are not susceptible to tetanus.

Clostridium tetani, the aetiological agent, is a straight, slender, anaerobic, Gram-positive rod. Spherical endospores, which are terminal and bulge mother cells, impart a characteristic ‘drumstick’ appearance to sporulated organisms (Fig. 22.1). The endospores are resistant to chemicals and boiling but are killed by autoclaving at 121°C for 15 minutes. *Clostridium tetani* has a swarming growth and is haemolytic on blood agar due to the production of tetanolysin. Ten sero-logical types of *C. tetani* can be distinguished by their flagellar antigens. The neurotoxin, tetanospasmin, is antigenically uniform irrespective of serotype, and antibodies induced by the neurotoxin of any one of the serotypes neutralize the neurotoxins produced by the others.

Table 22.1 Production, mode of action and effects of the neurotoxins of *Clostridium tetani* and *C. botulinum*.

Feature of neurotoxin	***Clostridium tetani***	***Clostridium botulinum***
Site of production	In wounds	In carcasses, decaying vegetation, canned foods. Occasionally in wounds or in intestine (toxico-infections)
Genes which regulate production	In plasmids	In chromosome, plasmids or bacteriophages (depending on type)
Antigenic type	One antigenic type (tetanospasmin)	Seven antigenically distinct toxins, types A to G
Mode of action	Synaptic inhibition	Inhibition of neuromuscular transmission
Clinical effect	Muscular spasms	Flaccid paralysis

Infection occurs when endospores are introduced into traumatized tissue from soil or faeces. Common sites of infection include deep penetrating wounds in the horse, castration and docking wounds in sheep, abrasions associated with dystocia in cows and ewes, and the umbilical tissues in all young animals. The presence of necrotic tissue, foreign bodies and contaminating facultative anaerobes in wounds may create the anaerobic conditions in which *C. tetani* spores can germinate. The clostridial organisms may replicate more readily in the tissues when the haemolytic toxin, tetanolysin, is released. Vegetative bacteria multiplying in necrotic tissues produce the potent tetanospasmin which is responsible for the clinical signs of tetanus.

Pathogenesis

Structurally, tetanus toxin consists of two chains joined by a disulphide bridge. The light chain is the toxic moiety and the heavy chain is responsible for receptor binding and internalization of the toxin. The neurotoxin binds irreversibly to ganglioside receptors on motor neuron terminals and is transported to the nerve cell body and its dendritic processes in the central nervous system in toxin-containing vesicles, by retrograde intra-axonal flow. Toxin is transferred trans-synaptically to its site of action in the terminals of inhibitory neurons and enters these cells by endocytosis. The low pH of the endosome induces a conformational change in the neurotoxin which allows it to penetrate the endosome membrane and form a pore (Cai and Singh, 2007). Following this change, the light chain, a zinc endopeptidase, enters the cytosol of the inhibitory neuron where it blocks presynaptic transmission of inhibitory signals by hydrolysis of synaptobrevins, protein components of vesicles containing neurotransmitters (Sanford, 1995). As a consequence of this, the vesicles can no longer dock on to the plasma membrane to release their contents. Because release of inhibitory neurotransmitters is prevented, spastic paralysis results. Toxin can also be blood-borne, especially when produced in large amounts, and can then bind to motor terminals throughout the body prior to transfer to the central nervous system. Bound toxin is not neutralized by antitoxin.

Clinical signs

The incubation period of tetanus is usually between 5 and 10 days but may extend to 3 weeks. When the development of clinical signs is delayed, the wound at the site of infection may have healed and the condition is then referred to as latent tetanus. The clinical effects of the neurotoxin are similar in all domestic animals. However, the nature and severity of the clinical signs are dependent on the anatomical site of the replicating bacteria, the amount of toxin produced and species susceptibility. Wounds on or near the head are usually associated with a shorter incubation period and an increased tendency to generalized tetanus. Localized tetanus, which usually affects less susceptible species such as dogs, presents as stiffness and spasm of muscles close to the site of injury as a result of the effect of toxin on local nerve endings.

Clinical signs include stiffness, localized spasms, altered heart and respiratory rates, dysphagia and altered facial expression. Comparatively mild tactile or auditory stimuli may precipitate tonic contraction of muscles. Spasm of masticatory muscles may lead to ‘lockjaw’. Generalized muscle stiffness can

result in a ‘saw-horse’ stance especially in horses. Animals that recover from tetanus are not necessarily immune because the amount of toxin that can induce clinical disease is usually below the threshold required to stimulate the production of neutralizing antibodies.

Diagnostic procedures

The diagnosis of tetanus is usually presumptive and is based on the clinical signs and a history of recent trauma in unvaccinated animals.

- Differentiation from strychnine poisoning is necessary, particularly in dogs. Gram-stained smears of material from lesions may reveal the characteristic ‘drumstick’ forms of *C. tetani* (Fig. 22.1).
- Anaerobic culture of *C. tetani* from necrotic wound tissue may be attempted but is often unsuccessful.
- PCR-based techniques, including real-time PCR, for the detection of the neurotoxin genes can be used to assist in diagnosis of disease (Akbulut *et al*., 2005).
- Serum from affected animals may be used to demonstrate circulating neurotoxin, using mouse inoculation.

Treatment

- To neutralize unbound toxin, antitoxin should be administered promptly, either intravenously or into the subarachnoid space on three consecutive days.
- Toxoid may be given subcutaneously to promote an active immune response even in those animals that have received antitoxin.
- Large doses of penicillin are administered intramuscularly or intravenously to kill toxin-producing vegetative cells of *C. tetani* in the lesion.
- Surgical debridement of wounds and removal of foreign bodies, followed by flushing with hydrogen peroxide, produces aerobic conditions which help to inhibit bacterial replication at the site of injury. This aspect of treatment is extremely important as studies in humans showed that, without debridement, it was possible to isolate *C. tetani* from patients after 16 days of treatment with intravenous penicillin (Campbell et al., 2009).
- Affected animals should be housed in a quiet dark environment. Fluid replacement therapy, sedatives, muscle relaxants and good nursing can minimize clinical discomfort and maintain vital functions.

Control

- Farm animals should be vaccinated routinely with tetanus toxoid. A booster dose of vaccine may be advisable if a vaccinated animal sustains a deep wound.
- In horses, prompt surgical debridement of wounds is desirable.
- Unvaccinated animals that have sustained deep wounds or are presented for surgery should be given antitoxin. This passive protection usually lasts about 3 weeks.

Botulism

Botulism is a serious, potentially fatal intoxication usually acquired by ingestion of pre-formed toxin. *Clostridium botulinum*, the aetiological agent, is an anaerobic Gram-positive rod which produces oval, subterminal endospores. The endospores of *C. botulinum* are distributed in soils and aquatic environments worldwide. Seven types of *Clostridium botulinum* are recognized, based on the antigenic properties of the toxins (A, B, C, D, E, F, G) which they produce. These neurotoxins, which are inactivated by boiling for up to 20 minutes, induce similar clinical signs but differ in their antigenicity and potency. Some *C. botulinum* types are confined to particular geographical regions. Germination of endospores, with growth of vegetative cells and toxin production, occurs in anaerobic locations such as rotting carcasses, decaying vegetation and contaminated canned foods. Toxico-infectious botulism, an uncommon form of disease in animals, occurs when spores germinate in wounds or in the intestinal tract. Intestinal toxico-infectious botulism has been recorded in foals (shaker-foal syndrome), pups (Farrow *et al*., 1983), broiler chickens and turkey poults. This form of the disease is of increasing importance in illicit drug users following contamination of needles. Spores of *C. botulinum* which are inadvertently introduced during subcutaneous injection of drugs germinate and produce toxin. In addition, thereis circumstantial evidence that equine grass sickness, a dysautonomia, may be due to toxico-infection with *C. botulinum* types C and D (McCarthy *et al*., 2004a,b).

Clostridium botulinum types C and D cause most outbreaks of botulism in domestic animals. Outbreaks of disease occur most commonly in waterfowl, cattle, horses, sheep, mink, poultry and farmed fish. Pigs and dogs are relatively resistant to the neurotoxins, and botulism is rare in domestic cats. Botulism in

cattle has been associated with ingestion of poultry carcasses present in ensiled poultry litter used as bedding or spread on pasture (McLoughlin *et al*., 1988). Poor-quality baled silage and silage or hay containing rodent carcasses have been linked to outbreaks of botulism in horses and ruminants. Pica, arising from starvation or phosphorus deficiency in herbivores on ranches in South Africa, USA and Australia, may induce affected animals to chew bones or carcasses containing botulinum toxin. The resultant botulism is known as lamsiekte in South Africa, bulbar paralysis in Australia and loin disease in the USA. Contaminated raw meat and carcasses are often sources of toxin for carnivores. Waterfowl and other birds can acquire toxin from dead invertebrates and decaying vegetation or from the consumption of maggots containing toxin (Hariharan and Mitchell, 1977; Quinn and Crinion, 1984). The usual sources of the toxins of C. botulinum types A–G for susceptible species are summarized in Table 22.2.

Pathogenesis

The neurotoxins of *C. botulinum* are the most potent biological toxins known. One minimum lethal dose, which is sufficient to kill a mouse, is equivalent to 10 pg of toxin for botulinum toxin type A (AOAC International, 2001). Preformed toxin in food, absorbed from the gastrointestinal tract, circulates in the bloodstream and acts at the neuromuscular junctions of cholinergic nerves and at peripheral autonomic synapses. Its structure and mode of action are similar to those of tetanus toxin. The heavy chain mediates binding to receptors on the nerve endings. The light chain or toxic moiety enters the cytosol of the cell following endocytosis and pore formation. As with tetanus toxin, hydrolysis of synaptobrevins and other so-called SNARE proteins causes irreversible interference with the release of the neurotransmitter, acetylcholine in this instance, resulting in flaccid paralysis. Death results from paralysis of respiratory muscles. The difference between the effects of tetanus and botulinum toxins is due to their different sites of action. Tetanus toxin travels up the nerve axon to the ventral horn whereas botulinum toxin remains at the neuromuscular junction.

Table 22.2 Toxins of *Clostridium botulinum*.

Toxin	Source	Susceptible species
Type A	Meat, canned products	Humans
	Toxico-infection	Infants
	Meat, carcasses, soil	Mink, dogs, pigs, rarely cattle

Type B	Meat, canned products	Humans
	Toxico-infection	Infants
	Toxico-infection, forage	Foals (up to 2 months of age), adult horses, cattle
Type C	Dead invertebrates, maggots, rotting vegetation and carcasses of poultry	Waterfowl, poultry
	Ensiled poultry litter, baled silage (poor quality), hay or silage contaminated with rodent carcasses	Cattle, sheep, horses
	Meat, especially chicken carcasses	Dogs, mink, lions, monkeys
Type D	Carcasses, bones	Cattle, sheep
	Feed contaminated with carcasses	Horses
Type E	Dead invertebrates, sludge in earth-bottomed ponds	Farmed fish
	Fish	Fish-eating birds, humans
Type F	Meat, fish	Humans
Type G	Soil-contaminated food	Humans (in Argentina)

Ingested spores of *C. botulinum* are normally excreted in the faeces. In toxico-infectious botulism, however, germination of spores in the intestine results in toxin production by the vegetative organisms. The factors that predispose to toxico-infectious botulism are not known. The shaker-foal syndrome, a form of toxico-infectious botulism in foals up to 2 months of age, has been attributed to the impact of stress on the dam leading to increased corticosteroid levels in the milk (Swerczek, 1980).

Clinical signs

The clinical signs of botulism, which develop 3 to 17 days after ingestion of toxin, are similar in all species. Dilated pupils, dry mucous membranes, decreased salivation, tongue flaccidity and dysphagia are features of the disease in farm animals. Incoordination and knuckling of the fetlocks is followed by flaccid paralysis and recumbency. Paralysis of respiratory muscles leads to abdominal breathing. Body temperature remains normal and affected animals are alert. Death may occur within days of the emergence of clinical signs. In birds, there is progressive flaccid paralysis which initially affects legs and wings. Paralysis of muscles of the neck (limberneck) is evident only in long-necked species.

Diagnostic procedures

Suspect carcasses and material should be handled with caution as large amounts

of potent neurotoxin may be present.

- Clinical signs and a history of access to contaminated food may suggest botulism as the cause of an outbreak of an ill-defined neurological disease.
- Confirmation requires the demonstration of toxin in the serum of affected animals. The traditional method for demonstrating toxin is by mouse inoculation. Injected mice develop a characteristic 'wasp-waist' appearance, a consequence of abdominal breathing following paralysis of respiratory muscles. Serum collected from dead animals is unsuitable for mouse inoculation. Mouse bioassay is still the only accepted method of confirming botulism because of its superior sensitivity although the procedure is expensive and takes up to 4 days (Cai and Singh, 2007). However, probably because of their extreme susceptibility, botulinum toxin is rarely detected in the serum of affected cattle (Hogg *et al*., 2008). Gastrointestinal contents collected immediately after death and frozen to prevent post-mortem proliferation of any *C. botulinum* organisms may be a better sample in which to demonstrate toxin in cattle.
- The polymerase chain reaction and nucleic acid probe-based methods have been used for the detection of *C. botulinum* toxin genes. However, detection of genes does not prove production of biologically active toxin and is thus an aid to diagnosis only.
- Immunological methods using ELISA or chemiluminescent assays are sensitive and specific procedures for toxin detection and have the advantages of speed and the possibility of automation. Despite the development of different amplification methods for signal detection, the sensitivity of these tests does not equal that of the mouse bioassay method. Existing and possible future methods for the diagnosis of botulism are reviewed by Cai and Singh (2007).
- Toxin neutralization tests in mice, using monovalent antitoxins, can be employed to identify the specific toxin involved if required. Sera should be collected from a number of affected animals because failure to demonstrate toxin in individual animals does not exclude botulism.
- Identification of the toxin in feedstuffs may be of value in epidemiological studies.
- Typing of *C. botulinum* isolates can be carried out using a number of molecular typing techniques. These techniques, which are usually carried out during epidemiological studies, include multilocus variable-number

tandem repeat analysis (Macdonald *et al*., 2008) and random amplified polymorphic DNA analysis (Hyytiä *et al*., 1999).

Treatment

- If available, polyvalent antiserum is effective in neutralizing unbound toxin early in the course of disease. Cost and availability limit the extent to which it can be used therapeutically.
- Therapeutic agents such as tetraethylamide and guanidine hydrochloride, which enhance transmitter release at neuromuscular junctions, may be of value when given intravenously.
- Mildly affected animals often recover over a period of weeks without therapy.
- Good nursing should complement the therapeutic regime.
- As the cellular pathogenesis of botulism is further clarified, particularly the binding mechanism of the toxin, it may be possible to develop novel effective antidotes in the future.

Control

- Vaccination of cattle with toxoid may be indicated in endemic regions in South Africa and Australia. Routine vaccination of farmed mink and foxes may be advisable.
- Suspect foodstuffs should not be fed to domestic animals.
- Where feasible, provision of a balanced diet prevents pica in herbivores when grazing on ranges during periods of drought.

Clinical conditions caused by histotoxic clostridia

Histotoxic clostridia produce a variety of lesions in domestic animals (Table 22.3). The exotoxins elaborated by replicating bacteria induce both local tissue necrosis and systemic effects which may be lethal. Some histotoxic clostridia are present in the tissues as latent spores which can germinate and produce specific clinical diseases. These include *C. chauvoei* and occasionally *C. septicum* in muscle tissue, and *C. novyi* type B and *C. haemolyticum* in the liver. Histotoxic clostridia introduced into wounds, often as mixed infections, can cause

malignant oedema and gas gangrene. The clostridial species involved include *C. chauvoei*, *C. septicum*, *C. novyi* type A, *C. perfringens* type A and, occasionally, *C. sordellii*. The abomasitis caused by *C. septicum* in sheep (braxy) is an example of a local histotoxic effect.

Usual habitats

Endospores of histotoxic clostridia are widely distributed in the environment and can persist for long periods in soil. The endospores of particular clostridial species are often found in certain localities and in well defined geographical regions.

Pathogenesis

It is probable that the majority of ingested endospores are excreted in the faeces but some may leave the intestine and become distributed in the tissues where they remain dormant. Activation of dormant spores in muscle or liver results in endogenous infections which include blackleg, infectious necrotic hepatitis and bacillary haemoglobinuria. The sequence of events which lead to endospore distribution in tissues is unclear. Spores originating in the intestinal lumen may be transported to the tissues in phagocytes. Tissue injury leading to reduced oxygen tension is required for spore germination and replication of vegetative bacteria. Local necrosis produced by the exotoxins of the replicating bacteria allows further proliferation of the organisms in the tissues with extension of the necrotizing process.

Table 22.3 Histotoxic clostridia and the diseases they produce in domestic animals. Identified toxins, many of which contribute to disease production, are listed.

Clostridium species	Disease	Toxin	
		Name	Biological activity
C. chauvoei	Blackleg in cattle and sheep	α	Oxygen-stable haemolysin, lethal, necrotizing
		β	Deoxyribonuclease
		γ	Hyaluronidase
		δ	Oxygen-labile haemolysin
C. septicum	Malignant oedema in cattle, pigs and sheep. Abomasitis in sheep (braxy) and occasionally in calves	α	Lethal, haemolytic, necrotizing
		β	Deoxyribonuclease, leukocidin
		γ	Hyaluronidase
		δ	Oxygen-labile haemolysin
C. novyi type A	'Big head' in young rams. Wound infections	α	Cytotoxin, glucosylation of small GTPases, necrotizing, lethal
C. perfringens type A*	Gas gangrene. Necrotic enteritis and gangrenous dermatitis in chickens, necrotizing enterocolitis in pigs	α	A phospholipase; haemolytic, necrotizing, lethal, lecithin digestion
		θ	Perfringolysin O, a thiol-activated cytolysin
		NetB	Role unclear, essential virulence component in some strains causing necrotic enteritis in chickens
C. sordellii	Myositis in cattle, sheep and horses. Abomasitis in lambs	α	Lecithinase, haemolytic
		β	Cytotoxin, glucosylation of small GTPases, lethal
C. novyi type B	Infectious necrotic hepatitis (black disease) in sheep and occasionally in cattle	α	Cytotoxin, glucosylation of small GTPases, necrotizing, lethal
		β	Necrotizing, haemolytic, lethal, lecithinase
C. haemolyticum	Bacillary haemoglobinuria in cattle and occasionally in sheep	β	Necrotizing, haemolytic, lethal, phospholipase C

* *Clostridium perfringens* type A may be involved in wound infections and also in some enteric conditions.

The exogenous infections, malignant oedema and gas gangrene, result from the introduction of clostridial organisms into wounds. The anaerobic environment in necrotic tissue is conducive to replication of the clostridia which are often present together with facultative anaerobes in mixed infections. Extension of local tissue destruction results from exotoxin production. The generalized clinical signs in both exogenous and endogenous clostridial infections are manifestations of toxaemia. The major toxins produced by the histotoxic clostridia and their biological activites are listed in Table 22.3. In many instances, toxins that are similar in structure and function are produced by different species of histotoxic clostridia although nomenclature is not consistent across the species. The α toxin of one species is not necessarily the same toxin as the α toxin of another species. The α and β toxins of *C. sordellii*, the α toxin of *C. novyi* and the α and β toxins of *C. difficile* (an enteropathogenic *Clostridium*) belong to a family of structurally and functionally related exotoxins known as the large clostridial cytotoxins. These toxins are enzymes that inactivate low molecular weight GTP-binding proteins by glucosylation. Inactivation of these

proteins disrupts the cytoskeleton leading to opening of tight junctions and cell death. The α toxin of *C. chauvoei* is an oxygen-stable haemolysin and the α toxin of *C. perfringens* A is a phospholipase.

Clinical infections

The clinical infections produced by histotoxic clostridia include blackleg, malignant oedema, gas gangrene, braxy, infectious necrotic hepatitis and bacillary haemoglobinuria. These disease conditions tend to recur on certain farms in the absence of suitable vaccination programmes. Histotoxic clostridial infection should be considered when individual animals die suddenly. Gross post-mortem findings may further indicate clostridial involvement.

Blackleg

Blackleg, an acute disease of cattle and sheep caused by *C. chauvoei*, occurs worldwide. In cattle, the disease is most often encountered in young thriving animals from 3 months to 2 years of age and infection is usually endogenous, the latent spores in muscle becoming activated through traumatic injury. The disease may affect sheep of any age, and in many instances exogenous infection occurs through skin wounds. In both cattle and sheep, gangrenous cellulitis and myositis caused by exotoxins produced by the replicating organisms usually lead to rapid death. The large muscle masses of the limbs, back and neck are frequently affected. Skeletal muscle damage is manifest by lameness, swelling and crepitation due to gas accumulation. Lesions in the muscles of the tongue and throat may produce dyspnoea. Myocardial and diaphragmatic lesions may cause sudden death without premonitory signs. Fluorescent antibody techniques or PCR techniques applied to specimens from lesions are rapid and sensitive confirmatory methods.

Malignant oedema and gas gangrene

Malignant oedema and gas gangrene are exogenous necrotizing soft-tissue infections. The bacteria most commonly implicated are *C. septicum* in malignant oedema and *C. perfringens* type A in gas gangrene. However, *C. novyi* type A, *C. chauvoei* and, rarely, *C. sordellii* have also been incriminated either alone or in association with other clostridial species. Other aerobic and anaerobic opportunistic invaders may be present in the lesions also. Infection can follow contamination of wounds, parturition injuries or injection sites. Tissue

devitalization associated with trauma provides the low redox potential, the alkaline pH and the protein breakdown products required for clostridial proliferation.

Malignant oedema manifests as cellulitis with minimal gangrene and gas formation. Tissue swelling due to oedema, and coldness with discoloration of the overlying skin are obvious clinical features. Generalized signs of toxaemia include depression and prostration. Death may follow rapidly when lesions are extensive.

Gas gangrene is characterized by extensive bacterial invasion of damaged muscle tissue. Gas production is detectable clinically as subcutaneous crepitation. The clinical features of toxaemia in gas gangrene are similar to those encountered in malignant oedema.

In rams, clostridial infection of head wounds caused by fighting is termed ‘big head’. There is oedematous swelling of subcutaneous tissues of the head, neck and cranial thorax. Death may be rapid. The clinical signs are attributed to the necrotizing lethal α toxin of *C. novyi* type A.

Braxy

Braxy, an abomasitis of sheep, is caused by the exotoxins of *C. septicum*. The disease, which occurs in winter during periods of heavy frost or snow, has been recorded in parts of northern Europe and occasionally elsewhere in the world. It has been suggested that ingestion of frozen herbage may cause local devitalization of abomasal tissue at its point of contact with the rumen, allowing invasion by *C. septicum*. The course of the disease is rapid and most animals die without premonitory signs. Anorexia, depression and fever may be evident immediately before death. *Clostridium septicum* may be demonstrated in specimens from the abomasal lesion by the fluorescent antibody technique.

Infectious necrotic hepatitis

Infectious necrotic hepatitis (black disease) is an acute disease that affects sheep and occasionally cattle. Rare cases have been described in horses and pigs. The hepatic necrosis is caused by exotoxins of *C. novyi* type B replicating in liver tissue which has been damaged by immature *Fasciola hepatica* or other migrating parasites. Although the condition is considered to be endogenous, it is possible that the migrating flukes may carry the bacteria or their spores to the liver. Death is rapid with no premonitory signs and the disease requires

differentiation from acute fasciolosis. The term ‘black disease’ relates to the dark discoloration of the skin caused by the marked venous congestion of subcutaneous tissues observed at post-mortem examination. The fluorescent antibody technique may be used to demonstrate *C. novyi* type B in specimens from liver lesions.

Bacillary haemoglobinuria

Bacillary haemoglobinuria occurs primarily in cattle and occasionally in sheep. In this endogenous infection with *C. haemolyticum*, the clostridial endospores are dormant in the liver, probably in Kupffer cells. As in infectious necrotic hepatitis, the main factor that facilitates spore germination and clostridial replication is fluke migration. The α toxin, a phospholipase produced by vegetative cells, causes intravascular haemolysis in addition to hepatic necrosis. Hae moglobinuria, a major clinical feature of the disease, is a consequence of extensive red cell destruction. The aetiological agent may be demonstrated in specimens from hepatic lesions by the fluorescent antibody technique.

Diagnostic procedures

- Histotoxic clostridia contributing to these conditions can be identified by fluorescent antibody techniques.
- *Clostridium perfringens* is cultured anaerobically on blood agar at 37°C for 48 hours.
- Colonies of *C. perfringens* type A are up to 5 mm in diameter, circular, flat, greyish and surrounded by a zone of double haemolysis (Fig. 22.3).
- Apositive CAMP test occurs with *Streptococcus agalactiae* A diffusible factor released by *S. agalactiae* enhances the partial haemolysis produced by the α toxin of *C. perfringens*. The pattern of haemolysis is similar to that observed in the *S. agalactiae* reaction with the β haemolysin of *Staphylococcus aureus* (see Fig. 15.4).
- The Nagler reaction, a plate neutralization test, identifies the α toxin of *C. perfringens*, which has lecithinase activity (Fig. 22.4).
- For many histotoxic species, PCR techniques have been developed for the identification of the organisms in tissues, including *C. chauvoei* (Kuhnert *et al*., 1997; Uzal *et al*., 2003) and *C. septicum* (Sasaki *et al*., 2001).

Treatment and control of histotoxic clostridial diseases

- Because the pathogenesis of the diseases caused by the histotoxic clostridia is similar, the procedures relevant to their treatment and control are also similar.
- Although treatment is usually ineffective, penicillin or broad-spectrum antibiotics administered to animals early in the disease may be of value.
- Vaccination, usually with bacterin and toxoid components in adjuvant, is the most effective method for preventing these diseases. Multicomponent vaccines, which induce protection against several pathogenic clostridial species, may be required on some farms. Animals should be vaccinated at 3 months of age and given a booster injection approximately 3 weeks later. Annual revaccination is recommended.

Figure 22.3 Double haemolysis on blood agar around a colony of *Clostridium perfringens*.

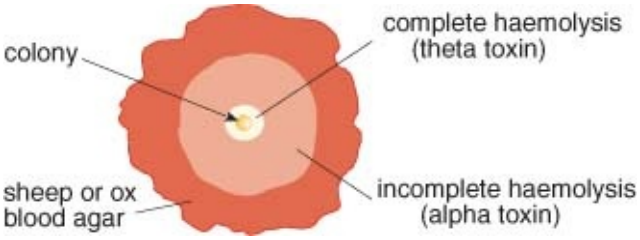
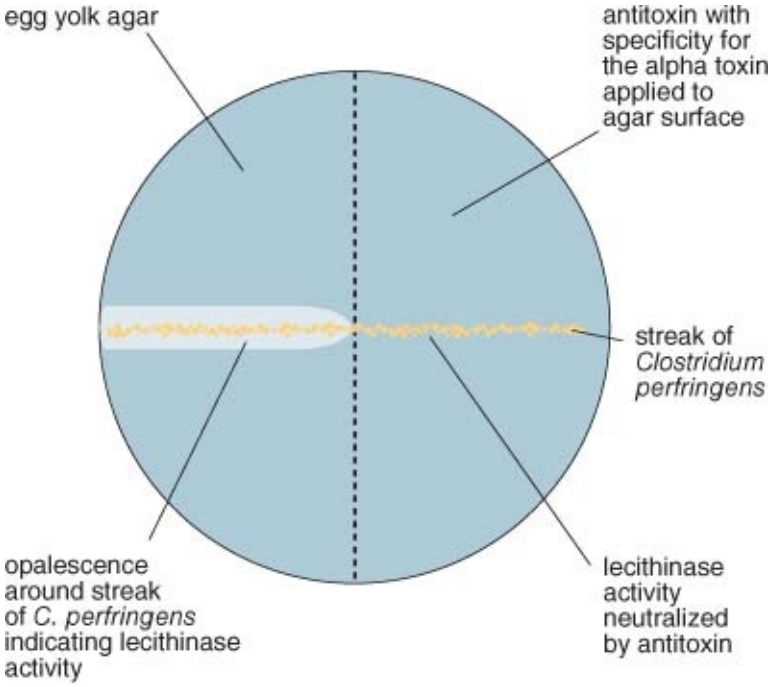


Figure 22.4 Nagler reaction produced by *Clostridium perfringens* growing on egg yolk agar. Antitoxin with specificity for the α toxin is applied to the surface of one half of an egg yolk agar plate and allowed to dry. *Clostridium perfringens* is streaked across the plate which is incubated anaerobically at 37°C for 24 hours. Although the organism grows on both halves of the plate, lecithinase activity is evident only on the half without antitoxin.



Enteropathogenic and enterotoxaemia-producing clostridia

Clostridia that produce enterotoxaemia and enteropathy replicate in the intestinal tract and elaborate toxins that produce both localized and generalized effects. Pathogenic strains of these clostridia may be present in the intestinal flora of animals but only produce disease in defined circumstances.

Clostridium perfringens

Types A, B, C and D of *Clostridium perfringens* are of particular significance in domestic animals. Factors that predispose to clostridial proliferation in the intestine include inappropriate husbandry methods, sudden dietary changes and local environmental influences. Thus, many diseases caused by these bacteria may be regarded as multifactorial and prevention involves appropriate management strategies.

Usual habitat

Clostridium perfringens is found in soil, in faeces, and in the intestinal tracts of animals and humans. *Clostridium perfringens* types B, C and D may survive in soil as spores for several months. *Clostridium perfringens* type A, which

constitutes part of the normal intestinal flora, is widely distributed in soil.

Pathogenesis and pathogenicity

Clostridium perfringens types A to E produce a number of potent, immunologically distinct exotoxins which cause the local and systemic effects encountered in enterotoxaemias. There are four major toxins produced, α, β, ε,and ι; the pattern of production varies with each *C. perfringens* type and determines the clinical syndrome observed. The toxins produced by *C. perfringens* types A to E, their biological activities and associated diseases are presented in Table 22.4. A range of minor toxins, some of which may enhance virulence, is also recognized. These include haemolysins, a collagenase, perfringolysin O, a β2 toxin and a hyaluronidase. In addition, enterotoxin, which has cytotoxic properties, is produced by all strains and is important in enteric disease caused by *C. perfringens* in animals.

The relative importance of the different toxins, which is known for only some *C. perfringens* types, is indicated in Table 22.4. Although α toxin is produced by all types of *C. perfringens* and is known to play a major role in gas gangrene, its role as a virulence factor in enteric conditions produced by type A strains is less clear. Some studies on necrotic enteritis of chickens even suggest that the toxin is not an essential pathogenicity factor as a type A mutant strain which did not produce α toxin retained full virulence in an *in vivo* model (Keyburn *et al*., 2006).

Predisposing intestinal and dietary factors allow opportunistic overgrowth of *C. perfringens* in sheep (Box 22.1). Sustained high levels of clostridial exotoxins are usually required for the development of systemic clinical signs.

Clinical infections

The diseases associated with *C. perfringens* types A to E are listed in Table 22.4. Although *C. perfringens* type A is primarily associated with gas gangrene in humans and domestic animals and with food poisoning in humans, it is also associated with necrotizing enterocolitis in unweaned and feeder pigs, necrotic enteritis in broiler chickens, canine haemorrhagic gastroenteritis and typhlocolitis in horses. *Clostridium perfringens* type E causes enteritis in rabbits and occasionally haemorrhagic enteritis in calves. In sheep, *C. perfringens* types B, C and D cause lamb dysentery, ‘struck’ and pulpy kidney disease respectively. *Clostridium perfringens* types A and C cause haemorrhagic enteritis in neonatal

piglets. In other species, comparable diseases are described periodically.

Lamb dysentery

This disease of young lambs, caused by *C. perfringens* type B, has been reported in parts of Europe and South Africa. Morbidity in flock outbreaks of lamb dysentery can be up to 30% with high mortality rates. Affected lambs, usually in the first week of life, may show abdominal distension, pain and blood-stained faeces. Many die suddenly without premonitory signs. The high susceptibility of this age group can be attributed to the absence of microbial competition and low proteolytic activity in the neonatal intestine (Box 22.1). β Toxin is extremely sensitive to trypsin digestion but in the absence of proteolytic activity it retains its potency and produces disease. In contrast, ε toxin, which is also produced by *C. perfringens* type B, requires proteolysis via trypsin for activation. It is suggested that prevailing intestinal conditions at the time of infection may select for predominant activity by one or other toxin (Uzal and Songer, 2008). At post-mortem, extensive haemorrhagic enteritis with areas of ulceration is present in the small intestine. Increased capillary permeability induced by the toxin results in fluid accumulation in the peritoneal cavity and in the pericardial sac.

Box 22.1 Factors which predispose to the development of enterotoxaemias associated with *Clostridium perfringens* in sheep.

- Low proteolytic activity in the neonatal intestine:
  - – Presence of trypsin inhibitors in colostrum
  - – Low level of pancreatic secretion
- Incomplete establishment of normal intestinal flora in neonates
- Dietary influences in older animals:
  - – Abrupt change to a rich diet
  - – Gorging on energy-rich diet
  - – Intestinal hypomotility, a consequence of overeating

Table 22.4 Types of *Clostridium perfringens* and their major toxins.

Clostridium perfringens	Disease	Toxin	
		Name	Biological activity
Type A*	Necrotic enteritis in chickens, necrotizing enterocolitis in pigs, canine haemorrhagic gastroenteritis	α	A phospholipase, haemolytic, necrotizing, lecithin digestion
		NetB toxin	Present in necrotic enteritis strains. Role unclear, essential virulence component in some strains causing necrotic enteritis in chickens
Type B	Lamb dysentery, haemorrhagic enteritis in calves and foals	α	A phospholipase, haemolytic, necrotizing, lecithin digestion
		β (significant toxin)	Lethal, necrotizing
		ε (exists as a prototoxin and requires activation by proteolytic enzymes)	Increases intestinal and capillary permeability, lethal
Type C	'Struck' in adult sheep, sudden death in goats and feedlot cattle, necrotic enteritis in chickens, haemorrhagic enteritis in neonatal piglets, foals, calves and lambs	α	A phospholipase, haemolytic, necrotizing, lecithin digestion
		β (significant toxin)	Lethal, necrotizing
		Enterotoxin	Cytotoxic
Type D	Pulpy kidney in sheep, enterotoxaemia in calves, adult goats and kids	α	A phospholipase, haemolytic, necrotizing, lecithin digestion
		ε (significant toxin, exists as a prototoxin and requires activation by proteolytic enzymes)	Increases intestinal and capillary permeability, lethal
Type E	Haemorrhagic enteritis in calves, enteritis in rabbits	α	A phospholipase, lethal, haemolytic, necrotizing, lecithin digestion
		ι (significant toxin)	Dermonecrotic, lethal

* *Clostridium perfringens* type A may be involved in wound infections and also in some enteric conditions.

Pulpy kidney disease

This disease, caused by *C. perfringens* type D, occurs in sheep worldwide. The condition is also described as 'over-eating disease' because gorging on a high grain diet or on succulent pasture predisposes to its development (Box 22.1). Ingestion of excessive quantities of food leads to 'carry-over' of partially digested food from the rumen into the intestine. The high starch content in the partially digested food is a suitable substrate for rapid clostridial proliferation. Sustained production of ε toxin, which exists as a prototoxin and requires activation by proteolytic enzymes, leads to toxaemia and the development of clinical signs.

Thriving lambs from 3 to 10 weeks of age are commonly affected. The course of the disease is usually short and lambs are often found dead. Clinical signs include dullness, opisthotonos, convulsions and terminal coma. Central nervous system signs such as blindness and head pressing may be present in subacute

disease. Bloating may be evident in the later stages of illness. Hyperglycaemia and glycosuria are constant features of the disease. Affected adult sheep, which have survived for several days, may exhibit diarrhoea and staggering.

In acute disease, the only post-mortem findings may be scattered hyperaemic areas in the intestines and fluid accumulation in the pericardial sac. Rapid kidney autolysis which leads to pulpy cortical softening is a typical post-mortem finding. Focal symmetrical encephalomalacia, a manifestation of the subacute effects of ε toxin on the vasculature, is characterized by symmetrical haemorrhagic lesions in the basal ganglia and midbrain.

Clostridium perfringens type C infection in sheep

Infection with *C. perfringens* type C causes ‘struck’, an acute enterotoxaemia in adult sheep in defined geographical regions such as the Romney Marsh district in England. The disease, which occurs in sheep at pasture, manifests as sudden death although some animals may be found in terminal convulsions. β Toxin plays the major role in the pathogenesis of the disease. Post-mortem findings include jejunal ulceration, patchy hyperaemia in the small intestine and accumulation of fluid in the peritoneal cavity along with congestion of peritoneal vessels and petechial haemorrhages.

Haemorrhagic enteritis in piglets

This peracute enterotoxaemia caused by *C. perfringens* type C has been described worldwide in newborn piglets. Often, entire litters are affected with mortality rates up to 80%. Infection is probably acquired from the sow’s faeces. Poor husbandry may be a predisposing factor in some outbreaks.

The clinical course of the disease is short, death occurring within 24 hours of onset. Recent immunohistochemical studies showed that binding of β toxin to vascular endothelial cells occurs early in the course of disease and this may induce the vascular necrosis which contributes to the pathological lesions observed (Miclard *et al*., 2009). Older piglets up to 2 weeks of age, which are occasionally affected, develop a more chronic form of the disease. The clinical signs include dullness, anorexia and, terminally, blood-stained faeces and perianal hyperaemia. Necrosis of the intestinal mucosa and blood-stained contents are present at post-mortem examination. The lesions are usually found in the terminal small intestine, caecum and colon. Excess serosanguineous fluid is present in the pleural and peritoneal cavities. A less severe necrotizing

enterocolitis in unweaned and feeder pigs is caused by *C. perfringens* type A strains.

Necrotic enteritis of chickens

Necrotic enteritis, caused by *C. perfringens* type A and, less frequently, by type C strains, primarily affects broilers up to 12 weeks of age. It is an acute enterotoxaemia characterized by sudden onset and high mortality. Confluent necrotic areas in the mucosa of the small intestine are found post mortem. Dietary changes, intestinal hypomotility and mucosal damage caused by coccidia and other enteric pathogens predispose to the development of disease. Mortality in birds concurrently infected with *Eimeria* species is 25% higher than in those affected by necrotic enteritis alone (Drew *et al*., 2004). The importance of necrotic enteritis to the poultry industry has increased in recent years in areas such as the EU in particular, owing to the ban on the use of antimicrobial growth promoters. For example, the incidence of disease in France rose from 4% in 1995 to 12% in 1999 (Casewell *et al*., 2003). A newly identified toxin, NetB, is now considered to be an important virulence factor of necrotic enteritis strains, and α toxin may be of lesser importance, although the latter appears to be capable of inducing a protective immune response (van Immerseel *et al*., 2009).

Infections caused by C. perfringens t ypes B, C and D in other domestic animals

Enterotoxaemias caused by *C. perfringens* type B have been recorded in newborn foals, in calves and in adult goats. In these species, the condition is rapidly fatal, and severe haemorrhagic enteritis is a common post-mortem finding.

Clostridium perfringens type C has been associated with a disease in feedlot cattle similar to ‘struck’ in adult sheep. In calves, lambs and foals, infections with *C. perfringens* type C result in acute enterotoxaemia along with a haemorrhagic enteritis resembling that produced by the infection in newborn piglets.

Enterotoxaemia caused by *C. perfringens* type D has been reported in kids and adult goats. The clinical and pathological features of acute disease in kids are similar to those of pulpy kidney disease in lambs. Although subacute forms of the disease have been described, focal symmetrical encephalomalacia has not been reported in goats.

Diagnostic procedures

- Sudden deaths in groups of unvaccinated animals on farms where outbreaks of clostridial enterotoxaemia have previously been recorded may suggest the involvement of *C. perfringens* types B, C or D.
- In recently dead animals, post-mortem findings may be of value. The presence of focal symmetrical encephalomalacia is indicative of *C. perfringens* type D involvement (Buxton *et al*., 1978).
- Direct smears from the mucosa or contents of the small intestine of recently dead animals which contain large numbers of thick Gram-positive rods are consistent with clostridial enterotoxaemia.
- Isolation of large numbers of *C. perfringens* types B and C from recently dead animals, especially in pure culture, is supportive of a diagnosis. PCR genotyping can be used to type *C. perfringens* isolates as an alternative to *in vivo* toxin neutralization tests.
- Glycosuria is a constant finding in pulpy kidney disease.
- Toxin neutralization tests using mouse and guinea-pig inoculation can definitively identify the toxins of *C. perfringens* present in the intestinal contents of recently dead animals. Because of the lability of some of these toxins, particularly the β toxin, failure to demonstrate their presence in intestinal contents does not necessarily exclude a diagnosis of clostridial enterotoxaemia. The supernatant from centrifuged ileal contents is generally used for the test. Antitoxins with specificity for each *C. perfringens* type are added to supernatant fluid to produce a mixture of three parts test fluid to one part known antitoxin. Saline added to supernatant is used as a positive control for the presence of toxin. To allow neutralization of toxin, each mixture is held at room temperature for 1 hour before intravenous injection into mice or intradermal injection into guinea-pigs. It is usual to inject 0.3 ml of the mixture into mice and 0.2 ml into guinea-pigs. The pattern of cross-neutralization observed in the mouse or guinea-pig tests indicates the specific *C. perfringens* type which is the cause of the enterotoxaemia.
- ELISA can be used as an alternative to *in vivo* assays for demonstrating toxin in intestinal contents (Songer, 1997). The sensitivity and specificity of ELISA for detection of *C. perfringens* toxins approaches that of mouse or guinea-pig inoculation methods. Indeed the sensitivity of ELISA methods is frequently greater than that of *in vivo* methods and this can lead to a

misdiagnosis in the absence of other diagnostic indicators. Misdiagnosis can occur because ELISA can detect low levels of toxin in the intestinal contents of normal animals (Uzal and Songer, 2008).

Treatment and control

- Hyperimmune serum, if available, may be of value in some instances. Because of the acute nature of the disease, antibiotic therapy is generally ineffective.
- Vaccination is the principal control method. Ewes should be vaccinated with toxoid 6 weeks before lambing to ensure passive protection for lambs up to 8 weeks of age. Ewes being vaccinated for the first time should be given two doses of vaccine 1 month apart. Annual revaccination is recommended.
- For the prevention of pulpy kidney disease, lambs should be vaccinated with toxoid before they are 2 months old and a booster injection should be given 1 month later.
- Sudden dietary changes and other factors predisposing to enterotoxaemias should be avoided (Box 22.1).

Clostridium difficile

Infection with *Clostridium difficile* has been reported in dogs with chronic diarrhoea (Berry and Levett, 1986) and in haemorrhagic enterocolitis in new-born foals (Jones *et al*., 1988). Disease in foals can occur in the absence of prior antimicrobial therapy (Jones *et al*., 1987) but disease in other animals and in humans is usually associated with antimicrobial drug therapy. Antimicrobial treatment suppresses the normal flora in the intestine with persistence of the clostridial spores. Germination and proliferation of *C. difficile* is followed by toxin production and diarrhoea. An emerging epidemic human nosocomial strain, ribotype 027, is of major importance in human healthcare and has also been reported recently in horses (Songer *et al*., 2009). *Clostridium difficile* is documented as an important cause of neonatal diarrhoea in pigs in the United States. The organism produces two major toxins, A and B, both of which belong to the family of large clostridial cytotoxins. Toxin A is an enterotoxin and B is a potent cytotoxin. Both act by inactivating low molecular weight GTP-binding molecules leading to disruption of the cytoskeleton. *Clostridium difficile* and its toxins can be detected in the faeces of normal animals and many aspects of

disease pathogenesis remain unclear. However, recent studies indicated that diarrhoeic horses with toxin A and/or *C. difficile* in faeces had more severe disease than those without *C. difficile* (Ruby *et al*., 2009). Diagnosis is based on the demonstration of toxin in faeces using ELISA or cytotoxicity assays. Commercial ELISA kits are available for humans although their suitability for testing specimens from animals has not been evaluated thoroughly. Chouicha and Marks (2006) found that sensitivity of ELISA was unsatisfactory for canine samples. Prevention of diarrhoea associated with *C. difficile* is based on good antimicrobial prescribing practices, alleviation of stress and thorough cleaning and disinfection (Båverud, 2004).

Clostridium colinum

This clostridial organism has been implicated in enteritis in quails (quail disease), chickens, turkeys, pheasants and grouse. *Clostridium colinum* is shed in the faeces of clinically affected and carrier birds. Mortality may approach 100% in susceptible quails but is usually less than 10% in chickens. Intestinal ulceration and, in some instances, hepatic necrosis are present at post-mortem examination.

Antibiotics are used therapeutically in drinking water or in feed. Contaminated litter should be removed regularly as part of a control programme.

Clostridium spiroforme

Clostridium spiroforme, a clostridial organism with atypical coiled morphology, has been implicated in spontaneous and antibiotic-induced enteritis in rabbits. This enterotoxaemia-like condition may be fatal within 48 hours. Predisposing factors include oral administration of antibiotics and low-fibre diets. Antibiotic administration adversely affects the intestinal flora of the rabbit, which is composed predominantly of Gram-positive bacteria. A toxin elaborated by *C. spiroforme* is neutralized by antitoxin to the ι toxin of *C. perfringens* type E as it is structurally similar to this toxin (Borriello and Carman, 1983). It is a binary toxin with cytolytic activity and is encoded by the genes sas and *sbs*. PCR assays for the detection of the organism and the toxin-encoding genes have been described (Drigo *et al*., 2008).

Clostridium piliforme

This spore-forming, filamentous Gram-variable intracellular pathogen is an

atypical member of the clostridia. It has not been cultured on artificial media and grows only in tissue culture or in fertile eggs. Although originally named B*acillus piliformis*, DNA sequencing demonstrated its relatedness to the clostridia (Duncan *et al*., 1993). The source of the organism is usually an infected animal.

Infection with *C. piliforme*, Tyzzer's disease, results in severe hepatic necrosis. The condition was originally described in mice and other laboratory animals (Sparrow and Naylor, 1978). It has been reported to occur sporadically in foals and rarely in calves, dogs and cats. Stress or immunosuppression may predispose to infection.

Affected foals are usually under 6 weeks of age and many are found comatose or dead. Following oral infection, the incubation period is up to 7 days. Clinical signs include depression, anorexia, fever, jaundice and diarrhoea. Hepatomegaly with extensive areas of necrosis is the principal post -mortem finding. Diagnosis is based on the histological demonstration of the organisms in hepatocytes using the Warthin-Starry silver impregnation technique. PCR-based techniques for detection of the organism have been described also (Borchers *et al*., 2006). Because of the acute nature of the disease, it is usually not possible to administer specific therapy.

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Chapter 23

Mycobacterium species

Mycobacteria are aerobic, non-spore-forming, non-motile, rod shaped, acid-fast bacilli. Individual species differ in size; the rods of *Mycobacterium bovis* and *M. avium* subsp. *avium* are slender and up to 4 (µm in length, whereas those of *M. avium* subsp. *paratubercu-losis* are broad and are usually less than 2 (µm long.

Although mycobacteria are cytochemically Grampositive, the high lipid and mycolic acid content of their cell walls prevents uptake of the dyes employed in the Gram stain. The cell wall lipids bind carbol fuchsin which is not removed by the acid–alcohol decolorizer used in the Ziehl-Neelsen (ZN) staining method. Bacilli that stain red by this method are called acid-fast or ZN-positive.

The mycobacteria include diverse species ranging from environmental saprophytes and opportunistic invaders to obligate pathogens.

Although some pathogenic mycobacteria exhibit particular host preferences, they can occasionally infect other species (Table 23.1). Mycobacterial diseases in domestic animals are usually chronic and progressive. Members of the *M. tuberculosis* complex are closely related and cause similar pathological changes in a number of mammalian hosts (Table 23.1).

Usual habitat

Lipid-rich walls render mycobacteria hydrophobic and resistant to adverse environmental influences. Environmental mycobacteria are found in soil, on vegetation and in water. Obligate pathogens, shed by infected animals, can also survive in the environment for extended periods (Morris *et al*., 1994).

Key points

- Acid-fast (ZN-positive) rods
- Cell walls rich in complex lipids and waxes containing mycolic acids
- Complex egg-enriched media required for growth of pathogenic species
- Aerobic, non-motile, non-spore-forming

- Genus includes obligate pathogens, opportunistic pathogens and saprophytes
- Pathogenic species grow slowly, colonies visible after several weeks
- Some mycobacteria produce carotenoid pigments
- Resistant to chemical disinfectants and environmental influences but susceptible to heat treatment (pasteurization)
- Multiply intracellularly and cause chronic, granulomatous infections
- Major diseases include tuberculosis, Johne's disease and feline leprosy

Differentiation of pathogenic mycobacteria

The ZN staining method is used to differentiate mycobacteria from other bacteria. Differentiation of pathogenic mycobacteria relies on cultural characteristics, biochemical tests, animal inoculation and chromatographic analyses. In addition, molecular techniques are increasingly used for identification of isolates. Mycobacteria associated with opportunistic infections can be differentiated on the basis of pigment production, optimal incubation temperature and growth rate (Table 23.2).

Table 23.1 Mycobacteria which are pathogenic for animals and humans.

Mycobacterium species	Main hosts	Species occasionally infected	Disease
M. tuberculosis complex			
M. tuberculosis	Humans, captive primates	Dogs, cattle, psittacine birds, canaries	Tuberculosis (worldwide)
M. bovis	Cattle	Deer, badgers, possums, humans, cats, other mammalian species	Tuberculosis
M. africanum	Humans		Tuberculosis (mainly West Africa)
'M. canettii'	Humans		Tuberculosis (mainly East Africa)
M. microti	Voles	Occasionally other mammalian species	Tuberculosis
M. caprae	Goats	Cattle	Tuberculosis
M. pinnipedii	Seals, sea-lions	Occasionally other mammalian species including humans	Tuberculosis
M. avium complex[a]	Most avian species except psittacines	Pigs, cattle	Tuberculosis
M. marinum	Fish	Humans, aquatic mammals, amphibians	Tuberculosis
M. ulcerans	Humans	Koalas, possums	Buruli ulcer
M. leprae	Humans	Armadillos, chimpanzees	Leprosy
M. lepraemurium	Rats, mice	Cats	Rat leprosy, feline leprosy
M. avium subsp. *paratuberculosis*	Cattle, sheep, goats, deer	Other ruminants	Paratuberculosis (Johne's disease)
Unspecified acid-fast bacteria[a]	Cattle		Associated with skin tuberculosis
M. senegalense, M. farcinogenes	Cattle		Implicated in bovine farcy

a, cattle infected with these mycobacteria often exhibit sensitivity to tuberculin.

- Safety precautions, including the use of a biohazard cabinet, must be implemented when working with material containing mycobacteria.
- Pathogenic mycobacteria grow slowly on solid media, and colonies are not evident until cultures have been incubated for 3 to 6 weeks. In contrast, the colonies of rapidly growing saprophytes are visible within days. Commercially available liquid culture systems such as BACTEC™ (Becton Dickinson and Company, USA) show improved isolation times for pathogenic mycobacteria, ranging from approximately 10 to 20 days.
- *Mycobacterium bovis, M. tuberculosis* and M.*avium* subsp.*paratuberculosis* have an optimal incubation temperature of 37°C. Mycobacteria belonging to the *M. avium* complex grow in the temperature range 37 to 43°C.
- Cultural features:
  – Pathogenic species of mycobacteria can be distinguished by their colonial appearance on egg-based media.
  – The influence of glycerol and sodium pyruvate on growth rate is used to

differentiate pathogenic species.

– Supplementation of media with mycobactin is required for M. *avium* subsp. *paratuberculosis*. Mycobactin is extracted from laboratory-maintained, rare, non-mycobactin-dependent isolates of M. *avium* subsp. *paratuberculosis*.

- Biochemical differentiation, based on specific test methods, aids in the identification of *M. tuberculosis*, *M. bovis* and *M. avium*. Some mycobacterial isolates cannot be assigned to a given species using biochemical differentiation as their biochemical profiles are difficult to interpret (Gunn-Moore *et al*., 1996).
- Guinea-pig and rabbit inoculation was used in the past to differentiate *M. tuberculosis* from *M. bovis* and *M. avium*. Guinea-pigs are highly susceptible to infection with *M. tuberculosis* and *M. bovis*. Rabbits are highly susceptible to infections with *M. bovis* and *M. avium*.
- Chromatographic analyses of the lipid composition of some mycobacterial species are used in specialized laboratories.
- Pigment production and photoreactivity for opportunistic mycobacteria:

  – Non-chromogens produce colonies devoid of orange, carotenoid pigments.

  – Photochromogens, when cultured in the dark, produce non-pigmented colonies which become pigmented after a period of exposure to light.

  – Scotochromogens produce pigment when cultured in the dark or in light.
- Molecular techniques:

  – DNA probes, complementary to species-specific sequences of rRNA, are commercially available for the *M. tuberculosis* complex, the *M. avium* complex and *M. kansasii*.

  – Nucleic acid amplification procedures, including the polymerase chain reaction, are being developed as sensitive and rapid methods for the detection of mycobacteria in tissue samples (Aranaz *et al*., 1996). Although a number of commercial and in-house methods are available, the reliability of many of the tests remains to be confirmed and further development is required (Anon., 2008).

  – DNA-based typing methods are used in epidemiological studies (Collins *et al*., 1994). Spoligotyping, which identifies polymorphisms in the spacer units in the direct repeat region of the chromosome, is the most common method used in studies of *M. bovis*. This method, together with variable

number tandem repeat (VNTR) typing, is currently considered the most satisfactory for epidemiological investigation of *M. bovis* strains (Hewinson *et al.*, 2006). Typing of *M. avium* subsp. *paratuberculosis* strains is frequently done by restriction fragment length polymorphism (RFLP) analysis.

Table 23.2 Clinical significance, growth characteristics and biochemical differentiation of pathogenic mycobacteria.

	M. tuberculosis	*M. bovis*	*M. avium* complex	*M. avium* subsp. *paratuberculosis*
Significance of infection	Important in humans and occasionally in dogs	Important in cattle and occasionally in other domestic animals and humans	Important in free-range domestic poultry, opportunistic infections in humans and domestic animals	Important in cattle and other ruminants
Cultural characteristics and requirements				
Growth rate	Slow (3–8 weeks)	Slow (3–8 weeks)	Slow (2–6 weeks)	Very slow (up to 16 weeks)
Optimal incubation temperature	37°C	37°C	37 to 43°C	37°C
Atmospheric requirements	Aerobic	Aerobic	Aerobic	Aerobic
Colonial features	Rough, buff, difficult to break apart	Cream-coloured, raised with central roughness, break apart easily	Sticky, off-white, break apart easily	Small, hemispherical; some pigmented
Essential growth supplement	None	None	None	Mycobactin
Effect of added glycerol	Enhanced growth (eugonic)	Growth inhibited (dysgonic)	Enhanced growth (eugonic)	
Effect of added pyruvate	No effect	Enhanced growth	No effect	
Biochemical differentiation				
Niacin accumulation	+	–	–	
Pyrazinamidase production	+	–	+	
Nitrate reduction	+	–	–	
Susceptibility to TCH (10 µg/ml)[a]	Resistant	Susceptible	Resistant	

a, TCH, thiophen-2-carboxylic acid hydrazine.

Clinical infections

The diseases caused by pathogenic mycobacteria are presented in Table 23.1. The major pathogenic *Mycobacterium* species that affect domestic animals

exhibit a considerable degree of host specificity although they can produce sporadic disease in a number of other hosts.

Diseases in domestic animals caused by mycobacteria include tuberculosis in avian and mammalian species, paratuberculosis in ruminants, and feline leprosy. Two other clinical conditions, skin tuberculosis and bovine farcy, are associated with the presence of acid-fast bacteria in lesions. In skin tuberculosis of cattle, nodular lesions are located along the course of lymphatics in the limbs. Unspecified acid-fast bacilli have been demonstrated in these lesions. *Mycobacterium senegalense* and *M. farcinogenes* have been isolated from the lesions of bovine farcy. Their aetiological role in this condition, however, is uncertain.

Granulomatous lesions which develop following opportunistic infections with environmental sapro-phytic mycobacteria are encountered occasionally in domestic animals. These saprophytic mycobacteria are grouped on the basis of pigment production and growth rate (Box 23.1). Members of the *M. avium* complex are grouped with those that produce opportunistic infection because they are occasionally involved in mammalian infections.

Tuberculosis in cattle

Bovine tuberculosis, caused by *M. bovis*, occurs worldwide. Because of the zoonotic implications of the disease and production losses owing to its chronic progressive nature, eradication programmes have been introduced in many countries. The presence of *M. bovis* reservoirs in wildlife makes disease eradication difficult to achieve. When eradication programmes are successful, infections in cattle caused by members of the *M. avium* complex and by other saprophytic mycobacteria are occasionally encountered. The incidence of human infection with *M. bovis* has been reduced to low levels in countries where tuberculosis eradication programmes have been implemented in cattle. In addition, pasteurization of milk has eliminated exposure of humans to infection from dairy products. Cross-infection with *M. tuberculosis* from infected humans has been recorded in cattle on rare occasions.

Box 23.1 A classification of mycobacteria of environmental origin which infrequently produce opportunistic infections (based on

Runyon, 1959).

- Photochromogens
  - *M. kansasii*
  - *M. marinum*
- Scotochromogens
  - *M. scrofulaceum*
- Non-chromogens
  - *M. avium complex*
  - *M. genavense*
- Rapid growers
  - *M. chelonae group*
  - *M. fortuitum group*
  - *M.phlei*
  - *M. smegmatis*

Epidemiology

Although *M. bovis* can survive for several months in the environment, transmission is mainly through aerosols generated by infected cattle. Experimental studies have established that infection of cattle by aerosol requires fewer than 10 organisms whereas approximately 10^7 organisms are necessary to establish infection by the oral route. Dairy cattle in particular are at risk because husbandry methods allow close contact between animals at milking and when housed during winter months. Calves can become infected by ingesting contaminated milk and ingestion is the probable route of transmission to pigs and cats. Wildlife reservoirs of *M. bovis* are major sources of infection for grazing cattle in some countries. They include the badger in Europe, the brush-tailed possum in New Zealand and the Cape buffalo and other ruminants in Africa. Deer, both wild and farmed, are particularly susceptible and may act as reservoirs of infection for cattle. The relative importance of wildlife species as reservoirs of infection depends on a number of factors including whether or not the species is a maintenance or spillover host, the levels of bacteria excreted by the wildlife host and the degree of contact with cattle. In a review by Corner (2006), feral pigs in Australia are given as an example of spillover hosts in which infection is not perpetuated within the population in the absence of contact with infected cattle. Feral pigs are infected by the oral route and have small lesions containing few bacilli and are thus deemed of low risk as a source

of infection in cattle. In contrast, badgers are maintenance hosts for *M. bovis*. Lesions occur primarily in the badger lung, leading to the generation of infective aerosols. In addition, there is increased contact between cattle and badgers with advanced disease because such badgers exhibit abnormal behaviour such as straying close to grazing cattle during daylight hours. Badgers are therefore an important source of infection for cattle in particular regions of the world such as the United Kingdom and Ireland.

Pathogenesis and pathogenicity

The virulence of *M. bovis* relates to its ability to survive and multiply in host macrophages (Fig. 23.1). Specific toxins have not been identified, but rather virulence is a consequence of many pathogen factors working in concert to establish infection. Following entry to the host via the respiratory tract, mycobacteria are engulfed by macrophages and by dendritic cells. Mycobacteria engulfed by dendritic cells travel to the draining lymph nodes. The initial response to infection is non-specific and triggered by the foreign body effect of waxes and lipids in the mycobacterial cell wall. Survival within the phagosome of macrophages is promoted by interference with phagosome-lysosome fusion, probably through retarding maturation of the phagosome and failure of lysosomal digestion. Bacilli released from dead macrophages are engulfed by surrounding viable phagocytes. Migration of macrophages containing viable mycobacteria can disseminate infection.

The complex lipid and waxy composition of the mycobacterial cell wall contributes not only to virulence but also, in association with *M. bovis* proteins, to the immunogenicity on which the development of the host responses and the lesions depends. Before activation, macrophages permit the survival and replication of mycobacteria. Infected macrophages accumulate in the alveolus as the primary site of infection and secrete a range of cytokines which recruit lymphocytes to the lung, thus aiding granuloma formation and containment of the organisms. The cytokines TNF-α and IFN-γ are essential in the development of resistance to mycobacteria, and their production is stimulated through both the innate and adaptive immune responses. These cytokines activate infected macrophages and enhance their ability to destroy contained mycobacteria. Given that activated dendritic cells are unable to kill engulfed mycobacteria but limit their replication, these cells may act as a reservoir for mycobacteria, in particular within the lymph nodes (Hope and Villarreal-Ramos, 2008).

With the development of cell-mediated immunity some weeks after infection, macrophage recruitment accelerates under the influence of cytokines produced by T lymphocytes sensitized to *M. bovis* cellular constituents. Both $CD4^+$ and $CD8^+$ T cells are necessary for immunity to mycobacteria although studies in cattle have shown that $CD8^+$ T cells may also play a role in the immunopathology of *M. bovis* infection. The gradual accumulation of macrophages around the developing lesion and the formation of a central necrotic core result in a tubercle or granuloma, the typical host response to *M. bovis* infection (Fig. 23.2). The architecture of the granuloma is important as it facilitates close interaction between the constituent macrophages, dendritic cells and T cells and containment or destruction of the pathogen. In addition, the layered structure helps to physically separate the necrotic core from the surrounding tissue. Granulomas may be histologically visible as early as 3 weeks after experimental infection of cattle with high doses of *M. bovis* (Cassidy *et al*., 1998).

Frequently, only a small number of cattle within a herd show positive reactions to the tuberculin test and develop pathological lesions. It is likely that these animals represent one end of the spectrum of responses to exposure to *M. bovis*. Other animals in the herd may clear infection without becoming sensitized to the tuberculin test (Cassidy, 2008). Animals which test positive but in which no lesions can be detected may be latently infected or may have cleared the infection but remain sensitized to tuberculin.

Clinical signs and pathology

Clinical signs are evident only in advanced disease, and cattle with extensive lesions can appear to be in good health. Loss of condition may become evident as the disease progresses. In advanced pulmonary tuberculosis, animals may eventually develop a cough and intermittent pyrexia. Involvement of mammary tissue may result in marked induration of affected quarters, often accompanied by supramammary lymph node enlargement. Tuberculous mastitis facilitates spread of infection to calves and cats. Consumption of unpasteurized milk is of major public health importance.

Figure 23.1 The possible consequences of *Mycobacterium bovis* infection in cattle, acquired via aerosols.

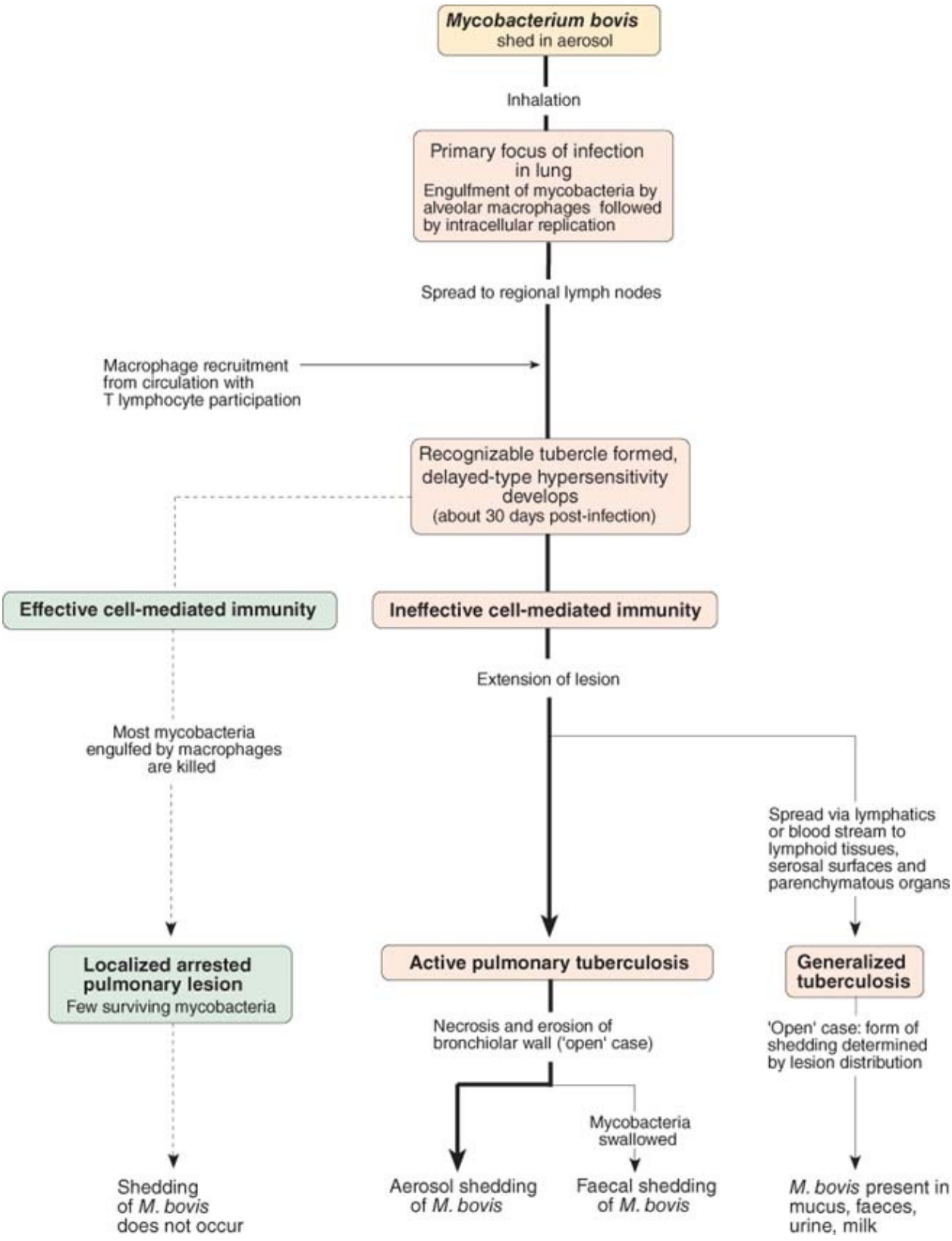
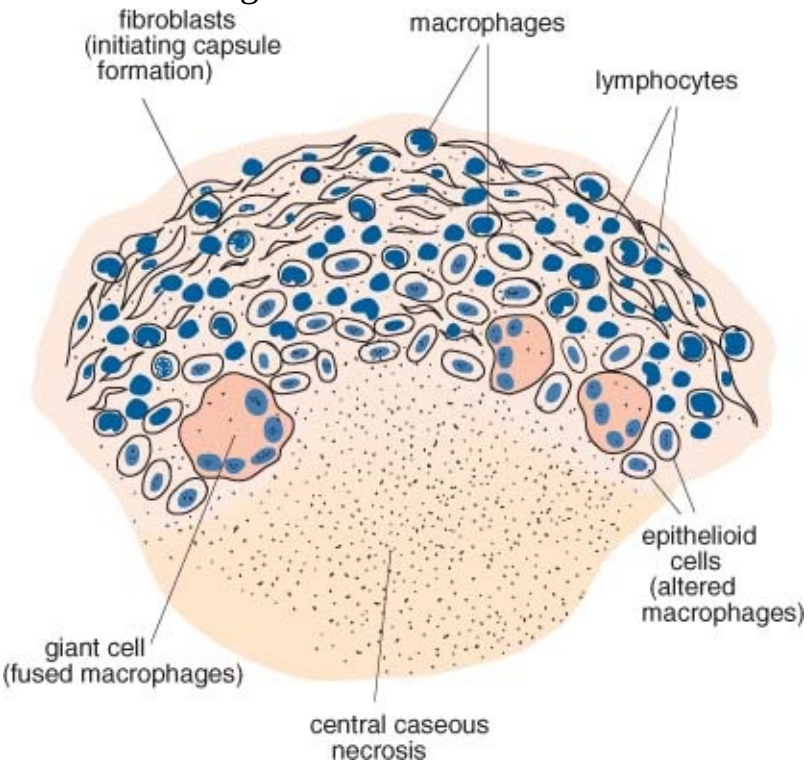


Figure 23.2 Microscopic appearance of part of a typical bovine tuberculous

lesion. The tubercle consists of a peripheral zone of mononuclear cells, fibroblasts and giant cells with central caseous necrosis.



In the early stages of the disease, lesions may be difficult to detect at post-mortem examination. These small lesions are composed of aggregates of altered macrophages, termed epithelioid cells. Multinucleate Langhans' giant cells, formed from the fusion of macrophages, may also be present. In older lesions, fibro-plasia produces early capsule formation and there is an area of central caseous necrosis, with the appearance and consistency of soft cheese. The characteristic histological appearance of a typical tubercle is illustrated in Fig. 23.2.

Diagnostic procedures

- The tuberculin test, based on a delayed-type hyper-sensitivity to mycobacterial tuberculin, is the standard ante-mortem test in cattle. The test can be adapted for use in pigs and farmed deer. Reactivity in cattle is usually detectable 30 to 50 days after infection (Monaghan *et al*., 1994). Tuberculin, prepared from mycobacteria and called purified protein derivative (PPD), is injected intradermally to detect sensitization. Two main methods of tuberculin testing are employed:
  – In the single intradermal (caudal fold) test, 0.1 ml of bovine PPD is

injected intradermally into the caudal fold of the tail. The injection site is examined 72 hours later and a positive reaction is characterized by a hard or oedematous swelling.

– In the comparative intradermal test, 0.1 ml of avian PPD and 0.1 ml of bovine PPD are injected intradermally into separate clipped sites on the side of the neck about 12 cm apart. Skin thickness at the injection sites is measured with calipers before injection of tuberculins and after 72 hours. An increase in skin thickness at the injection site of bovine PPD which exceeds that at the avian PPD injection site by 4 mm or more is interpreted as evidence of infection and the animal is termed a reactor.

- False positive reactions which occur in the tuberculin test may be attributed to sensitization to mycobacteria other than *M. bovis*. In countries in which a high proportion of false positive reactions occur, the comparative test is used in preference to the single intradermal test.
- False negative test results may be recorded:

  – Cattle tested before delayed-type hypersensitivity to tuberculin develops (at about 30 days postinfection) do not react.

  – In some cattle an unresponsive state, referred to as anergy, may accompany advanced tuberculosis. The mechanisms involved are incompletely understood.

  – A transient desensitization may follow injection of tuberculin. Reactivity usually returns within 60 days.

  – Immunosuppression due to stress, including that experienced early post partum or due to administration of immunosuppressive drugs, may contribute to the inability to respond to the tuberculin test.
- Blood-based tests that have been developed for use in conjunction with the tuberculin test include:

  – Gamma interferon assay. This test identifies animals at a slightly earlier stage of infection than the tuberculin test and is approved as a supplementary test for cattle in the EU, USA and New Zealand (de la Rua-Domenech *et al*., 2006). It is frequently used as a parallel test with the tuberculin test in persistently-infected herds or those showing extensive breakdowns. In addition, research is ongoing to investigate the use of this test for differentiation of BCG-vaccinated and naturally infected cattle through the identification of antigens which are not expressed by the BCG organism (Pollock *et al*., 2001). Such antigens could then be used in the

test instead of the mammalian and avian PPD which are used in the commercial tests currently available.

– ELISA for detecting circulating antibodies. Antibodies are produced in the later stages of infection. These tests may be most useful in countries with a high prevalence of bovine TB where there are a large number of animals with chronic disease, and which require low-cost methods of detection (de la Rua-Domenech *et al*., 2006).

– Lymphocyte transformation and related assays.

- Specimens suitable for laboratory examination include lymph nodes, tissue lesions, aspirates and milk.
- The low numbers of mycobacteria present in bovine lesions can render visual confirmation difficult using the ZN stain. In contrast, large numbers of acid-fast bacilli are usually present in smears of specimens from deer and badgers (Fig. 23.3).
- Stained tissue sections usually reveal typical patterns of tubercle formation (Fig. 23.2).
- Isolation of *M. bovis* requires:

  – Decontamination of specimens to eliminate fast-growing contaminating bacteria. Ground-up specimens are treated for up to 30 minutes with 2 to 4% sodium hydroxide or 5% oxalic acid, followed by neutralization of the alkali or acid. Centrifugation is used to concentrate the mycobacteria, and the supernatant fluid is discarded.

  – Slants of Lowenstein-Jensen medium, without glycerol and containing 0.4% sodium pyruvate, are inoculated with the centrifuged deposit and incubated aerobically at 37°C for up to 8 weeks.
- Identification criteria for isolates:

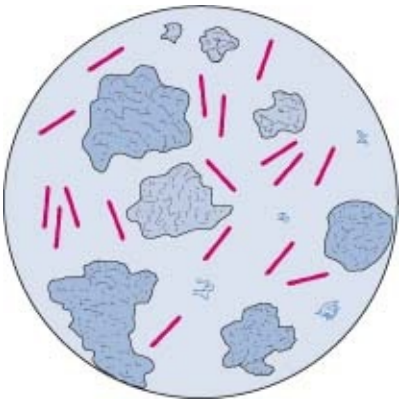
  – Growth rate and colonial appearance

  – Positive ZN staining of bacilli in smears from colonies

  – Biochemical profile (Table 23.2)

  – Analytical and molecular techniques which are now routinely used for identification of isolates include spoligotyping and use of various gene probes (Anon., 2008).
- Commercially available, rapid, automated systems can be used for isolating pathogenic mycobacteria of the *M. tuberculosis* complex (Yearsley *et al*., 1998) and provide shorter isolation times than culture on conventional solid media.

Figure 23.3 Thin rods of *Mycobacterium bovis* as they appear in a smear from a tuberculous lesion from deer or badgers. Organisms are sparse in lesions from cattle. Using the Ziehl-Neelsen method, the mycobacteria stain red (acid-fast) and other lesion material stains blue.



Control

- Treatment is inappropriate in control programmes for cattle and testing for susceptibility of *M. bovis* strains to antimicrobial agents is not usually carried out. Where studies have been completed, organisms are usually susceptible to most agents. However, antimicrobial resistance is a major problem in organisms of the *M. tuberculosis* complex that cause disease in humans, and poses huge challenges for the treatment of human tuberculosis in many countries.
- In many countries, tuberculin testing of cattle followed by isolation, slaughter of reactors and disinfection of farm buildings forms the basis of national eradication schemes.
- Routine meat inspection is an essential part of the surveillance programme for bovine tuberculosis in many countries.
- Wildlife reservoirs such as badgers and possums are major obstacles to disease eradication in some countries. Culling of wildlife has been successful in reducing the prevalence of *M. bovis* infection in cattle in some countries (Corner, 2006). However, in the UK, increased prevalence of infection in cattle was recorded in areas adjacent to those in which badger culling was carried out (White *et al*., 2008).
- Research is ongoing worldwide in order to develop a vaccine for use in cattle and also in wildlife. Strategies include the improvement of the effectiveness of the BCG (Bacille Calmette-Guérin) vaccine, which has been used in humans for decades, possibly by using heterologous prime-

boost strategies (Hope and Villarreal-Ramos, 2008). A variety of subunit vaccines consisting of either protein or DNA have been reported to enhance resistance against *M. tuberculosis* and also against *M. bovis*. Co-administration of DNA vaccines with BCG vaccine enhanced protection against aerosol challenge with virulent *M. bovis*. Heterologous prime-boost immunization strategies using two different types of vaccine vectors, each expressing the same antigen, have been evaluated for protection against *M. tuberculosis* in humans. Studies using prime-boost strategies in cattle employing three DNA vaccines encoding mycobacterial proteins followed by BCG boosting induced significant enhancement of protection compared with BCG alone (Hope and Villarreal-Ramos, 2008).

Tuberculosis in poultry and other avian species

Avian tuberculosis, which occurs worldwide, is usually caused by members of the *M. avium* complex, serotypes 1 to 3. The disease is encountered most often in free-range adult birds. Bacilli, excreted in the faeces of birds with advanced lesions, can survive for long periods in soil.

Non-specific clinical signs including dullness, emaciation and lameness develop in affected birds only when the disease is at an advanced stage. At postmortem examination, granulomatous lesions are characteristically present in the liver, spleen, bone marrow and intestines. Diagnosis is based on the post-mortem findings and on the demonstration of large numbers of ZN-positive bacilli in smears from lesions. Ante-mortem diagnosis of avian tuberculosis in free-range poultry is based on tuberculin testing, using avian PPD injected into the skin of a wattle. *Mycobacterium tuberculosis* occasionally infects parrots and canaries and *M. genavense* has been isolated from pet birds (Hoop *et al*., 1993).

Members of the *M. avium* complex cause infections in immunocompromised humans. Rare cases of generalized disease in cats, dogs and horses caused by members of the complex have been reported. Pigs infected through the ingestion of uncooked swill contaminated with *M. avium* often develop small tuber-cles in the retropharyngeal, submaxillary and cervical lymph nodes.

Feline leprosy

It is generally considered that feline leprosy, a cutaneous disease of worldwide distribution, is caused by *M. lepraemurium*, the aetiological agent of rat leprosy. Sporadic transmission of the organism to cats probably occurs through bites

from infected rodents, the wildlife reservoirs. Nodular lesions, involving subcutaneous tissues, may be solitary or multiple and are usually confined to the head region or the limbs. The nodules, which are fleshy and freely movable, tend to ulcerate. Large numbers of ZN-positive bacilli are present in smears from the lesions. Histopathological examination demonstrates many infiltrating macrophages which contain densely packed mycobacteria.

Mycobacterium lepraemurium, a slow-growing fastidious organism, requires a specially formulated culture medium for growth. It does not appear to be infectious for other species of domestic animals or for humans. Diagnosis is based on the histopathological features of the lesions and negative cultural results for *M. bovis* and opportunistic mycobacteria, which can also cause granulomatous dermatitis in cats. Surgical excision of lesions is the preferred treatment.

Paratuberculosis (Johne's disease)

Paratuberculosis is a chronic, contagious, invariably fatal enteritis which can affect domestic and wild ruminants. The aetiological agent, *M. avium* subsp. *paratuberculosis*, is an acid-fast organism formerly referred to as *Mycobacterium johnei*.

Uncertainty exists regarding the association between infection with *M. avium* subsp. *paratuberculosis* and Crohn's disease, a chronic enteritis in humans (Waddell *et al*., 2008).

Epidemiology

The epidemiology of the disease has been studied in cattle and the pattern of infection and spread in other species is assumed to be similar. Infection is acquired by calves at an early age through ingestion of organisms shed in the faeces of infected animals. Shedding of *M. avium* subsp. *paratuberculosis* in colostrum and milk has been recorded (Taylor *et al*., 1981; Streeter *et al*., 1995). Feeding of pooled colostrum is considered to be a risk factor for the acquisition of *M. avium* subsp. *paratuberculosis* by calves in infected herds although the magnitude of the risk is not clear (Nielsen *et al*., 2008; Stabel, 2008). *Mycobacterium avium* subsp. *paratu-berculosis* may remain viable in the environment for up to a year under suitable conditions.

Although the organism has been isolated from the genital organs and semen of infected bulls (Larsen *et al*., 1981), venereal transmission is unimportant

epidemiologically. *In utero* transmission has been reported but its importance as a means of spread is unknown. Whittington and Windsor (2009) conducted a review and meta-analysis of the literature and calculated the estimated incidence of calves infected by this route for herds with a range of different values for within-herd prevalence. They suggested that incidence could range from 0.44 to 9.3 calves infected *in utero* per 100 cows per year for within-herd prevalence ranging from 5% to 40%. Such incidence rates could be significant in herds in which hygienic calf rearing is practised with separation of calves at birth and feeding of pasteurized colostrum and artificial milk replacer. There are three major subtypes of *M. avium* subsp. *paratubercu-losis*, bovine (type I), ovine (type II) and intermediate (type III) strains: ovine strains have been isolated from cattle and bovine strains from sheep. In addition, *M. avium* subsp. *paratuberculosis* has been isolated from many wildlife species, including rabbits, deer, ferrets and mice. Molecular typing techniques show that strains infecting wildlife and domestic animals sharing the same environment are frequently indistinguishable but the degree to which *M. avium* subsp. *paratuberculosis* strains exhibit host specificity or host preference is unclear at present (Motiwala *et al*., 2006). However, rabbits are considered a key element in the persistence of infection in the environment and may contribute in part to difficulties in controlling Johne's disease (Daniels *et al*., 2003 ; Davidson *et al*., 2009).

Calves under 1 month of age are particularly susceptible to infection and are more likely to develop clinical disease than animals infected later in life. The incubation period of paratuberculosis is protracted and variable. Clinical disease is rarely encountered in cattle under 2 years of age. Signs of disease do not develop in all infected animals; some become subclinical carriers and shed mycobacteria intermittently in their faeces.

Pathogenesis and pathogenicity

Mycobacterium avium subsp. *paratuberculosis* is an intra-cellular pathogen and cell-mediated reactions are mainly responsible for the enteric lesions. Ingested mycobacteria are taken up by M cells over Peyer' s patches. Uptake is through the interaction of fibronectin attachment proteins with fibronectin, followed by binding to integrins on the surface of the M cells. The organisms cross the intestinal epithelial layer and are engulfed by macrophages in which they survive and replicate. Interference with maturation of the phago-some and prevention of phagosome–lysosome fusion appears to be important for intracellular survival of *M. avium* subsp. *paratuberculosis* as is the case for *M. bovis*. As the disease

progresses, an immune-mediated granulomatous reaction develops, with marked lymphocyte and macrophage accumulation in the lamina propria and submucosa. The resulting enteropathy leads to loss of plasma proteins and malabsorption of nutrients and water. The macrophages in the intestinal wall and in the regional lymph nodes contain large numbers of mycobacteria. Two types of lesion are recognized, multibacillary (lepromatous) and paucibacillary (tuberculoid), which appear to be correlated with host immune response. High levels of IL-10 gene expression were detected in cattle with extensive pathological changes and high numbers of bacteria, whereas up-regulation of IFN-γ was recorded in the intestinal tissues of cows with subclinical disease (Sweeney *et al*. 1998' Khalifeh and Stabel, 2004). The prevailing view of the immune response to *M. avium* subsp. *paratuberculosis* is that a T_H1 response predominates early in the post-infection period when disease is subclinical whereas a T_H2 response occurs later in infection during clinical disease (Stabel, 2007).

Clinical signs and pathology

Clinical signs develop in most ruminant species after a prolonged subclinical phase of infection. Affected cattle are usually more than 2 years of age when signs are first observed. The disease is clinically evident only in mature sheep and goats. Clinical signs may develop rapidly in farmed deer and may be evident by 1 year of age.

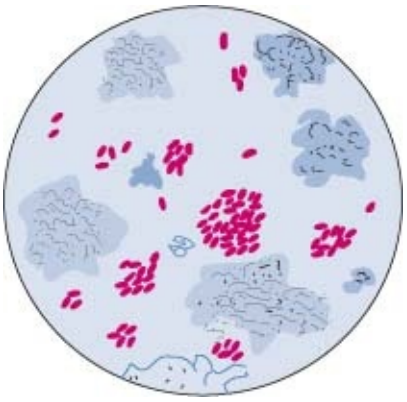
The main clinical feature in cattle is diarrhoea, initially intermittent but becoming persistent and profuse. Progressive weight loss results without loss of appetite, and affected animals seldom survive for more than a year after initial detection.

In sheep and goats, diarrhoea is less marked and may be absent. In some infected deer, there may be rapid weight loss and sudden onset of diarrhoea with death in 2 to 3 weeks. In others, extreme emaciation may develop over a period of months without evidence of diarrhoea (Gilmour and Nyange, 1989).

In cattle, the mucosa of affected areas of the terminal small intestine and the large intestine is usually thickened and folded into transverse corrugations. The mesenteric and ileocaecal lymph nodes are enlarged and oedematous. Thickening of the intestinal mucosa is less marked in sheep, and necrosis and caseation may be present in the regional lymph nodes. Lesions in deer are similar to those in sheep.

Figure 23.4 Clusters of *Mycobacterium avium* subsp. *paratuberculosis* in a

rectal scraping from a cow with Johne's disease. Using the Ziehl-Neelsen method, the short mycobacterial rods, present in clumps, stain red (acid-fast). Faecal and rectal debris stain blue.



Diagnosis

- Paratuberculosis requires differentiation from other chronic wasting diseases in ruminants.
- Specimens for direct microscopy from live animals include scrapings or pinch biopsies from the rectum. Faeces may be submitted for culture and serum for serological tests.
- Post-mortem specimens for histopathological examination from cattle include tissue from affected regions of the intestines and from regional lymph nodes.
- Specimens for microscopical examination should be stained by the ZN technique (Fig. 23.4).
- Isolation of *M. avium* subsp. *paratuberculosis* from faeces or tissues is a sensitive diagnostic procedure but it is difficult and time-consuming. After decontamination of the specimen with 0.3% benzalkonium chloride and concentration by centrifugation, slants of Herrold's egg-yolk medium with and without mycobactin are inoculated with the deposit. Slants are incubated aerobically at 37°C for up to 16 weeks and examined weekly for evidence of growth.
- More rapid isolation techniques, which are based on growth in liquid media and detection of growth using radiometric methods (e.g. BACTEC), are available. These commercial systems are suitable for large specialized laboratories.
- Identification criteria for isolates:

– Colonies less than 1 mm in diameter, usually colourless and hemispherical, appear in 5 to 16 weeks. Isolates from sheep may be pigmented and usually grow considerably more slowly than isolates cultured from cattle and goats.

– Smears from colonies are ZN-positive.

– Medium containing mycobactin supports growth.

– PCR techniques for the detection of insertion sequence IS900 which is specific for *M. avium* subsp. *paratuberculosis*.

- Serological tests:

  – Complement fixation tests have been used but are laborious and relatively insensitive.

  – The agar-gel immunodiffusion test has low sensitivity but may be useful for confirming clinical infection.

  – Several ELISA tests have been developed for the detection of antibodies to *M. avium* subsp. *paratuberculosis*, some of which are commercially available. Nielsen and Toft (2008) evaluated the sensitivity and specificity of these tests and found them to be highly variable. Sensitivity was higher in clinically-affected cattle shedding organisms compared with those infected but not shedding organisms in faeces. ELISA for use with milk is also available.

- Cell-mediated responses:

  – Johnin, the counterpart of tuberculin PPD, may be used as a field test. The preparation is inoculated intradermally or intravenously into cattle. The reliability of the test is questionable and, in addition, it may sensitize cattle to tuberculin.

  – The gamma interferon assay is widely used for early detection of infected animals. As with ELISA, reported sensitivity and specificity of the test are variable (Nielsen and Toft, 2008).

  – Assays based on lymphocyte stimulation appear to be of limited value and are infrequently used.

- DNA probes, which are highly sensitive, are being used to detect *M. avium* subsp. *paratuberculosis* in faeces. Several real-time PCR methods have now been published and are of comparable sensitivity to culture but much more rapid.

Control

- Animals with clinical signs suggestive of paratu-berculosis should be isolated. If the condition is confirmed, affected animals should be slaughtered promptly as they shed large numbers of mycobacteria which can contaminate buildings and pasture.
- Detection and elimination of subclinically affected animals are challenging for clinicians and laboratory staff. Testing should be carried out on a herd or flock basis. Subclinical excretors may be detected by faecal culture at intervals of 6 months or by detection of *M. avium* subsp. *paratuberculosis* in faeces using DNA probes. Serology, using the absorbed ELISA, may detect subclinical infection.
- In problem herds, appropriate hygiene and husbandry measures should be instituted to prevent infection of young susceptible animals. Calves should be separated from their dams at birth and raised on pasteurized milk. They should remain isolated from the herd for 2 years.
- Inactivated adjuvanted and live attenuated whole-cell vaccines are available. In cattle, vaccination may reduce the number of clinical cases but cannot be relied on to eliminate the disease from a herd. Because vaccinated animals usually become sensitized to tuberculin, vaccine use in some countries is subject to regulatory control. The development of new subunit vaccines for the prevention of paratu-berculosis infections in animals offers improved options for future control measures (Rosseels and Huygen, 2008). These vaccines are based on immunodominant protein antigens which stimulate a strong T_H1 response, and some vaccines may enable serological differentiation of vaccinated and naturally infected animals as naturally infected animals produce a poor antibody response to these particular protein antigens. However, limited data are available to date on the performance of these newer vaccines in experimentally infected animals or from field trials.

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Chapter 24

Enterobacteriaceae

Bacteria belonging to the family *Enterobacteriaceae* are Gram-negative rods up to 3 μm in length (Fig. 24.1) which ferment glucose and a wide range of other sugars and are oxidase-negative. They are catalasepositive, non-spore-forming facultative anaerobes which grow well on MacConkey agar because they are not inhibited by the bile salts in the medium. These enteric organisms reduce nitrates to nitrites, and some species, notably *Escherichia coli*, ferment lactose. The motile enterobacteria have peritrichous flagella. The family contains more than 40 genera and over 180 species. Less than half of the genera are of veterinary importance (Fig. 24.2). The term 'coliform', formerly only used to describe enterobacteria capable of fermenting lactose, is now sometimes used to describe other members of the family.

Enterobacteria can be arbitrarily grouped into three categories: major pathogens, opportunistic pathogens and non-pathogens. Those without pathogenic significance for animals, such as *Hafnia* and *Erwinia*, can be isolated from faeces and from the environment and may contaminate clinical specimens. Opportunistic pathogens occasionally cause clinical disease in locations other than the alimentary tract. The major animal pathogens *E. coli*, *Salmonella* species and *Yersinia* species can cause both enteric and systemic disease.

Usual habitat

Bacteria belonging to the *Enterobacteriaceae* have a worldwide distribution, inhabit the intestinal tract of animals and humans and contaminate vegetation, soil and water. Some members of the family constitute part of the normal flora of the intestinal tract whereas some of the pathogenic organisms are disseminated by clinical and subclinical excretors and by survival in the environment.

Key points

- Gram-negative rods

- Growth on non-enriched media
- Oxidase-negative
- Facultative anaerobes, catalase-positive
- Most are motile by peritrichous flagella
- Ferment glucose, reduce nitrate to nitrite
- Enteric bacteria which tolerate bile salts in MacConkey agar
- Cause a variety of clinical infections
- Major enteric and systemic pathogens:
  – *Escherichia coli*
  – *Salmonella* serotypes
  – *Yersinia* species
- Opportunistic pathogens:
  – *Proteus* species
  – *Enterobacter* species
  – *Klebsiella* species
  – Some other members of the *Enterobacteriaceae*

Differentiation of the *Enterobacteriaceae*

Gram-negative rods which are oxidase-negative, facultative anaerobes and grow on MacConkey agar are presumed to be members of the *Enterobacteriaceae*. The main criteria for differentiating pathogenic members are presented in Table 24.1. Few enterobacteria, apart from some strains of *E. coli*. produce haemolysis on blood agar.

- Lactose fermentation in MacConkey agar:
  – The colonies of lactose fermenters and the surrounding medium are pink due to acid production from lactose.
  – The colonies of non-lactose fermenters and the surrounding medium have a pale appearance and are alkaline due to utilization of peptones in the medium.
- Reactions on selective/indicator media:
  – A number of commonly used media, including brilliant green (BG) agar and xylose–lysine– deoxycholate (XLD) agar, are used to differentiate *Salmonella* from other enteropathogens. On BG agar, *Salmonella* colonies and the surrounding medium show a red alkaline reaction. On XLD medium the colonies of most *Salmonella* serotypes are red (alkaline reaction) with black centres due to hydrogen sulphide (H_2S) production.

– A wide range of chromogenic agars have been developed for the easy identification and enumeration of *E. coli* in clinical samples and food specimens. Examples include eosin–methylene blue (EMB) agar on which the colonies of some isolates of *E. coli* have a unique metallic sheen. Harlequin™ TBGA (Lab M, Bury, Lancashire, UK), which can be used for identification and enumeration of *E. coli*, is based on β-glucuronidase activity, an enzyme which is highly specific for *E. coli*. On this medium colonies develop a blue– green colour.

- Colonial morphology:

  – Mucoid colonies are typical of *Klebsiella* and *Enterobacter* species while rare isolates of *E. coli* are mucoid.

  – *Proteus* species produce characteristic swarming on non-inhibitory media such as blood agar.

  – *Serratia marcescens* is unique among the opportunistic pathogens in its ability to produce red pigment.

- Reactions in triple sugar iron (TSI) agar:

  – This is a non-inhibitory indicator medium used primarily to confirm that colonies isolated on BG, XLD or other selective media are those of *Salmonella*. Other members of the *Enterobacteriaceae* isolated on BG or XLD media can be differentiated by their reactions in TSI. Triple sugar iron agar contains 0.1% glucose, 1% lactose and 1% sucrose and chemicals to indicate H_2S production. Phenol red is used as an indicator for pH change (red at pH 8.2, yellow at pH 6.4). A black precipitate of ferrous sulphide is indicative of H_2S production. An inoculum from a single isolated colony of the organism under test is stabinoculated with a straight wire into the butt of the TSI agar, and on withdrawal the slant surface is inoculated. Loosely capped tubes are incubated for 18 hours at 37°C. The reactions in this medium of the more important members of the *Enterobacteriaceae* are presented in Table 24.2.

- Additional biochemical tests:

  – The lysine decarboxylase production test is used to distinguish *Proteus* species from *Salmonella* species as these organisms have similar reactions in TSI agar. *Proteus* species are negative in the test, whereas *Salmonella* species invariably produce the enzyme. Production of lysine decarboxylase is indicated by a purple colour in the liquid medium; in a negative test, the medium is yellow.

– Urease production distinguishes *Proteus* species from *Salmonella* species. *Proteus* species produce urease whereas *Salmonella* species do not.

– The IMViC (indole production, methyl red test, Voges-Proskauer test, citrate utilization) tests are a group of biochemical reactions used to differentiate *E. coli* from other lactose fermenters (Table 24.1).

– Tests for motility allow differentiation of *Klebsiella* species (non-motile) from *Enterobacter* species (motile). Both species produce similar mucoid colonies which are difficult to distinguish visually (Table 24.1).

- Commercial biochemical tests:

  – A number of commercial biochemical test systems are available for differentiating enterobacteria. Some of these systems incorporate a wide range of biochemical tests and results can be matched against computer-generated numerical profiles to identify isolates to a species level.

- Serotyping of *E. coli*, *Salmonella* and *Yersinia* species:

  – Slide agglutination tests with antisera are used to detect O (somatic) and H (flagellar) antigens in all three species and sometimes detection of K (capsular) antigens is carried out (Fig. 24.3). Serotyping allows identification of the organisms involved in disease outbreaks and has applications in epidemiological investigations.

- Molecular techniques, usually based on PCR, are increasingly used both for detection of enterobacteria in clinical or food samples and for identification of suspect isolates.

- Molecular typing techniques are now widely used for epidemiological tracing of members of the *Enterobacteriaceae* and it is likely that these techniques will eventually replace older methods such as serotyping. Digestion by restriction enzymes followed by pulsed-field gel electrophoresis (PFGE) is used for tracing the source of outbreaks and is extensively used in the investigation of *Salmonella* infections in humans. The Pulse-Net programme of the Center for Disease Control in the USA is a system whereby PFGE profiles of isolates from around the world can be compared using an electronic computerized database. Multilocus sequence typing does not provide the same degree of discrimination as PFGE-based techniques but it can be used for comparing the genetic relatedness of isolates from different geographical regions worldwide.

Figure 24.1 Medium-sized rods of members of the *Enterobacteriaceae*,

morphologically indistinguishable from some other Gram-negative organisms.

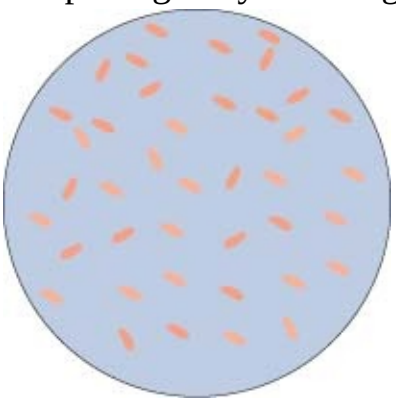


Figure 24.2 Members of the *Enterobacteriaceae* of veterinary importance.

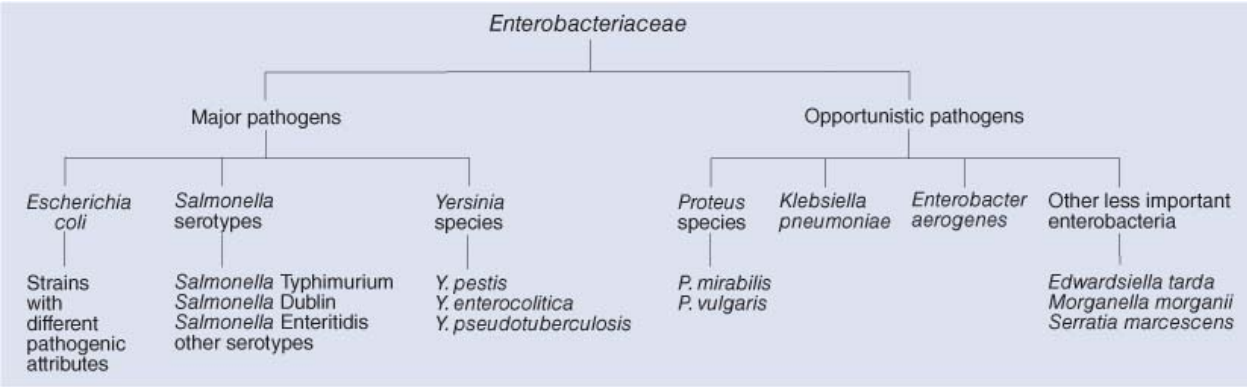


Table 24.1 The clinical relevance, growth characteristics and biochemical reactions of members of the *Enterobacteriaceae* which are of veterinary importance.

	Escherichia coli	*Salmonella* serotypes	*Yersinia* species	*Proteus* species	*Enterobacter aerogenes*	*Klebsiella pneumoniae*
Clinical importance	Major pathogen	Major pathogens	Major pathogens	Opportunistic pathogens	Opportunistic pathogen	Opportunistic pathogen
Cultural characteristics	Some strains haemolytic	–	–	Swarming growth[a]	Mucoid	Mucoid
Motility at 30°C	Motile	Motile	Motile[b]	Motile	Motile	Non-motile
Lactose fermentation	+	–	–	–	+	+
IMViC tests						
Indole production	+	–	v	±[c]	–	–
Methyl red test	+	+	+	+	–	–
Voges-Proskauer	–	–	–	v	+	+
Citrate utilization test	–	+	–	v	+	+
H_2S production in TSI agar	–	+	–	+	–	–
Lysine decarboxylase	+	+	–	–	+	+
Urease activity	–	–	+[b]	+	–	+

a, when cultured on non-inhibitory medium.

b, except *Y. pestis*.

c, *P. vulgaris* +; *P. mirabilis* −.

v, reaction varies with individual species.

Table 24.2 Reactions of the *Enterobacteriaceae* of veterinary importance in triple sugar iron (TSI) agar.[a]

Species	pH change[b]		H_2S production
	Slant	Butt	
Salmonella serotypes[c]	Red	Yellow	+[d]
Proteus mirabilis	Red	Yellow	+
P. vulgaris	Yellow	Yellow	+
Escherichia coli	Yellow	Yellow	–
Yersinia enterocolitica	Yellow	Yellow	–
Y. pseudotuberculosis and *Y. pestis*	Red	Yellow	–
Enterobacter aerogenes	Yellow	Yellow	–
Klebsiella pneumoniae	Yellow	Yellow	–

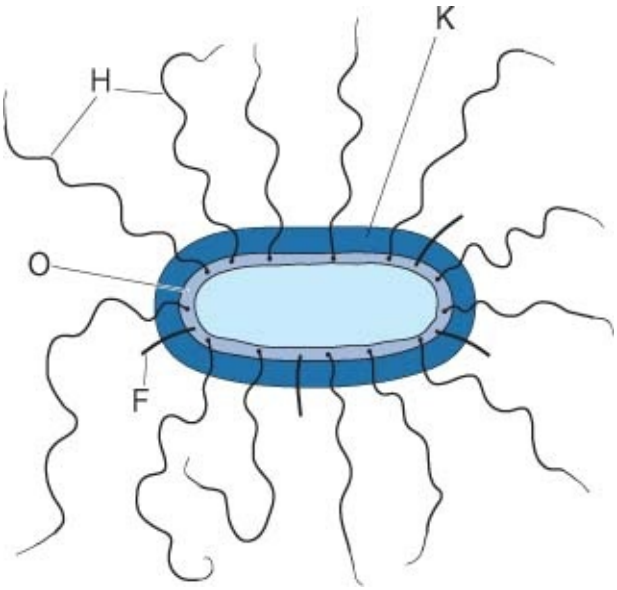
a, the majority of strains give the reactions indicated.

b, red, alkaline; yellow, acid.

c, *Salmonella* serotypes and *Proteus* species can be differentiated by lysine decarboxylase production and urease activity (see Table 24.1).

d, exceptions include *S.* Choleraesuis.

Figure 24.3 Schematic diagram of a typical member of the *Enterobacteriaceae* indicating the K (capsular), O (somatic), F (fimbrial) and H (flagellar) antigens used for serotyping isolates.



Escherichia coli

Escherichia coli is usually motile with peritrichous flagella and is often fimbriate. This lactose fermenter produces pink colonies on MacConkey agar and has characteristic biochemical reactions in IMViC tests (Table 24.1). Some strains produce colonies with a metallic sheen when grown on eosin–methylene blue agar. Haemolytic activity on blood agar is a characteristic of certain strains of *E. coli*.

Somatic (O), flagellar (H) and sometimes capsular (K) antigens are used for serotyping *E. coli*. The somatic antigens are lipopolysaccharide in nature and located at the surface of the cell wall. The specificity of these antigens is determined by carbohydrate side chains. The flagellar antigens are protein in nature and the capsular antigens are composed of polysaccharides. Proteinaceous fimbrial (F) antigens are present in many strains and act as adhesins facilitating attachment to mucosal surfaces.

Colonization of the mammalian intestinal tract by *E. coli* from environmental sources occurs shortly after birth. These organisms persist as important members of the normal flora of the intestine throughout life. Most strains of *E. coli* can be regarded as commensal organisms and are of low virulence but may cause opportunistic infections in extraintestinal locations such as the mammary gland and urinary tract. Strains of *E. coli* which produce extraintestinal disease frequently colonize the intestinal tract of normal animals also. Strains that cause enterocolitis are not usually part of the normal flora of healthy animals and

infection results from direct contact with clinically or subclinically infected animals or by ingestion of contaminated food or water. Pathogenic strains of *E. coli* possess virulence factors which allow them to colonize mucosal surfaces and subsequently produce disease. Predisposing factors that permit colonization and render animals susceptible to the development of clinical disease include age, immune status, nature of diet and heavy exposure to pathogenic strains.

The main categories of pathogenic strains of *E. coli* in animals and their clinical effects are presented in Figs 24.4 and 24.5. In general, strains can be divided into those causing enteric disease and those responsible for extraintestinal infections. However, not all strains conform strictly to these categories in that strains in one category may induce pathogenic effects similar to those induced by strains in the other category.

In recent years, *E. coli* O157:H7 and other enterohaemorrhagic serotypes have emerged as major food-borne, zoonotic pathogens in humans, responsible for the haemorrhagic colitis–haemolytic uraemic syndrome.

Pathogenesis and pathogenicity

The virulence factors of pathogenic strains of *E. coli* include capsules, endotoxin, structures responsible for adherence and colonization, enterotoxins and other secreted substances.

- Capsular polysaccharides, which are produced by some *E. coli* strains, interfere with the phagocytic uptake of these organisms. Capsular material, which is weakly antigenic, also interferes with the antibacterial effectiveness of the complement system.
- Endotoxin, a lipopolysaccharide (LPS) component of the cell wall of Gram-negative organisms, is released on death of the bacteria. It is composed of a lipid A moiety, core polysaccharide and specific side chains. The role of LPS in disease production includes pyrogenic activity, endothelial damage leading to disseminated intravascular coagulation, and endotoxic shock. These effects are of greatest significance in septicaemic disease.
- Fimbrial adhesins which are present on many strains of *E. coli* allow attachment to mucosal surfaces in the small intestine and in the lower urinary tract. Firm attachment to the mucosa facilitates colonization by diminishing the expulsive effects of peristalsis and the flushing effect of urine. Many fimbrial adhesins have been identified. The most significant adhesins in strains of *E. coli* producing disease in domestic animals are K88

(F4), K99 (F5), 987P (F6), F18 and F41. The more recent system of nomenclature uses ‘F’ and a number to identify particular fimbriae. The reason for the ‘K’ used under the older system is because originally some of the fimbrial adhesins were mistakenly thought to be capsular (K) antigens. The use of ‘P’ derives from the term pilus, because fimbriae are sometimes known as pili. The most common adhesin present in strains of *E. coli* infecting pigs is K88. The receptor for the K88 antigen is encoded by a dominant gene and thus if a sow is homozygous recessive for the gene, her colostrum contains no anti-K88 antibody, resulting in highly susceptible piglets. F41 adhesins occur in calves and K99 in calves and lambs. Although the numbers of receptors for K88 adhesins on pig enterocytes decline with age, $K88^{+}$*E. coli* strains may cause diarrhoea in piglets after weaning. Receptors for F18 are found only in older piglets and thus $F18^{+}$ strains are important in postweaning diarrhoea and oedema disease. Although neonatal piglets are susceptible to strains of *E. coli* bearing F6 adhesins, resistance to colonization develops by 3 weeks of age. Both K88 and K99 adhesins are encoded by plasmids.

- An adhesin termed intimin is associated with attaching and effacing *E. coli* (AEEC). This adhesin is one of the products of genes encoded in a pathogenicity island termed the locus of enterocyte effacement (LEE). Intimin is encoded by the enterocyte attaching and effacing (*eae*) gene and is an outer membrane protein of AEEC strains. Intimin binds to the translocated intimin receptor (Tir) and this receptor is also encoded by the LEE and is translocated into the host cell by the bacterium where it forms a receptor to which intimin can bind.
- AEEC produce characteristic attaching and effacing lesions in which the bacteria are intimately adherent to the host epithelial cell membrane with marked rearrangement of the cytoskeleton. In addition, the lesion involves pedestal formation, effacement of microvilli, premature enterocyte exfoliation and villous distortion. Although AEEC strains possess the LEE, the exact mechanisms which result in the attaching and effacing lesion are unclear. The LEE is known to encode several secreted proteins, a type III secretion apparatus, intimin and the intimin receptor Tir, but the detailed events at cellular level which result in lesion production are not yet fully elucidated.
- The pathological effects of infection with pathogenic *E. coli*, other than those attributed to endotoxin, derive mainly from the production of

enterotoxins, shigatoxins or verotoxins or cytotoxic necrotizing factors (Figs 24.4 and 24.5). Unlike enterotoxins which affect only the functional activity of enterocytes, shigatoxins and cytotoxic necrotizing factors can produce demonstrable cell damage at their sites of action.

– Two types of enterotoxins, heat-labile (LT) and heat-stable (ST), have been identified. Each type of enterotoxin has two subgroups. Many strains of enterotoxigenic *E. coli* (ETEC) from pigs produce LT1 which induces hypersecretion of fluid into the intestine through stimulation of adenylate cyclase activity. Most ETEC isolates which produce LT1 also possess K88 adhesins. A second heat-labile toxin, LT2, has been demonstrated in some ETEC strains isolated from cattle. One of the heat-stable enterotoxin subgroups, STa, has been identified in strains of ETEC isolated from porcine, bovine, ovine and human specimens. This toxin induces increased guanylate cyclase activity in enterocytes, and the resultant increase in intracellular guanosine monophosphate stimulates fluid and electrolyte secretion into the small intestine and inhibits fluid absorption from the intestine. The heat-stable enterotoxin STb also causes secretion of chloride and bicarbonate ions as well as inhibition of absorption of sodium ions through mechanisms which differ from those of STa and LT1.

- Enteroaggregative heat stable toxin 1 (EAST1) is found in some enterotoxigenic *E. coli* (ETEC) and enteropathogenic *E. coli* (EPEC) strains, and is found in all enterohaemorrhagic *E. coli* (EHEC) strains. It also acts through the stimulation of cyclic GMP but strains producing EAST1 alone are incapable of causing diarrhoea (Nagy and Fekete, 2005).

  – Verotoxins (VT), also known as shigatoxins (ST), are similar structurally, functionally and antigenically to the shigatoxin of *Shigella dysenteriae*. These toxins are heat-labile and lethal for cultured Vero cells. Shigatoxigenic *E. coli* (STEC) colonizing the intestines can damage enterocytes and, when shigatoxin is absorbed into the bloodstream, it exerts a deleterious effect on endothelial cells in relatively defined anatomical locations such as the central nervous system in pigs. Shigatoxins inhibit protein synthesis in eukaryotic cells but the relatively greater degree of damage induced in certain tissues may relate to differences in receptors for these toxins. Vascular damage can lead to oedema, haemorrhage and thrombosis. The shigatoxin ST2e is implicated in oedema disease of pigs.

  – Cytotoxic necrotizing factors, CNF1 and CNF2, and recently CNF3

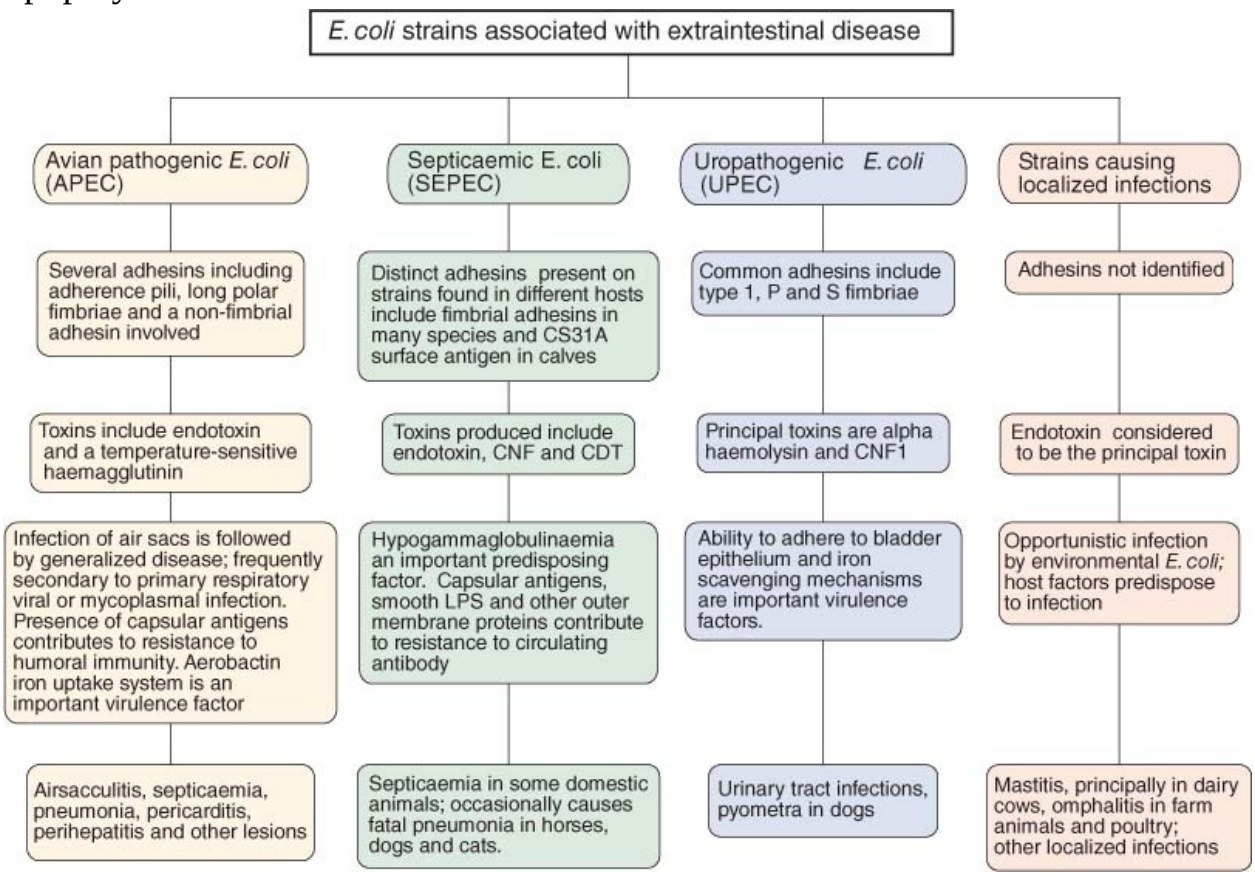
(Orden *et al*., 2007), have been demonstrated in extracts of strains of *E. coli* isolated from cases of extraintestinal *E. coli* infections in animals and humans. It is known that CNF1 is encoded chromosomally whereas CNF2 is encoded by a transmissible plasmid known as Vir. Although these toxins have been shown to induce pathological changes in laboratory animals and culture cells, the exact role of CNF toxins in the production of disease in domestic animals is still uncertain.

- Alpha-haemolysin, although often a useful marker for virulence in certain strains of *E. coli*, does not appear to contribute directly to their virulence but is closely linked with the expression of other virulence factors. Haemolysin production is often a feature of strains of *E. coli* isolated from pigs with oedema disease and diarrhoea. It has been suggested that the action of alpha-haemolysin may increase the availability of iron for invading organisms.
- Siderophores, iron-binding molecules such as aerobactin and enterobactin, are synthesized by certain pathogenic strains of *E. coli*. When available iron levels in the tissues are low, these iron-binding molecules may contribute to bacterial survival.

Figure 24.4 Pathotypes of *E. coli* which produce enteric conditions in animals and humans.

Figure 24.5 Pathotypes of *E. coli* which produce extraintestinal clinical

conditions in animals. Where characterized, their virulence factors are indicated. CNF, cytotoxic necrotizing factor; CDT, cytolethal distending toxin; LPS, lipopolysaccharide.



Clinical infections

The main categories of disease produced by pathogenic strains of *E. coli* are indicated in Figs 24.4 and 24.5. Clinical infections in young animals may be limited to the intestines (enteric colibacillosis, neonatal diarrhoea), or may manifest as septicaemia (colisepticaemia, systemic colibacillosis) or toxaemia (colibacillary toxaemia). In older pigs, post-weaning enteritis and oedema disease are manifestations of toxaemia. Extraintestinal localized infections in adult animals, many due to opportunistic invasion, can involve the urinary tract, mammary glands and uterus.

Enteric colibacillosis

Enteric colibacillosis primarily affects newborn calves, lambs and piglets. Oral infection with a pathogenic strain of *E. coli*, colonization of the intestine and

toxin production are prerequisites for the development of this condition. The incidence and severity of the disease increases under intensive systems of management. This may reflect heavy exposure of young animals to pathogenic strains of *E. coli* as a result of build-up of infection in the environment. Factors that may predispose young farm animals to infection by strains of pathogenic *E. coli* are summarized in Box 24.1. Enterotoxigenic strains of ETEC, possessing fimbrial adhesins such as K88 and K99, are of particular importance in neonatal diarrhoea. These strains colonize the distal small intestine by attaching to receptors which are present on the enterocytes of neonates. They produce enterotoxins (LT and STa) which stimulate hypersecretory diarrhoea and interfere with fluid absorption without major morphologically detectable damage to enterocytes. In contrast, necrosis of enterocytes with stunting and fusion of villi are features of enteric colibacillosis caused by strains of attaching/ effacing *E. coli*, which colonize the lower small intestine and the colon. These strains induce diarrhoea through maldigestion and malabsorption of nutrients in the small intestine and by reducing the absorptive capacity of the colonic mucosa.

Box 24.1 Factors which may predispose young farm animals to infection with pathogenic *Escherichia coli* strains

- Insufficient or no colostral immunity
- Build-up of pathogenic *E.coli* strains
- Overcrowding and poor hygiene, facilitating increased transmission of organisms
- Normal flora of neonates not fully established
- Naive immune system in neonates
- Receptors for ETEC adhesins are present only during first week of life in calves
- Pigs retain receptors for some adhesins past weaning age (post-weaning diarrhoea)
- Digestive tract of young pigs equipped only for easily digested foods. Accumulation of undigested and unabsorbed nutrients encourages replication of *E. coli*
- Stress factors such as cold ambient temperatures and frequent mixing of animals

In enteric colibacillosis in calves, diarrhoea develops within the first few days of life. Faecal consistency is somewhat variable. In some cases faeces are profuse and watery, in others they are pasty, white or yellowish and rancid. This rancid faecal material may accumulate on the tail and hind limbs. Depression becomes marked as dehydration and acidosis develop. Mildly affected animals may recover spontaneously. Without treatment, severely affected calves die within a few days.

Piglets may succumb to enteric colibacillosis within 24 hours of birth. Often, an entire litter is affected and, as the disease progresses, piglets refuse to suck. A profuse watery diarrhoea rapidly leads to dehydration, weakness and death. Although enteric colibacillosis occasionally affects lambs, the septicaemic form of the disease is more common.

Colisepticaemia

Systemic infections with *E. coli* are relatively frequent in calves, lambs and poultry. Septicaemic strains of *E. coli* have special attributes for resisting host defence mechanisms. They invade the bloodstream following infection of the intestines, lungs or umbilical tissues (navel ill).

Septicaemic spread throughout the body commonly occurs in calves with low levels of maternally-derived antibodies and the severity of the disease corresponds to the degree of hypogammaglobulinaemia (Penhale *et al*., 1970). Colisepticaemia often presents as an acute fatal disease with many of the clinical signs attributable to the action of endotoxin. Pyrexia, depression, weakness and tachycardia, with or without diarrhoea, are early signs of the disease. Hypothermia and prostration precede death which may occur within 24 hours. Meningitis and pneumonia are commonly encountered in affected calves and lambs. Postsepticaemic localization in the joints of calves and lambs results in arthritis with swelling, pain, lameness and stiff gait.

Watery mouth occurs in lambs up to 3 days of age and has been associated with systemic invasion by *E. coli* (King and Hodgson, 1991; Sargison *et al*., 1997). It is characterized by severe depression, loss of appetite, profuse salivation and abdominal distension. The condition is encountered in lambs born in confined lambing areas. Morbidity rates may exceed 20% and mortality in affected lambs is high, many dying within 24 hours of clinical onset. Death is attributed to endotoxic shock.

In poultry, airsacculitis and pericarditis may develop following septicaemia. Coligranuloma (Hjärre's disease) is characterized by chronic inflammatory changes resembling tuberculous lesions which are encountered at post-mortem examination in laying hens.

Oedema disease of pigs

Oedema disease is a toxaemia which usually occurs 1 to 2 weeks after weaning in rapidly growing pigs. The aetiology of the disease is complex, with nutritional and environmental changes and other stress factors contributing to its development. A limited number of haemolytic *E. coli* serotypes have been isolated from the intestinal tract in cases of the disease. These noninvasive strains replicate in the tract and produce a shigatoxin (ST2e) which is absorbed into the bloodstream and damages endothelial cells with consequent perivascular oedema.

The onset of oedema disease is sudden, with some animals found dead without showing clinical signs. Characteristic signs include posterior paresis, muscular tremors and oedema of the eyelids and the front of the face. The squeal may be hoarse due to laryngeal oedema. The faeces are usually firm. Flaccid paralysis precedes death which typically occurs within 36 hours of the onset of clinical signs. Animals that recover frequently have residual neurological dysfunction. The characteristic post-mortem lesions are oedema of the greater curvature of the stomach and the mesentery of the colon. Perivascular oedema in the central nervous system, detectable on histological examination, accounts for the neurological dysfunction. Cerebrospinal angiopathy, in which there is marked fibrinoid necrosis in vessel walls, may develop in animals surviving acute oedema disease.

Post-weaning diarrhoea of pigs

This condition occurs within a week or two after weaning, often following changes in feeding regimens or in management and with possible involvement of rotaviruses. The majority of outbreaks are associated with ETEC strains but EPEC may be involved. Clinical signs vary from an afebrile disease with inappetence to watery diarrhoea in severe cases. Diarrhoea and purplish discoloration of areas of the skin are often observed. Some animals may die suddenly (van BéersSchreurs *et al*., 1992).

Coliform mastitis

Infection of the mammary glands of cows and sows by members of the *Enterobacteriaceae*, including *E. coli*, occurs opportunistically. In dairy cows, the source of infection is faecal contamination of the skin of the mammary gland, and relaxation of the teat sphincter following milking increases vulnerability to infection. Host factors are of major importance in determining whether disease occurs (see Chapter 93). Cows with low somatic cell counts are particularly susceptible to infection. This form of mastitis has not been linked to specific serotypes of *E. coli*. The acute form of the disease is characterized by endotoxaemia and can be life-threatening. Peracute disease may be fatal within 24 to 48 hours. Affected animals are severely depressed with drooping ears and sunken eyes. Mammary secretions are watery and contain white flecks.

Urogenital tract infections

Opportunistic ascending infections of the urinary tract by certain uropathogenic strains of *E. coli* result in cystitis, especially in bitches. These strains possess virulence factors such as fimbriae which facilitate mucosal colonization.

Invasion of hyperplastic endometrium by opportunistic strains of *E. coli* is a critical factor in the pathogenesis of canine pyometra. Prostatitis in dogs is also associated with invasion by opportunistic *E. coli* strains.

Diagnostic procedures

The age and species of the affected animal, the clinical signs and the duration of illness may suggest the type of infection and the category of disease. The selection of specimens, the laboratory procedures for diagnosis and appropriate treatment and control measures are influenced by the history, the progress of the disease and the system or organ affected.

- Suitable specimens include faecal samples from animals with enteric disease, tissue specimens from cases of septicaemia, mastitic milk, samples of midstream urine and cervical swabs from suspected cases of pyometra or metritis.
- Specimens cultured on blood and MacConkey agar are incubated aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates:
  - On blood agar, colonies are greyish, round and shiny with a

characteristic smell. Colonies may be haemolytic or non-haemolytic.

– On MacConkey agar, colonies are bright pink.

– IMViC tests can be used for confirmation (Table 24.1).

– The colonies of some *E. coli* strains have a metallic sheen on EMB agar.

– A full biochemical profile may be necessary to identify isolates from coliform mastitis or cystitis.

– Some serotypes are found in association with certain disease conditions. Slide agglutination tests for O and H antigens are employed for serotype identification.

- In suspected cases of colisepticaemia, isolation of the organism in pure culture from the blood or from parenchymatous organs is considered confirmatory.
- When enterotoxigenic strains of *E. coli* are suspected, the presence of either enterotoxins or fimbrial antigens can be confirmed by immunological methods or molecular techniques such as the polymerase chain reaction.

  – Enterotoxins in the small intestine can be detected, using methods employing monoclonal antibodies (Carroll *et al*., 1990). Some of these reagents are available commercially.

  – For expression of fimbrial antigens, isolates should be subcultured on Minca medium. Fimbrial antigens can be identified using ELISA or latex agglutination (Thorns *et al*., 1989).

  – PCR techniques using primers specific for genes encoding heat-labile and heat-stable enterotoxins may be used to identify enterotoxigenic strains of *E. coli*. Gene probes and PCR primers for the detection of enterotoxins and other virulence factors of *E. coli* of veterinary significance are reviewed by DebRoy and Maddox (2001).
- Although the toxins produced by shigatoxigenic and necrotoxigenic strains can be detected by Vero cell assay (Wray *et al*., 1993), it is now more usual to use PCR-based methods using primers specific for genes encoding the toxins. PCR primers used for the identification and characterization of attaching and effacing *E. coli* have been developed (Fröhlicher *et al*., 2008).

Treatment

The nature and duration of therapeutic measures are determined by the severity and duration of the disease process.

- In calves with neonatal diarrhoea, milk should be supplemented with fluids containing electrolytes. Severely dehydrated calves require parenteral fluid replacement therapy.
- Calves with hypogammaglobulinaemia can be given bovine gammaglobulin intravenously.
- In most domestic species, enteric diseases may be treated, if required, by oral administration of antimicrobial compounds which are active in the gastrointestinal tract. Systemic and localized infections require parenteral administration of therapeutic agents. Treatment should be based on susceptibility testing of isolates.
- Antimicrobial resistance is a major problem with respect to *E. coli* organisms, particularly those isolated from intensively-reared farm animals such as pigs and poultry. Multiple resistance to three or more classes of antimicrobial agents is common in isolates of *E. coli* from pigs and poultry at time of slaughter and in clinical isolates from all animals, both farm animals and pets (White *et al*., 2002; Anon., 2004a; Fairbrother *et al*., 2005; Lloyd, 2007). Increasing resistance is of importance because resistant *E. coli* may be transferred from animals to humans, either through food or by direct contact. In addition, multiple resistance in *E. coli* infections in animals is significant because it limits therapeutic options and may lead to treatment failure in some cases. Antimicrobial resistance to newer agents is a particular concern in animal isolates of *E. coli*, and isolates producing extended spectrum β-lactamases have been reported in many countries and in most domestic animal species (Li *et al*., 2007). Resistance to fluoroquinolones is an emerging problem in animals, particularly in samples from clinical cases (Anon., 2004a,b).
- Because of the extensive local tissue damage, intramammary treatment of coliform mastitis is often of limited value. Therapy is aimed at counteracting shock and eliminating toxic material from the mammary gland by frequent stripping of affected quarters.

Control

- Newborn animals should receive ample amounts of colostrum shortly after birth. Colostral antibodies can prevent colonization of the intestine by pathogenic *E. coli*. Absorption of gammaglobulin from the intestine declines progressively after birth and is negligible by 36 hours.

- A clean, warm environment should be provided for newborn animals.
- Dietary regimes may contribute to the development of oedema disease and other post-weaning conditions. To avoid factors that may contribute to the occurrence of disease, new feed should be introduced gradually.
- Vaccination is of value for a limited number of the diseases caused by *E. coli*. Vaccination methods used for prevention of enteric disease in piglets and calves include:

  – Vaccination of pregnant cows with purified *E. coli* K99 fimbrial or whole-cell preparations, often combined with rotavirus antigen, can be used to enhance colostral protection (Snodgrass, 1986).

  – Commercially available killed vaccines containing prevalent pathogenic *E. coli* serotypes can be given to pregnant sows. Research is ongoing into the development of oral vaccines for postweaning diarrhoea in pigs which contain live attenuated *E. coli* strains carrying the particular fimbrial adhesins involved in disease production (Fairbrother *et al*., 2005).

  – A commercial vaccine is available for protection against *E. coli* mastitis in cows (see Chapter 93).
- Breeding for disease resistance can be employed and has been used commercially to reduce the number of susceptible pigs. In Switzerland, breeding policies have been implemented which greatly reduced the number of pigs which were susceptible to *E. coli* F18-associated diarrhoea (Fairbrother *et al*., 2005).
- Control of *E. coli* infections in animals is important not only for the prevention of disease in animals but also for public health reasons. Much research has been carried out into methods of controlling enterohaemorrhagic *E. coli* O157 in cattle. To date, only the feeding of a probiotic product containing *Lactobacillus acidophilus* has been consistently shown to reduce the shedding of *E. coli* O157 (LeJeune and Wetzel, 2007). This product is available commercially in the United States.

Salmonella serotypes

Salmonellae are usually motile and do not ferment lactose (Table 24.1). Rarely, lactose-fermenting strains are encountered. The genus *Salmonella* contains more than 2,500 serotypes, based on a system devised by Kaufmann and White in which somatic (O) and flagellar (H) antigens are identified. Occasionally,

capsular (Vi) antigens may be detected. In a modification of this scheme, two species were proposed, *S. enterica* and *S. bongori*, and this system was accepted by the Judicial Commission of the International Committee on Systematics of Prokaryotes and published as Opinion 80 in 2005 (Anon., 2005). *Salmonella enterica* has been divided into six subspecies (Le Minor and Popoff, 1987; Reeves *et al*., 1989). The majority of salmonellae of veterinary importance belong to *S. enterica* subspecies *enterica*. The subspecies are further qualified by the serotype to give a final designation such as *S. enterica* subspecies *enterica* serotype Typhimurium. A phage typing system has been developed for a small number of serotypes such as Typhimurium and Enteritidis and is used for epidemiological investigation of isolates.

Salmonella serotypes occur worldwide. They infect many mammals, birds and reptiles and are excreted mainly in faeces. Ingestion is the main route of infection in salmonellosis although it can also occur through the mucosae of the upper respiratory tract and conjunctiva (Fox and Gallus, 1977). Colonization and persistence in the tonsils appear to be important in pigs (Boyen *et al*., 2008). Organisms may be present in water, soil, animal feeds, raw meat and offal, and vegetable material. The source of environmental contamination is invariably faeces. In poultry, some serotypes such as *Salmonella* Enteritidis infect the ovaries, and the organisms can be isolated from eggs. Salmonellae can survive in damp, shaded soil for up to 9 months (Carter *et al*., 1979), in empty poultry houses for at least a year and over 2 years in poultry feed (Davies and Wray, 1996).

Pathogenesis and pathogenicity

Most serotypes of *Salmonella enterica* can infect a wide range of host animals but host adaptation occurs with some serotypes and is observed with particular phage types also. The molecular basis of host specificity is not fully elucidated but host-adapted serotypes tend to cause more severe disease than non-adapted sero-types. The virulence of *Salmonella* serotypes relates to their ability to invade and replicate in epithelial cells. Survival within macrophages is necessary for development of systemic disease. Many of the virulence features of salmonellae are encoded on *Salmonella* pathogenicity islands (SPI) and on virulence plasmids. *Salmonella* pathogenicity islands comprise clusters of virulence genes situated on the bacterial chromosome or on plasmids, and 18 of these ‘islands’ have been described to date (Hensel, 2004Fuentes *et al*., 2008).

Not all serovars contain all 18 SPIs, and the exact functions of each pathogenicity island have not yet been clarified.

Once ingested, salmonellae must survive the barrier of gastric acid, and the organism possesses a number of strategies to avoid or repair damage caused by acid stress. There are two principal types of acid tolerance response, one induced during the exponential growth phase and the other which operates during the stationary phase of growth. These systems protect against both organic and inorganic acid stress through the production of several acid-shock proteins. The organism may also be protected in the stomach by the food matrix in which it is contained, and the infectious dose appears to be low if the organism is contained in food with a high fat content (de Jong and Ekdahl, 2006). In the intestine, the organism adheres to the mucosa through fimbrial attachment. Salmonellae are known to produce several different types of fimbriae, including type 1 fimbriae and long polar fimbriae. The latter appear to be important in binding to the surface of Peyer's patches and M cells (Bäumler *et al*., 1996). Sef fimbriae, encoded by SPI-10, are found in only a limited number of serovars and are among the factors considered to determine host specificity (Hensel, 2004).

Following attachment to the surface of intestinal mucosal cells, the bacteria induce ruffling of cell membranes (Salyers and Whitt, 1994). This ruffling is part of the mechanism whereby the organisms are taken up into non-phagocytic cells and is now known to be one of the functions encoded by genes on SPI-1. This pathogenicity island is found in all serotypes of *S. enterica* analysed to date and one of its major effectors is a Type III secretion system (TTSS). The TTSS is a complex of proteins which forms a needle-like structure for the transfer of virulence factors from the bacterium into the host cell (Foley and Lynne, 2008). Some of these virulence factors are proteins that interact with the actin cytoskeleton of the host cell, causing rearrangements which lead to ruffle formation. The ruffles facilitate uptake of the bacteria into membranebound vesicles, termed *Salmonella*-containing vesicles (SCV) which often coalesce. The organisms replicate in these vesicles and are eventually released from the cells, which sustain only mild or transient damage. Other products transferred by the TTSS activate secretory pathways and alter ion balance within the cell (Wallis and Galyov, 2000). In addition, effector proteins result in neutrophil recruitment, and the resulting inflammation, together with the disturbance of fluid and ion balance, causes diarrhoea (Foley and Lynne, 2008). In cattle, genes encoded by SPI-5 as well as those encoded by SPI-1 are involved in inflammatory responses and ion/fluid secretion in the intestinal tract (Jones *et*

al., 1998).

Many of the genes responsible for systemic invasion by *Salmonella* serotypes are clustered in SPI-2. A TTSS is encoded by this pathogenicity island also but the genes are expressed only within the acidified SCV of the host cell. The effector proteins of this secretory system are involved in the formation of *Salmonella*induced filaments (Abrahams and Hensel, 2006). The exact function of these filaments is unknown but it is thought that they play a role in intracellular replication of salmonellae. Effector proteins also appear to protect intracellular organisms from bactericidal compounds produced by the host cell such as reactive oxygen intermediates. In addition to these mechanisms which protect intracellular bacteria, SPI-2 mediates a process which prevents phagolysosome fusion. Resistance to digestion by phagocytes and to the lethal action of complement components facilitates the spread of organisms within the host. Virulence genes encoded by plasmids, including the *spv* gene, which is important for intracellular multiplication and other genes, which encode serum resistance, play a role in systemic invasion (Foley and Lynne, 2008). Resistance to killing by complement is partly dependent on the length of O antigen chains of lipopolysaccharide (LPS). Long chains of LPS prevent the components of the membrane attack complex from interacting with and damaging the bacterial cell membrane (Salyers and Whitt, 1994). The LPS is also responsible for the endotoxic effects of infection with salmonellae. It may contribute to the local inflammatory response which damages intestinal epithelial cells and results in the development of diarrhoea. Bacterial cell wall LPS also mediates the endotoxic shock which may accompany septicaemic salmonellosis. Production of exotoxins by *Salmonella* serotypes has not been definitively demonstrated although an RTX (repeats in toxin)-like protein has been demonstrated in serovar Typhi (Parkhill *et al*., 2001).

Clinical infections

Salmonellosis is of common occurrence in domestic animals and the consequences of infection range from subclinical carrier status to acute fatal septicaemia. Some *Salmonella* serotypes such as *Salmonella* Pullorum and *Salmonella* Gallinarum in poultry, *Salmonella* Choleraesuis in pigs and *Salmonella* Dublin in cattle are relatively host-specific. In contrast, *Salmonella* Typhimurium has a comparatively wide host range. It is recognized that healthy adult carnivores are innately resistant to salmonellosis.

Salmonellae often localize in the mucosae of the ileum, caecum and colon and in the mesenteric lymph nodes of infected animals. Although most organisms are cleared from the tissues by host defence mechanisms, subclinical infection may persist with shedding of small numbers of salmonellae in the faeces. Latent infections, in which salmonellae are present in the gall bladder or mesenteric lymph nodes but are not excreted, also occur. Clinical disease may develop from subclinical and latent infections if affected animals are stressed. The stress factors which have been most often associated with the development of clinical salmonellosis are listed in Box 24.2. Some of these factors, such as transportation and overcrowding, have proved to be significant in outbreaks of the disease in young animals and in adult sheep and horses. When salmonellosis occurs in adult cattle it is usually sporadic and is also often associated with stress.

Other factors that determine the clinical outcome of infection include the number of salmonellae ingested, the virulence of the infecting serotype or strain and the susceptibility of the host. Host susceptibility may be related to immunological status, genetic make-up or age. Young and debilitated or aged animals are particularly susceptible and may develop the septicaemic form of the disease.

In most animal species, both enteric and septicaemic forms of salmonellosis are recorded. A number of serotypes have been associated with abortion in farm animals, often without other obvious clinical signs in dams. The *Salmonella* serotypes of importance in domestic animals and the consequences of infection are indicated in Table 24.3. *Salmonella* Dublin causes a variety of clinical effects in cattle (Table 24.4). Terminal dry gangrene and bone lesions are common manifestations of chronic infections with *Salmonella* Dublin in calves (Gitter *et al*., 1978).

Box 24.2 Stress factors which may activate latent or subclinical salmonellosis.

- Intercurrent infections
- Transportation
- Overcrowding
- Pregnancy
- Extreme ambient temperatures
- Water deprivation
- Oral antimicrobial therapy

- Sudden changes in rations altering the intestinal flora
- Surgical procedures requiring general anaesthesia

Table 24.3 *Salmonella* serotypes of clinical importance and the consequences of infection.

***Salmonella* serotype**	**Hosts**	**Consequences of infection**
Salmonella Typhimurium	Many animal species	Enterocolitis and septicaemia
	Humans	Food poisoning
Salmonella Dublin	Cattle	Many disease conditions
	Sheep, horses, dogs	Enterocolitis and septicaemia
Salmonella Choleraesuis	Pigs	Enterocolitis and septicaemia
Salmonella Pullorum	Chicks	Pullorum disease (bacillary white diarrhoea)
Salmonella Gallinarum	Adult birds	Fowl typhoid
Salmonella Arizonae	Turkeys	Arizona or paracolon infection
Salmonella Enteritidis	Poultry	Often subclinical in poultry
	Many other species	Clinical disease in mammals
	Humans	Food poisoning
Salmonella Brandenburg	Sheep	Abortion

Table 24.4 Infection with *Salmonella* Dublin in cattle.

Outcome of infection/Age group	**Comments**
Subclinical faecal excretors/All ages	Probable outcome of most infections. Small numbers of salmonellae excreted intermittently in faeces
Latent carriers/All ages	Salmonella present in gall bladder. No excretion of organisms
Acute or chronic enteric disease/All ages	Enterocolitis with foul-smelling diarrhoea containing blood, mucus and epithelial shreds or casts
Septicaemia/All ages	Potentially fatal disease with fever and depression. Diarrhoea or dysentery may be present. Dramatic drop in milk production in dairy cows. Calves surviving acute disease may develop arthritis (joint ill), meningitis or pneumonia
Abortion	A common cause of abortion in some European countries. No signs of illness may be evident
Joint ill/Calves	May follow septicaemia or umbilical infection
Osteomyelitis/Young animals	Often involves the cervical vertebrae or bones of the distal limb. In cervical osteomyelitis, nervous signs relate to spinal cord compression
Terminal dry gangrene/Calves	Disseminated intravascular coagulation due to endotoxaemia results in local ischaemia and gangrene of distal parts of hind limbs, ears and tail

Enteric salmonellosis

Enterocolitis caused by *Salmonella* organisms can affect most species of farm animals, irrespective of age. Acute disease is characterized by fever, depression, anorexia and profuse foul-smelling diarrhoea often containing blood, mucus and epithelial casts. Dehydration and weight loss follow and pregnant animals may abort. Severely affected young animals become recumbent and may die within a few days of acquiring infection. On farms with endemic salmonellosis, the

milder clinical signs often observed may be attributed to the influence of acquired immunity. Chronic enterocolitis can follow acute salmonellosis in pigs, cattle and horses. Intermittent fever, soft faeces and gradual weight loss leading to emaciation are common features of this condition.

Septicaemic salmonellosis

The septicaemic form can occur in all age groups but is most common in calves, in neonatal foals and in pigs less than 4 months of age. Onset of clinical disease is sudden with high fever, depression and recumbency. If treatment is delayed, many young animals with septicaemic salmonellosis die within 48 hours. Surviving animals can develop persistent diarrhoea, arthritis, meningitis or pneumonia.

In pigs with septicaemic *Salmonella* Choleraesuis infection, there is a characteristic bluish discoloration of the ears and snout. Intercurrent viral infections often predispose to severe clinical forms of the disease. The close clinical and pathological relationships which have been recognized in animals infected with *Salmonella* Choleraesuis (‘hog-cholera bacillus’) and with classical swine fever virus, either jointly or separately, exemplify both the importance of intercurrent infections and the difficulty of clinically distinguishing the diseases caused by these agents.

Salmonellosis in poultry

Salmonella Pullorum, *Salmonella* Gallinarum and *Salmonella* Enteritidis can infect the ovaries of hens and can be transmitted through eggs. The presence of *Salmonella* Enteritidis in undercooked egg dishes may result in human food poisoning (Cooper, 1994).

Pullorum disease or bacillary white diarrhoea (*Salmonella* Pullorum) affects young chicks and turkey poults up to 2 to 3 weeks of age. The mortality rate is high and affected birds huddle under a heat source, are anorexic and depressed and have whitish faecal pasting around their vents. Characteristic lesions include whitish nodes throughout the lungs and focal necrosis of liver and spleen.

Fowl typhoid (*Salmonella* Gallinarum) can produce lesions in young chicks and poults similar to those of pullorum disease. However, in countries where fowl typhoid is endemic, a septicaemic disease of adult birds occurs, often resulting in sudden deaths. Characteristic findings include an enlarged, friable, bile-stained liver and enlarged spleen. As *Salmonella* Pullorum and *Salmonella*

Gallinarum possess similar somatic antigens (Table 24.5), both have been eradicated from many countries by a serological testing and slaughter policy for pullorum disease.

Table 24.5 Somatic and flagellar antigens and the serogroups of selected *Salmonella* serotypes.

Serotype	Serogroup	Somatic (O) antigens	Flagellar (H) antigens	
			Phase 1	Phase 2
Salmonella Typhimurium	B	1, 4, [5], 12	i	1, 2
Salmonella Choleraesuis	C1	6, 7	c	1, 5
Salmonella Choleraesuis biotype Kunzendorf	C1	6, 7	[c]	1, 5
Salmonella Enteritidis	D1	1, 9, 12	g, m	[1, 7]
Salmonella Dublin*	D1	1, 9, 12	g, p	–
Salmonella Gallinarum	D1	1, 9, 12	–	–
Salmonella Pullorum	D1	9, 12	–	–
Salmonella Anatum	E1	3, 10	e, h	1,6

1, Presence dependent on phage conversion. [ ], Antigen may be present or absent. * *Salmonella* Dublin strains may have a capsular antigen termed Vi.

Paratyphoid is a name given to infections of poultry by non-host-adapted salmonellae such as *Salmonella* Enteritidis and *Salmonella* Typhimurium. These infections are often subclinical in laying birds.

Diagnostic procedures

- A history of previous outbreaks of the disease on the premises, the age group affected and the clinical picture may suggest salmonellosis.
- At post-mortem, enterocolitis with blood-stained luminal contents and enlarged mesenteric lymph nodes are commonly observed.
- Laboratory confirmation is required. Specimens for submission should include faeces and blood from live animals. Intestinal contents and samples from tissue lesions should be submitted from dead animals and abomasal contents from aborted ruminant foetuses.
- Isolation of salmonellae from blood or parenchymatous organs is deemed to be confirmatory for septicaemic salmonellosis.
- A heavy growth of salmonellae on plates directly inoculated with faeces, intestinal contents or foetal abomasal contents strongly suggests the aetiological involvement of the pathogen. Recovery of small numbers of salmonellae from faeces is usually indicative of a carrier state.

- Because clinical specimens for the diagnosis of enterocolitis due to *Salmonella* serotypes frequently contain other contaminating flora, specimens should be cultured directly on to BG and XLD agars and also added to selenite F, Rappaport or tetrathionate broth for selective enrichment and subsequent subculture (Fig. 24.6). The plates and enrichment broth are incubated aerobically at 37°C for up to 48 hours. Rappaport-Vassiliadis broth is incubated at 41.5°C. Subcultures are made from the enrichment broth at 24 and 48 hours. If animals are to be tested for subclinical carriage of *Salmonella* serotypes, or environmental samples are being investigated and small numbers of organisms are expected to be present, non-selective enrichment by overnight incubation in a broth such as buffered peptone water may be carried out before the selective enrichment step.
- Molecular techniques are now frequently used for the detection of salmonellae in clinical and environmental samples, a major advantage being the speed with which a result can be obtained. PCR and real-time PCR-based tests can be applied directly to samples or, if very small numbers of organisms are expected, samples can be enriched first and PCR performed on the overnight enrichment culture.
- Identification criteria for isolates:

  – On brilliant green agar, colonies and medium are red indicating alkalinity. On XLD agar, colonies are red (alkaline) with a black centre, indicating H_2S production.

  – Suspicious colonies, subcultured from the selective media into TSI agar and lysine decarboxylase broth, should be examined after incubation for 18 hours at 37°C to establish their biochemical identity as *Salmonella* (Tables 24.1 and 24.2).

  – If reactions in TSI agar and lysine decarboxylase broth are inconclusive, a biochemical profile using a battery of biochemical tests may allow definitive identification.

  –The isolates from the TSI agar slant are confirmed as *Salmonella* using commercially available antisera for O and H antigens in a slide agglutination test. Serotypes with O antigens in common are assigned to a serogroup (Table 24.5).

  – Serotypes which have flagellar (H) antigens in two phases, phase 1 (specific) and phase 2 (nonspecific), are termed diphasic. The antigens in

both phases must be determined. The majority of organisms in these serotypes usually possess H antigens in a single phase and are agglutinated by the appropriate antiserum. However, a minority of bacteria, invariably present in the alternative phase, can be selected by a procedure referred to as ‘phase changing’ (Fig. 24.7). Although the term ‘phase changing’ is used to denote the procedure illustrated in Fig. 24.7, ‘phase selection’ would be a more accurate term. When the alternative phase is isolated, the antigenic formula used for serotyping can be completed.

– Biotyping is required for serotypes which are antigenically indistinguishable such as *Salmonella* Pullorum and *Salmonella* Gallinarum (Table 24.6).

- Phage typing is used in epidemiological studies to identify isolates with specific characteristics such as multiple resistance to antibiotics and enhanced virulence. Examples of important phage types are *Salmonella* Typhimurium DT (definitive type) 104 which exhibits multiple resistance to antibiotics, and *Salmonella* Enteritidis PT (phage type) 4 which is found in poultry products and is a common cause of food poisoning in humans.
- Serological tests such as ELISA and agglutination techniques are of greatest value when used on a herd or flock basis. A rising antibody titre using paired serum samples is indicative of active infection.
- Molecular techniques have been developed for the identification of some serovars. For example, O’Regan *et al*. (2008) developed a multiplex PCR for the identification and differentiation of serovars Enteritidis, Gallinarum, Typhimurium, Kentucky and Dublin in poultry samples.

Figure 24.6 Procedures for the isolation and identification of salmonella serotypes from clinical specimens.

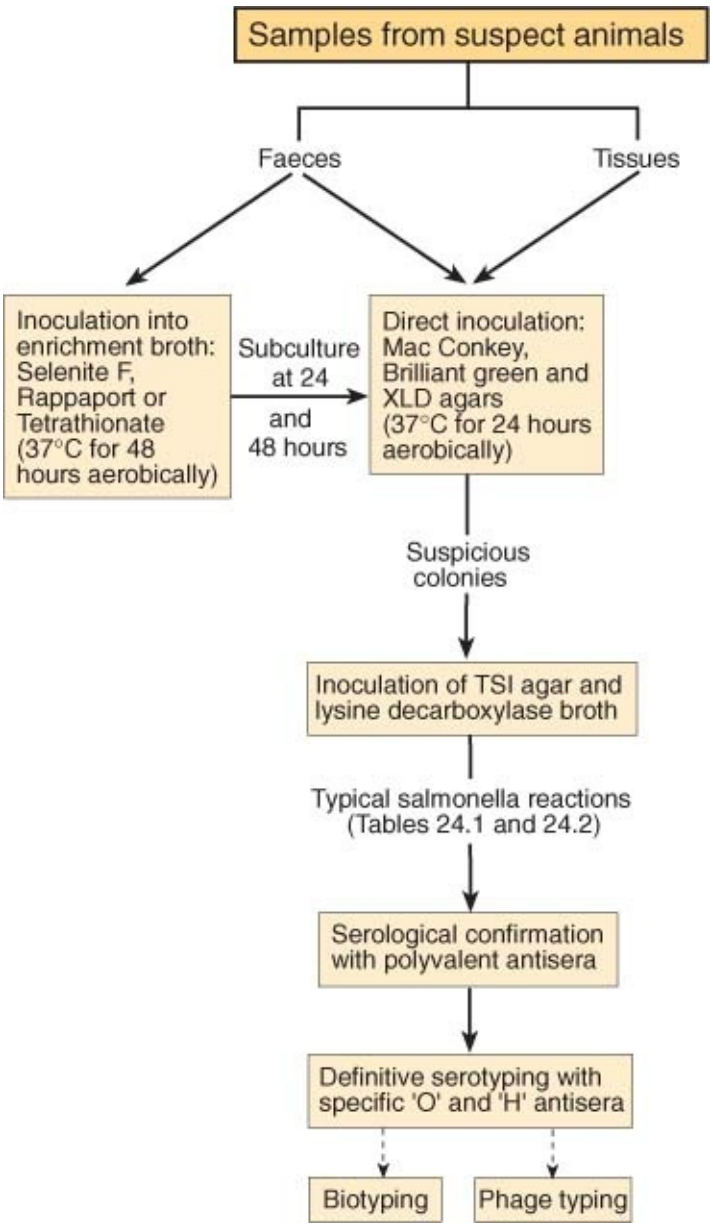


Figure 24.7 The Craigie tube method for 'phase-changing' salmonella isolates. The biphasic organism *Salmonella* Typhimurium illustrates the principle of the method. In phase 1 this bacterium has flagellar i antigens. The organism is inoculated into a Craigie tube placed in semisolid agar containing antiserum to the flagellar i antigen and incubated aerobically at 37°C for 24 hours. Salmonellae in phase 1 are agglutinated by the antiserum and immobilized. Those in phase 2 with flagellar 1,2 antigens are not immobilized. The motile phase 2 organisms which move out from the bottom of the Craigie tube can be sampled at the agar surface.

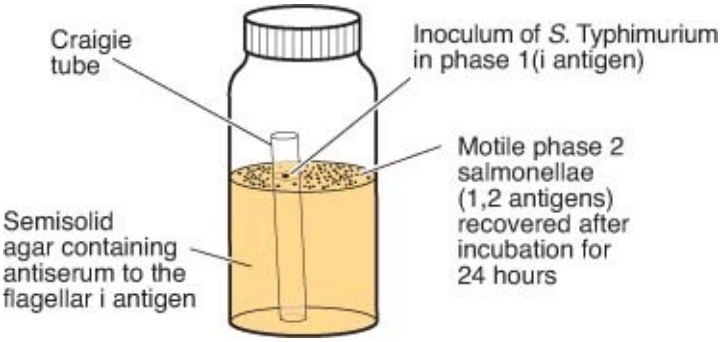


Table 24.6 Differentiation of the biotypes of *Salmonella* Pullorum and *Salmonella* Gallinarum.

	***Salmonella* Pullorum**	***Salmonella* Gallinarum**
Glucose (gas)	+	−
Dulcitol	−	+
Maltose	−	+
Ornithine decarboxylase	+	−
Rhamnose	+	−
Motility	−	−

Treatment

- Antibiotic therapy should be based on results of susceptibility testing because R-plasmids coding for multiple resistance are comparatively common in salmonellae.
- Oral antimicrobial therapy should be used judiciously for treating enteric salmonellosis because it may disturb the normal intestinal flora, extend the duration of *Salmonella* excretion and increase the probability of drug resistance developing. In the septicaemic form of the disease, intravenous antibiotic therapy must be used.
- Fluid and electrolyte replacement therapy is required to counteract dehydration and shock.
- As with other members of the *Enterobacteriaceae*, antimicrobial resistance, including resistance to multiple antimicrobial classes, is a major problem in both human and animal *Salmonella* isolates. Many countries document the occurrence of resistance in *Salmonella* isolates each year and resistance trends can be monitored. Reports from many countries record a high level of multiple resistance. In the period 1999 to 2005, a significant increase in resistance to tetracycline, chloramphenicol, ampicillin and sulphonamides was seen in *Salmonella* Typhimurium isolated from pigs in Denmark and coincided with an increase in the use of these antibacterial drugs in the pig

industry (DANMAP, 2005). In the USA, 44% of *Salmonella* isolates from abattoir and diagnostic sources were resistant to at least one antimicrobial agent (Anon., 2006). Recent increases in extended spectrum cephalosporin resistance in both animal and human isolates is of particular concern as ceftriaxone is an important drug for treating systemic salmonellosis in children under 16 years (Foley and Lynne, 2008). Antibacterial resistance can be transferred between salmonellae and related organisms. Of even greater concern is that plasmids encoding both antimicrobial resistance and virulence factors can be formed and strains containing such plasmids may be selected for by inappropriate antimicrobial usage. If such changes in resistance and virulence were to emerge, they could present many challenges for the veterinary and medical professions (Fluit, 2005).

Control

Control is based on reducing the risk of exposure to infection. Intensively reared food-producing animals are more likely to acquire infection than free-range birds or mammals and such infected animals are also a major source of human infection (Cooper, 1994).

- Measures for excluding infection from a herd or flock free of salmonellosis:
  – A closed-herd policy should be implemented when feasible.
  – Animals should be purchased from reliable sources and remain isolated until negative for *Salmonella* on three successive samplings at weekly intervals.
  – Steps should be taken to prevent contamination of foodstuffs and water. In this context, rodent control is important.
  – Protective clothing and footwear should be worn by personnel entering hatcheries and minimal disease pig units.
- Measures for reducing environmental contamination:
  – Effective routine cleaning and disinfection of buildings and equipment are essential.
  – Overstocking and overcrowding should be avoided.
  – Slurry should be spread on arable land where possible. An interval of at least 2 months should elapse before grazing commences on pastures following the application of slurry.
  – The continuous use of paddocks for susceptible animals should be avoided.

- Strategies for enhancing resistance and reducing the likelihood of clinical disease:

  – Vaccination procedures are used in cattle, sheep, poultry and pigs. Modified live vaccines which stimulate humoral immunity and cell-mediated immunity are more efficacious than bacterins but public perception is that inactivated or sub-unit vaccines are safer. Vaccination against serotypes which cause systemic disease is more successful than against non-host-adapted serotypes in which prevention of intestinal colonization is the goal. However, vaccination is used as a component of *Salmonella* control programmes in poultry in several countries. Modern molecular techniques are likely to lead to the development of more effective safer vaccines (Barrow, 2007).

  – Colonization inhibition or competitive exclusion, which involves the administration of adult gut flora preparations to newly hatched chicks, is successfully used to prevent *Salmonella* infection in poultry. In addition, research is ongoing to find a live attenuated *Salmonella* strain which could be administered orally to day-old chicks and which would achieve the same effect of preventing colonization by virulent *Salmonella* organisms (Barrow, 2007).

  – The impact of stress factors (Box 24.2) should be reduced by appropriate decisions relating to management of animals and surgical or therapeutic intervention.

  – Feeding of antimicrobial drugs for either prophylaxis or growth promotion should be avoided where possible.

- Measures for controlling outbreaks of salmonellosis:

  – Detection and elimination of the source of infection is essential.

  – Clinically affected animals should be isolated.

  – Movement of animals, vehicles and humans should be curtailed.

  – Foot baths containing suitable disinfectant, such as 3% iodophor, should be placed at strategic locations to limit spread of *Salmonella*.

  – Careful disposal of contaminated carcasses and bedding is mandatory.

  – Contaminated buildings and utensils should be thoroughly cleaned and disinfected. The choice of disinfectant is determined by the size and cleanliness of the building and the nature of the utensils. A3% concentration of sodium hypochlorite or iodophors is suitable for clean surfaces. Phenolic disinfectants are suitable for buildings with residual

organic matter. Fumigation with formaldehyde is the most effective method for disinfecting poultry houses.

– Herd vaccination may be of value for limiting the spread of infection during outbreaks of disease in cattle (Wray, 1991).

– Humans working with clinically-affected animals should be aware of the risk of acquiring infection.

Yersinia species

Yersinia species are non-lactose fermenters and with the exception of *Y. pestis* are motile (Table 24.1). Although there are more than 10 *Yersinia* species, only *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* are pathogenic for animals and humans (Table 24.7). *Yersinia ruckeri* causes perioral haemorrhagic inflammation in some species of fish. Growth of yersiniae tends to be less rapid than other members of the *Enterobacteriaceae*. They characteristically demonstrate bipolar staining in Giemsa-stained smears from animal tissues.

Serotyping and biotyping methods are used for identifying pathogenic yersiniae. Of the 21 serological groups of *Y. pseudotuberculosis*, serotypes I, II and III contain the majority of pathogenic isolates. There are five biotypes and more than 50 serotypes of *Y. enterocolitica*. Somatic antigens 2, 3, 5, 8 and 9 are present in isolates from clinical infections caused by this species. Serotype O:9 is of particular importance because it shares common antigens with *Brucella* species and it may induce false-positive reactions in brucella agglutination tests.

Table 24.7 The consequences of infection with *Yersinia* species.

***Yersinia* species**	**Hosts**	**Consequences of infection**
Y. enterocolitica	Pigs, other domestic animals, wildlife	Subclinical enteric infections, occasionally enteritis
	Ewes	Sporadic abortion
	Humans	Gastroenterocolitis
Y. pseudotuberculosis	Farmed deer, sheep, goats, cattle, buffaloes, pigs	Enteritis in young animals, subclinical infections common in older animals, mesenteric lymphadenitis
	Cattle, sheep, goats	Sporadic abortion
	Guinea-pigs, other laboratory animals	Focal hepatic necrosis, septicaemia
	Caged birds	Septicaemia
	Humans	Enterocolitis, mesenteric lymphadenitis
Y. pestis	Humans	Bubonic and pneumonic plague
	Rodents	Sylvatic plague
	Cats	Feline plague

Yersinia pseudotuberculosis and *Y. enterocolitica* are found in the intestinal

tract of a wide range of wild mammals, birds and domestic animals. All these animals may be reservoirs of infection. Many avian species may act as amplifier hosts and may also transfer the organisms mechanically (Cork *et al*., 1995). Both organisms can grow in a wide temperature range (5 to 42°C) and survive for long periods in cool wet conditions.

In endemic areas, wild rodents are important reservoirs of *Y. pestis*. Fleas, especially *Xenopsylla cheopis*, the Oriental rat flea, transmit the infection to humans and other animals. Following ingestion by the flea from a bacteraemic host, the organism multiplies within the gastrointestinal tract of the flea resulting in blockage of the tract. This effectively deprives the flea of nutrients, which drives it to continually seek other animals on which to feed. During feeding the flea regurgitates yersiniae into the bite wounds on these new hosts. *Yersinia pestis* is maintained by a sylvatic cycle in wild rodents and their associated fleas, and although dog and cat fleas may become infected with *Y. pestis* they do not transmit infection effectively.

Pathogenesis and pathogenicity

Pathogenic yersiniae are facultative intracellular organisms and survival within macrophages is important particularly in the early stages of infection. Later in the disease process, large numbers of extracellular organisms can be demonstrated and survival appears to be dependent in part on inhibition of phagocyte function. The three pathogenic *Yersinia* species possess virulence factors encoded on plasmids and chromosomes, some of which are common to all three species. However, *Y. pestis* produces extra virulence factors and is more pathogenic than *Yersinia pseudotuberculosis* and *Y. enterocolitica*, which rarely produce generalized infections. The pathogenetic mechanisms in enteric disease caused by *Y. enterocolitica* and *Y. pseudotuberculosis* are incompletely understood. Both organisms gain entry to the mucosa through M cells of Peyer’s patches. Adhesion to and subsequent invasion through these cells are facilitated by factors such as invasin and adhesion/invasion proteins which have an affinity for integrins on cell surfaces. Once in the mucosa, the bacteria are engulfed by macrophages in which they survive and are transported to the mesenteric lymph nodes (Brubaker, 1991). Survival within macrophages may be by prevention of acidification of the *Yersinia*containing vacuole, which has been shown to occur with *Y. pestis* (Pujol *et al*., 2009). Replication in the lymph nodes follows with the development of necrotic lesions and neutrophil infiltration. A key virulence

factor of all three pathogenic *Yersinia* species is a type III secretion system which is encoded on a plasmid called pYV. Similar to the TTSS found in *Salmonella* serotypes, this TTSS allows effector proteins to be translocated into host cells. These effector proteins are termed *Yersinia* outer proteins or Yops and several of these are delivered into phagocytes where they interfere with phagocytosis and production of reactive oxygen species (Cornelis, 2002).

Yersinia pestis, which is more invasive than *Y. pseudotuberculosis* and *Y. enterocolitica*, possesses additional virulence factors encoded on two plasmids which are specific to this pathogen. Production of an antiphagocytic protein capsule (Fraction 1) and a phospholipase D is encoded on one of the plasmids, and a plasminogen activator which aids systemic spread is encoded on the second plasmid. The phospholipase D enzyme is essential for survival of the organism in the midgut of fleas which transmit disease. Endotoxin, with properties similar to the endotoxin produced by other members of the *Enterobacteriaceae*, also contributes to the pathogenesis of disease.

Y. pestis and some serotypes of *Y. pseudotuberculosis* and *Y. enterocolitica* contain a chromosomal element, the high-pathogenicity island, which encodes genes facilitating iron acquisition.

Clinical infections

Yersinia pseudotuberculosis causes enteric infections in a wide variety of wild and domestic animals which are often subclinical. The septicaemic form of disease, known as pseudotuberculosis, can occur in laboratory rodents and aviary birds. Sporadic abortions caused by *Y. pseudotuberculosis* have been reported in cattle (Jerrett and Slee, 1989), sheep (Otter, 1996) and goats (Witte and Collins, 1985).

Wild and domestic animals may act as reservoirs of *Yersinia enterocolitica* which is primarily a human enteric pathogen. The pig is the natural reservoir for *Y. enterocolitica* serotype O3 biotype 4, which is an important pathogen in humans. Rare cases of enteric disease, precipitated by stress, may be encountered in pigs, farmed deer, goats and lambs. *Yersinia enterocolitica* has been implicated in sporadic ovine abortion (Corbel *et al*., 1990).

Yersinia pestis, the cause of human bubonic plague (‘black death’), can infect both dogs and cats in endemic areas. Cats, which are particularly susceptible, may be a source of infection for owners and attending veterinarians (Kaufmann *et al*., 1981; Orloski and Lathrop, 2003).

Enteric yersiniosis

Enteritis caused by *Y. pseudotuberculosis* is relatively common in young farmed deer in New Zealand and Australia (Henderson, 1983 ; Jerrett *et al*., 1990). Outbreaks of the disease have been reported also in buffaloes in Brazil (Riet-Correa *et al*., 1990). Enteric disease has been reported in sheep, goats and cattle under 1 year of age. Subclinical infection in many species is common, and clinical disease may be precipitated in the winter months by stress factors such as poor nutrition, weaning, transportation and cold wet conditions. There may be prolonged survival of *Y. pseudotuberculosis* on pasture in cold wet weather, facilitating faecal–oral transmission.

Enteritis in young deer and lambs is characterized by profuse watery diarrhoea, sometimes bloodstained, which may be rapidly fatal if untreated. The luminal contents of the small and large intestine are watery, and mucosal hyperaemia is evident at postmortem examination. Severely affected animals may show mucosal ulceration. The mesenteric lymph nodes are often enlarged and oedematous, and scattered pale necrotic foci may be present in the liver.

A clinically similar but less severe enterocolitis caused by *Y. enterocolitica* has been described in young ruminants.

Diagnosis

- The species and age group affected, especially during cold wet spells of weather, may suggest yersiniosis.
- Histological examination of intestinal lesions may reveal clusters of organisms in microabscesses within the mucosa.
- Confirmation requires isolation and identification of *Y. pseudotuberculosis* or, occasionally, *Y. enterocolitica*:

  – Samples from tissues can be plated directly on to blood agar and MacConkey agar and incubated aerobically at 37°C for up to 72 hours.

  – Faecal samples should be plated directly on to special selective media containing antibiotics which suppress the growth of contaminating organisms.

  – A cold enrichment procedure may facilitate recovery of yersiniae from faeces, especially if they are present in low numbers. A 5% suspension of faeces in phosphate buffered saline, held at 4°C for 3 weeks, is subcultured weekly on to MacConkey agar or selective agar.

- Serotyping may be necessary to establish whether or not the isolates belong to known pathogenic serotypes. As conventional serotyping is a laborious and expensive procedure, PCR-based identification techniques have been developed (Bogdanovich *et al*., 2003 ; Jacobsen *et al*., 2005).
- Many PCR-based methods have been published for detection of the enteropathogenic yersiniae, particularly for the detection of *Y. enterocolitica* which is an important food-borne pathogen in some parts of the world (Fredriksson-Ahomaa *et al*., 2000; Fukushima *et al*., 2003).

Treatment and control

- Fluid replacement therapy together with broad spectrum antimicrobial treatment should be initiated promptly in young animals.
- A formalin-killed *Y. pseudotuberculosis* vaccine composed of serotypes I, II and III, administered in two doses 3 weeks apart, has been shown to decrease the occurrence of clinical disease in young deer. Recent developments include the successful use of a recombinant intranasal vaccine which provides mucosal and systemic protection against *Y. pseudotuberculosis* in mice (Daniel *et al*., 2009).
- Stressful conditions should, where practicable, be mimimized.

Septicaemic yersiniosis

Septicaemia, caused by *Y. pseudotuberculosis*, occurs in birds kept in cages or aviaries. It is presumed that infection is acquired through contact with the faeces of wild birds or rodents, or through the feeding of contaminated leafy plants. In aviaries, overcrowding may predispose to the development of disease. Infected birds may die suddenly. Some may display ruffling of feathers and listlessness shortly before death. Pin-point white necrotic foci are present in the liver at post-mortem examination. Confirmation is based on the isolation and identification of *Y. pseudotuberculosis* from the liver and other internal organs.

Treatment is seldom feasible due to the acute nature of the disease. Control should be aimed at preventing faecal contamination of food and water by wild birds and rodents.

Pseudotuberculosis in laboratory animals

Infection with *Y. pseudotuberculosis* in colonies of guinea-pigs or rodents is

usually introduced through faecal contamination of food by wild rodents. Diarrhoea and gradual weight loss leading to emaciation and death are the signs most often observed in affected animals. Some animals may die suddenly from septicaemia.

At post-mortem examination, numerous white necrotic lesions are present in the liver. Affected mesenteric lymph nodes are enlarged and may show caseous necrosis.

Treatment is usually undesirable because some animals in the colony may become carriers and the organism is zoonotic. Depopulation, disinfection and restocking are the preferred control measures. Exclusion of wild rodents is an essential step in preventing infection with *Y. pseudotuberculosis*.

Feline plague

Cats usually acquire infection with *Y. pestis* by ingestion of infected rodents. Three clinical forms of the disease are recognized: bubonic, septicaemic and pneumonic. The most common form of the disease is characterized by enlarged lymph nodes (buboes) associated with lymphatic drainage from the site of infection. Clinical signs include fever, depression and anorexia. Affected superficial lymph nodes may rupture, discharging serosanguineous fluid or pus. Septicaemia may occur without lymphadenopathy and is potentially fatal. Pneumonic lesions may result from haematogenous spread.

Because cats with pneumonic lesions are a potential source of human infection through aerosol generation, they should be euthanized. Human infection can also be acquired through cat scratches and bites and possibly through the bites of fleas from infected cats. Care should be taken when handling infected animals.

Diagnosis

- Lymphadenopathy and severe depression in cats in endemic areas may suggest feline plague.
- Specimens from suspect cases should be sent to specialized reference laboratories. Suitable specimens include pus, blood and lymph-node aspirates.
- Giemsa-stained smears from abscesses or lymphnode aspirates may reveal large numbers of bipolarstaining rods.
- Direct fluorescent antibody tests are carried out in reference laboratories.
- PCR-based techniques, including real-time PCR, are available for detection

of *Y. pestis* in clinical and other samples, such as fleas.

- A passive haemagglutination test, using Fraction 1A antigen, can be used on paired serum samples taken 2 weeks apart from suspect cats. A substantial increase in the antibody level is usually indicative of active infection.

Treatment and control

- Cats with suspected plague should be kept in isolation and immediately treated for fleas to prevent those handling the animal from becoming exposed to flea bites. The bubonic form of the disease may respond to tetracyclines or chloramphenicol administered parenterally. Multidrug resistance, mediated by a transferable plasmid, has been reported in *Y. pestis* (Galimand *et al*., 1997), although the majority of isolates of the organism remain susceptible to most antibacterial agents (Galimand *et al*., 2006).
- In endemic areas, dogs and cats should be routinely treated for fleas.
- Rodent control measures should be implemented after flea control procedures are in place.

Opportunistic pathogens

This group of enterobacteria, which rarely cause enteric disease in domestic animals, are sometimes involved in localized opportunistic infections in diverse anatomical locations. Faecal contamination of the environment accounts for widespread distribution of the organisms and contributes to the occurrence of opportunistic infection. Predisposing factors include intercurrent infection, tissue devitalization and the inherent vulnerability of certain organs.

These opportunistic invaders have characteristics which may allow them to circumvent host defence mechanisms and colonize and survive in affected organs. *Klebsiella pneumoniae* and *Enterobacter* species produce abundant capsular material which may inhibit phagocytosis and enhance intracellular survival. Adhesins are of particular importance in those bacteria that colonize the lower urinary tract. Siderophores produced by some opportunistic pathogens contribute to bacterial survival when the supply of available iron in tissues is limited. Some toxic effects of these opportunistic pathogens are attributable to release of endotoxin from dead bacteria. This can induce local and systemic changes which include inflammatory responses, pyrexia, endothelial damage and microthrombosis.

Clinical infections

The clinical conditions arising from infections with opportunistic members of the *Enterobacteriaceae* are presented in Table 24.8. *Klebsiella pneumoniae* and *Enterobacter aerogenes* are two opportunistic pathogens commonly encountered in coliform mastitis of dairy cattle. These organisms usually gain entry to the mammary gland from contaminated environmental sources. Sawdust used for bedding, for example, may be the source of infection in coliform mastitis caused by *Klebsiella pneumoniae*. This bacterium is also reported to be one of the commonest causes of metritis in mares; capsule types 1, 2 and 5 are transmitted venereally. *Proteus* species and *Klebsiella* species cause infections of the lower urinary tract in dogs. *Proteus* species are often implicated in otitis externa in dogs and sometimes in cats. A variety of factors may predispose to this infection (see Chapter 45).

Table 24.8 Opportunistic pathogens in the *Enterobacteriaceae* and their associated clinical conditions.

Bacterial species	Clinical conditions
Edwardsiella tarda	Diarrhoea; wound infections in some animal species (rare)
Enterobacter aerogenes	Coliform mastitis in cows and sows
Klebsiella pneumoniae	Coliform mastitis in cows; endometritis in mares; pneumonia in calves and foals; urinary tract infections in dogs
Morganella morganii subsp. morganii	Ear and urinary tract infections in dogs and cats (uncommon)
Proteus mirabilis and *P. vulgaris*	Urinary tract infections in dogs and horses; associated with otitis externa in dogs
Serratia marcescens	Bovine mastitis (uncommon); septicaemia in chickens (rare)

The other opportunistic pathogens in this group, *Edwardsiella tarda*, *Morganella morganii* subsp. *morganii* and *Serratia marcescens*, are rarely associated with clinical disease in domestic animals.

Diagnostic procedures

When opportunistic pathogens are involved in a disease process, clinical signs are non-specific.

- Specimens for examination should be collected from the infected organ.
- Blood agar and MacConkey agar inoculated with the specimens are cultured aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates:
  - Gram-negative rods
  - Oxidase-negative, catalase-positive

  – Growth and appearance on MacConkey agar
  – Colonial appearance on blood agar.
- Appropriate biochemical profile for presumptive or definitive identification.

Treatment and control

- The type of treatment is determined by the location and severity of the infection.
- Antibiotic therapy should be based on antibiotic susceptibility testing.
- Predisposing causes and sources of infection should be identified and, if possible, eliminated.

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Chapter 25

Pseudomonas aeruginosa and Burkholderia species

Pseudomonas aeruginosa, Burkholderia mallei and *B. pseudomallei* are Gram-negative rods (0.5 to 1.0 × 1 to 5μm) which are obligate aerobes and oxidize carbohydrates. Most isolates are oxidase-positive and catalase-positive. They are motile by one or more polar flagella, with the exception of *B. mallei* which is non- motile. The majority of these organisms have no special growth requirements and grow well on MacConkey agar. *Burkholderia mallei* requires glycerol in media for optimal growth. *Pseudomonas aeruginosa*, characterized by the production of diffusible pigments, causes a variety of opportunistic infections in a wide range of animals. A number of other *Pseudomonas* species may be isolated from clinical specimens. *Pseudomonas fluorescens* and *P. putida* occasionally infect freshwater fish.

Burkholderia species, previously classified in the genus *Pseudomonas*, include *B. mallei*, the cause of glanders, and *B. pseudomallei*, the cause of melioidosis. Both diseases are zoonoses. Both species possess two circular chromosomes although those of *B. mallei* are smaller and contain fewer genes than those of *B. pseudomallei*, probably reflecting the host-adapted nature of *B. mallei*.

Usual habitat

Pseudomonas species are environmental organisms that occur worldwide in water, soil and on plants. *Pseudomonas aeruginosa* is also found on the skin, on mucous membranes and in faeces. *Burkholderia pseudomallei*, which is found in soils, occasionally infects animals and humans. Wild rodents can act as reservoirs of this organism. It is widely distributed in some tropical and subtropical regions of south-east Asia and Australia. Although *B. mallei* can survive in the environment for up to 6 weeks, its reservoir is infected *Equidae*.

Key points

- Medium-sized, Gram-negative rods
- Obligate aerobes
- Most isolates are oxidase-positive and catalase-positive
- *Pseudomonas* species and *Burkholderia pseudomallei* are motile by polar flagella
- *Burkholderia mallei* is non-motile and requires glycerol in media for optimal growth
- Diffusible pigments are produced by *P. aeruginosa*
- *Burkholderia mallei* causes glanders
- *Burkholderia pseudomallei* causes melioidosis
- *Pseudomonas aeruginosa* causes opportunistic infections

Differentiation of *Pseudomonas* and *Burkholderia* species

- The comparative colonial and biochemical features of these organisms are presented in Table 25.1.
- Many *Pseudomonas* species produce pigments. *Pseudomonas aeruginosa* strains can form up to four diffusible pigments (Box 25.1). Pyocyanin, unique to this organism, is produced by most strains and specifically identifies *P. aeruginosa*. Pyocyanin-enhancing media are available for isolates which are weak pyocyanin producers. Pigment production is observed most clearly on media without dyes such as nutrient agar. Pyorubin and pyomela-nin develop slowly and may be detectable only after incubation for 1 to 2 weeks. Colonies of *B. pseudomallei* and *B. mallei* become brownish with age but do not produce pigments.
- The majority of *Pseudomonas* and *Burkholderia* species are motile. Absence of motility distinguishes *B. mallei* from other members of the group.
- Several PCR-based methods for the detection and identification of *Pseudomonas* species and for the differentiation of *Burkholderia* species have been developed.

Table 25.1 Comparative features of *Pseudomonas aeruginosa*, *Burkholderia mallei* and *Burkholderia pseudomallei*.

Feature	*P. aeruginosa*	*B. mallei*	*B. pseudomallei*
Colonial morphology	Large and flat with serrated edges	White and smooth, becoming granular and brown with age	Ranges from smooth and mucoid to rough and dull, becoming yellowish brown with age
Haemolysis on blood agar	v	–	v
Diffusible pigment production	+	–	–
Colony odour	Grape-like	None	Musty
Growth on MacConkey agar	+	+[a]	+
Growth at 42°C	+	–	+
Motility	+	–	+
Oxidase production	+	–[b]	+
Oxidation of:			
glucose	+	+	+
lactose	–	–	+
sucrose	–	–	+[a]

a, 75% of strains positive.

b, 25% of strains positive.

v, variable.

Box 25.1 Pigments produced by *Pseudomonas aeruginosa*.

- Pyocyanin (blue green)
- Pyoverdin (greenish yellow)
- Pyorubin (red)
- Pyomelanin (brownish black)

Clinical infections

Burkholderia mallei, a major pathogen of *Equidae*, causes both acute and chronic disease. It manifests mainly as lesions in the skin and the respiratory tract. Infection with *B. pseudomallei* can cause chronic suppurative lesions in the lungs and other organs of a wide range of species. In contrast, *P. aeruginosa* is an opportunistic pathogen which may occasionally cause acute systemic disease.

Pseudomonas aeruginosa infections

Pseudomonas aeruginosa causes a wide range of opportunistic infections (Table 25.2). Although predisposing factors are associated with the occurrence of many of these infections, some species, such as farmed mink, appear to be particularly susceptible to the organism (Long *et al*., 1980). Haemorrhagic pneumonia and septicaemia, caused by *P. aeruginosa*, occurs sporadically in ranched mink with mortality rates up to 50% in some outbreaks. Bovine mastitis associated with this organism (Crossman and Hutchinson, 1995) is often linked to contaminated water used for udder washing or to the insertion of contaminated intramammary antibiotic tubes. Fleece rot of sheep, a condition associated with heavy or prolonged rainfall, has been reported from the UK and Australia. Maceration of the skin surface following water penetration of the fleece allows colonization by *P. aeruginosa* resulting in sup-purative dermatitis. The bluish green pyocyanin pigment produced by *P. aeruginosa* discolours the wool. Development of purulent rhinitis and otitis in sheep following showering with a 'shower wash' previously contaminated by use on sheep with *P. aeruginosa* dermatitis has been reported (Watson *et al*., 2003). *Pseudomonas aeruginosa* is often found in the oral cavity of snakes and can cause necrotic stomatitis in captive reptiles kept under poor husbandry conditions. *Pseudomonas aeruginosa* is a major nosocomial pathogen in human hospitals and is a particularly important pathogen of patients with cystic fibrosis (Kerr and Snelling, 2009).

Table 25.2 Clinical conditions arising from infection with *Pseudomonas aeruginosa*.

Host	Disease condition
Cattle	Mastitis, metritis, pneumonia, dermatitis, enteritis (calves)
Sheep	Mastitis, fleece rot, pneumonia, otitis media
Pigs	Respiratory infections, otitis
Horses	Genital tract infections, pneumonia, ulcerative keratitis
Dogs, cats	Otitis externa, cystitis, pneumonia, ulcerative keratitis
Mink	Haemorrhagic pneumonia, septicaemia
Chinchillas	Pneumonia, septicaemia
Reptiles (captive)	Necrotic stomatitis

Pathogenesis and pathogenicity

Because *P. aeruginosa* is an opportunistic organism, invasion of the host is preceded by a breach in host defences. Examples include breaks in the skin due to trauma, prolonged wetting as in the case of fleece rot in sheep, or the presence of urinary or intravenous catheters. The first stage of infection involves adherence and colonization. Attachment to host cells is mediated by fimbriae,

mainly Type IV pili, during the initial phases of attachment. Flagella and LPS also play a role in adherence. Colonization and replication are aided by antiphagocytic properties of exoenzyme S, extracellular slime and outer-membrane lipopoly-saccharides. The production of extracellular slime and biofilm formation are particularly important in the pathogenesis of infections associated with indwelling devices such as catheters. Resistance to complement-mediated damage and the ability to obtain iron from host tissues are additional virulence factors. The pigments produced by some strains of *Pseudomonas* function as siderophores.

Following invasion, tissue damage is caused by a variety of extracellular toxins and enzymes. These include toxins such as exotoxin A, phospholipase C and proteases. Exotoxin A is a bipartite toxin with binding and toxic components. The active component, once internalized in a cell, blocks protein synthesis by ADP-ribosylation and elongation of Factor 2 with resultant cell death. Phospholipase C is a haemolysin. Proteases, including elastase, mediate cell damage in the lungs and blood vessels. *Pseudomonas aeruginosa* has both Type II and Type III secretion systems. The Type III system is of major importance; its toxic effects result from the action of effector proteins which are injected into the host cells. Four proteins which have been identified to date cause cytotoxic effects in tissues and may also interfere with neutrophil and macrophage function (Kerr and Snelling, 2009). The pigment pyocyanin can directly accept electrons from reducing agents, transfer them to oxygen and generate reactive oxygen species, which results in damage to host cells (Liu and Nizet, 2009).

Infection may remain localized or it may become disseminated. Spread throughout the host is aided by exoenzyme S, and systemic toxicity is attributed to exotoxin A and endotoxin. Host defence mechanisms against *P. aeruginosa* include opsonizing antibodies and phagocytosis by neutrophils and macrophages.

Diagnostic procedures

- Specimens for laboratory examination include pus, respiratory aspirates, mid-stream urine, mastitic milk and ear swabs.
- Blood agar and MacConkey agar plates, inoculated with suspect material, are incubated aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates:

  - Colonial morphology and characteristic fruity, grape-like odour
  - Pyocyanin production
  - Lactose-negative, pale colonies on MacConkey agar
  - Oxidase-positive
  - Triple sugar iron agar unchanged.
- Biochemical profile (Table 25.1).
- Isolates may also be identified by PCR amplification and sequencing of the rRNA gene (Watson *et al*., 2003).
- Several molecular techniques can be used for strain typing of *Pseudomonas* isolates including PFGE and PCR-based techniques such as random amplification of polymorphic DNA (RAPD) (Pujana *et al*., 2000; Las Heras *et al*, 2002).

Treatment and control

- Predisposing causes and sources of infection should be identified and, where possible, eliminated.
- *Pseudomonas aeruginosa* is extremely resistant to many antibiotics and disinfectants and susceptibility testing should be carried out on isolates. Intrinsic resistance is attributable to low permeability of its outer membrane, multidrug efflux pumps and the production of chromosomally-encoded β-lactamases. In addition, organisms within biofilms are less susceptible to the action of antimicrobial agents (Clutterbuck *et al*., 2007). A combination of gentamicin or tobramycin with either carbenicillin or ticaricillin may be effective although the emergence of pan-resistant strains in human medicine has been reported (Souli *et al*., 2008).
- Vaccines may be required for farmed mink and chinchillas. As there are antigenic differences between strains, polyvalent or autogenous formalin-killed bacterins should be employed. Humoral antibody induced by a polyvalent exotoxin A-polysaccharide vaccine appears to be protective (Cryz *et al*., 1987).

Glanders

Glanders, caused by *B. mallei*, is a contagious disease of *Equidae* characterized by the formation of nodules and ulcers in the respiratory tract or on the skin. Humans and carnivores are also susceptible to infection. Once worldwide in

distribution, glanders has now been eradicated from most developed countries, but sporadic cases and small endemic foci of disease occur in parts of the Middle East, India, Pakistan and China.

Transmission follows ingestion of food or water contaminated by nasal discharges of infected *Equidae*. Less commonly, infection may be acquired by inhalation or through skin abrasions. Infection by inhalation requires extremely small numbers of organisms, which is one of the reasons the organism is classified as a Category B bioterrorist agent by the Centers for Disease Control, USA. An acute septicaemic form of the disease is characterized by fever, mucopurulent nasal discharge and respiratory signs. Death usually follows within a few weeks. Chronic disease is more common and presents as nasal, pulmonary and cutaneous forms, all of which may be observed in an affected animal. In the nasal form, ulcerative nodules develop on the mucosa of the nasal septum and lower part of the turbinates. A purulent, blood-stained nasal discharge and regional lymphadenopathy are usually present. The ulcers eventually heal leaving star-shaped scars. The respiratory form is characterized by respiratory distress and the development of tubercle- like lesions throughout the lungs. The cutaneous form, termed farcy, is a lymphangitis in which nodules occur along the course of the lymphatic vessels of the limbs. Ulcers develop and discharge a yellowish pus. Chronically affected animals may die after several months or may recover and continue to shed organisms from the respiratory tract or skin.

Carnivores may contract the disease by eating infected carcasses (Galati *et al*., 1974).

Pathogenesis

Glanders in the horse is usually a chronic, disseminated, debilitating disease. Much has been learned about the pathogenic mechanisms of *B. mallei* following the sequencing of its genome and the identification of genes predicted to be involved in virulence. The organism has a capsule, type III and type IV secretion systems, and quorum sensing mechanisms. In addition, genes encoding adhesion proteins and fimbriae have been identified. *Burkholderia mallei* is capable of intracellular survival and spread, and utilizes actinbased motility, similar to *Listeria monocytogenes* (Larsen and Johnson, 2009). The presence of *B. mallei* in the host gives rise to a hypersensitivity reaction, the basis of the mallein test.

Diagnostic procedures

- In regions where the disease is endemic, clinical signs may be diagnostic.
- Specimens for laboratory diagnosis should include discharges from lesions and blood for serology. Specimens must be processed in a biohazard cabinet.
- *Burkholderia mallei* grows best on media containing glycerol and most strains grow on MacConkey agar (Anon., 2008). Plates are incubated aerobically at 37°C for 2–3 days.
- Identification criteria for isolates:
  – Colonial characteristics
  – Majority of strains grow on MacConkey agar without utilizing lactose
  – Comparatively unreactive biochemically and non - motile (Table 25.1)
  – As commercially available biochemical test kits are not useful for confirmation of identification, PCR and real-time PCR techniques may be employed for this purpose (Anon., 2008).
- Suitable serological tests include the complement fixation test and agglutination techniques. A competitive ELISA has been developed and found to have comparable sensitivity and specificity to the CFT (Sprague *et al*., 2009).
- The mallein test is an efficient field test both for confirmation and for screening in-contact animals. Mallein, a glycoprotein extract of *B. mallei*, is injected intradermally (0.1ml) just below the lower eyelid. A positive reaction is indicated by local swelling and mucopurulent ocular discharge after 24 hours.

Treatment and control

- A test and slaughter policy is enforced in countries where the disease is exotic.
- In endemic areas, antibiotic therapy is inappropriate as treated animals often become subclinical carriers.
- Effective cleaning and disinfection of all contaminated areas must be carried out. Formalin (1.5%) or an iodophor (2.0%) can be used, with a contact time of 6 hours.
- Vaccines for use in humans are not currently available. Because of the threat of bioterrorism, research in this area is progressing (Larsen and

Johnson, 2009).

Melioidosis

Melioidosis, caused by *B. pseudomallei*, is endemic in tropical and subtropical regions of south-eastern Asia and Australia where the organism is widely distributed in soil and water. Infection may follow ingestion, inhalation or skin contamination from environmental sources. The bacterium is an opportunistic pathogen, and stress factors or immunosuppression may predispose to clinical disease. Many animal species, including humans, are susceptible and subclinical infections may occur. Because infection is usually disseminated, abscesses develop in many organs including lungs, spleen, liver, joints and central nervous system. Melioidosis is a chronic, debilitating, progressive disease, often with a long incubation period. Clinical signs, which are variable, relate to lesion severity and distribution. In horses melioidosis, which can mimic glanders, is often referred to as pseudoglanders.

Pathogenesis and pathogenicity

As with glanders, considerable progress in understanding the pathogenesis of melioidosis has been made recently. The aetiological agent shares many putative virulence factors with *B. mallei*, including a capsule and Type III and Type IV secretion systems (Larsen and Johnson, 2009). Extracellular products of *B. pseudomallei* such as an exotoxin, a dermonecrotic protease and a lecithinase have been implicated in disease production (Dance, 1990). Both strain virulence and host immunosuppression may influence the establishment and outcome of infection.

Diagnostic procedures

- In regions where the disease is encountered, gross pathological findings may aid diagnosis.
- Specimens for laboratory diagnosis should include pus from abscesses, affected tissues and blood for serology. A biohazard cabinet must be used for processing specimens.
- A fluorescent antibody technique for demonstrating the organism in tissue smears is available in some reference laboratories.
- Blood agar and MacConkey agar plates, inoculated with suspect material,

are incubated aerobically at 37°C for 24 to 48 hours.

- Identification criteria for isolates:
  – Colonial morphology and characteristic musty odour
  – Lactose utilized in MacConkey agar
  – Biochemical characteristics (Table 25.1)
  – Slide agglutination test using specific antiserum
  – PCR techniques may be used to confirm the identity of isolates.
- ELISA, complement fixation and indirect haemag-glutination tests can be used for detecting serum antibodies.

Treatment and control

- Confirmation of infection followed by slaughter of infected animals is mandatory in countries where the disease is exotic.
- Treatment is expensive and unreliable. Relapses can occur after antibiotic therapy is discontinued. As with *Pseudomonas* species, this organism is intrinsically resistant to many antimicrobial agents and a large number of drug resistance genes have been identified, including genes encoding drug efflux pumps, β-lactamases and aminoglycoside acetyl-transferases (Whitlock *et al*., 2008).
- Effective vaccines are not available at present but research in this area is progressing.

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Chapter 26

Actinobacillus species

Actinobacillus species are non-motile, Gram-negative rods (0.3 to 0.5 × 0.6 to 1.4 µm) which may be pleomorphic and frequently have a coccobacillary appearance. These facultative anaerobes ferment carbohydrates, producing acid but not gas. Most species are ureasepositive and oxidase - positive. Actinobacilli exhibit some host specificity and are mainly pathogens of farm animals. Currently, there are 18 species within the genus and those of veterinary importance are presented in Fig. 26.1.

The genus *Actinobacillus* is one of 15 genera within the family *Pasteurellaceae*. Based on phenotypic characteristics, classification of members of the family is difficult. Recent minimum standards for the description of members of the *Pasteurellaceae* have been described by Christensen *et al*. (2007). These include phylogenetic comparison of 16s rRNA gene sequences. *Actinobacillus seminis* is not closely related to other members of the genus but reclassification of this organism is awaiting further study (Euzby, 2005).

Usual habitat

Actinobacilli are commensals on mucous membranes of animals, particularly in the upper respiratory tract and oral cavity. As actinobacilli cannot survive for long in the environment, carrier animals play a major role in transmission.

Key points

- Medium-sized, non-motile, Gram-negative rods
- Facultative anaerobes
- Most species are oxidase-positive and produce urease
- Species of veterinary importance grow on MacConkey agar, apart from *Actinobacillus pleuropneumoniae*
- Commensals on mucous membranes
- Produce a wide range of disease conditions in domestic animals

Differentiation of *Actinobacillus* species

Actinobacillus species can be distinguished by colonial characteristics and biochemical reactions (Table 26.1) but definitive identification increasingly relies on molecular methods.

- On primary isolation on blood agar, colonies of *A. lignieresii, A. equuli*, and A. *suis* exhibit cohesive properties when touched with an inoculation loop. Colonies of *A. pleuropneumoniae, A. suis* and *A. equuli* subspecies *haemolyticus* are haemolytic.
- Growth and reactions on MacConkey agar:

  – *A. lignieresii, A. equuli* and most strains of *A. suis* grow well on MacConkey agar. Colonies of *A. lignieresii* are initially pale, turning pink after 48 hours. *Actinobacillus equuli* and *A. suis* ferment lactose, producing pink colonies.

  – *A. pleuropneumoniae* and *A. seminis* do not grow on MacConkey agar.
- Commercially available biochemical kits or specialized test methods can be used to differentiate *Actinobacillus* species. *Actinobacillus seminis*, which is catalase-positive, is relatively inactive biochemically.
- Serotyping of *A. pleuropneumoniae* isolates is based on differences in capsular polysaccharide antigens and is carried out using slide agglutination or geldiffusion tests. Molecular tests which can distinguish differences between certain serovars have been developed also. For example, Zhou *et al*. (2008) described a multiplex PCR that can differentiate serovars 3, 6 and 8 of *A. pleuropneumoniae*.
- Many PCR-based methods have been developed for identification and differentiation of actinobacilli (Frey *et al*., 1995; Schaller *et al*., 2001; vanden Bergh *et al*., 2008b).

Figure 26.1 *Actinobacillus* species and the conditions they cause in domestic animals.

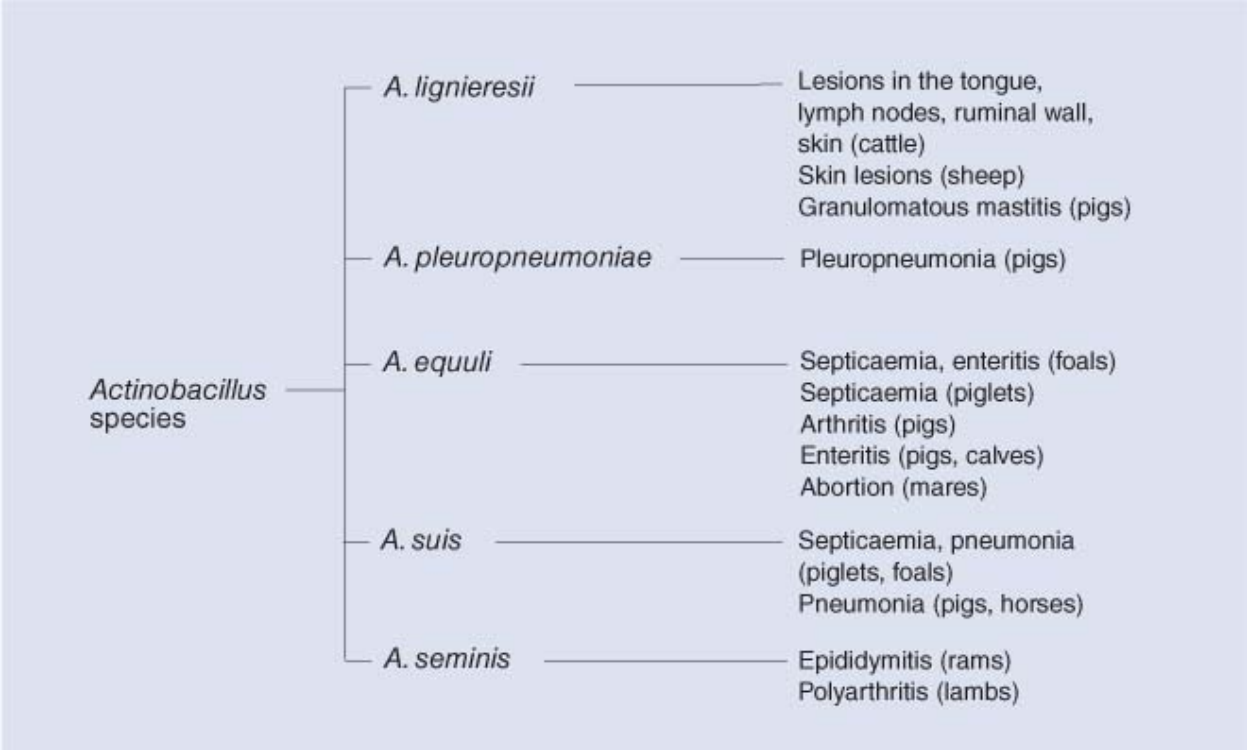

Table 26.1 Differentiating features of *Actinobacillus* species.

Feature	*A. lignieresii*	*A. pleuropneumoniae*	*A. equuli*	*A. suis*
Haemolysis on sheep blood agar	–	+	v[a]	+
Colony type on blood agar	Cohesive	Variable	Cohesive	Cohesive
Growth on MacConkey agar	+	–	+	+
CAMP test with *S. aureus*	–	+	–	–
Oxidase production	+	v	+	+
Catalase production	+	v	v	+
Urease production	+	+	+	+
Hydrolysis of aesculin	–	–	–	+
Acid from:				
L-arabinose	v	–	–	+
lactose	+[b]	–	+	+
maltose	+	+	+	+
mannitol	+	v	+	–
melibiose	–	–	+	+
salicin	–	–	–	+
sucrose	+	+	+	+
trehalose	–	–	+	+

+, over 90% isolates positive. −, less than 10% isolates positive. v, variable reaction.

a, *A. equuli* subspecies *haemolyticus* are haemolytic.

b, slow reaction.

Pathogenesis and pathogenicity

With the exception of *A. pleuropneumoniae*, the cause of pleuropneumonia in pigs, the virulence factors and pathogenic mechanisms associated with the actinobacilli are poorly defined.

Clinical infections

Actinobacilli can cause a variety of infections in farm animals including timber (wooden) tongue in cattle, pleuropneumonia in pigs and systemic disease in foals and piglets (Fig. 26.1).

Actinobacillosis in cattle

Actinobacillosis, a chronic pyogranulomatous inflammation of soft tissues, is most often manifest clinically in cattle as induration of the tongue, referred to as timber tongue. Potentially important lesions occur in the oesophageal groove and the retropharyngeal lymph nodes. The aetiological agent, *Actinobacillus lignieresii*, is a commensal of the oral cavity and the intestinal tract. It can survive for up to 5 days in hay or straw. The organisms enter tissues through erosions or lacerations in the mucosa and skin. A localized pyogranulomatous response is associated with club colonies containing the bacteria. In addition, spread through the lymphatics to the regional lymph nodes may induce pyogranulomatous lymphadenitis. Virulence mechanisms are unknown. Although the gene for production of a repeats - in - structural - toxin (RTX) has been identified in the genome, it does not appear to be expressed.

Bovine actinobacillosis is usually a sporadic disease, although herd outbreaks of limited extent can occur (Campbell *et al*., 1975). Animals with timber tongue have difficulty in eating and drool saliva. Involvement of the tissues of the oesophageal groove can lead to intermittent tympany, and enlargement of the retropharyngeal lymph nodes can cause difficulty in swallowing and stertorous breathing. Lesions of cutaneous actinobacillosis may be found on the head, thorax, flanks and upper limbs. Animals with ulcerated discharging lesions can contaminate the environment. Localized pyogranulomatous lesions in the retropharyngeal lymph nodes are often found at slaughter. Isolates of *A.*

lignieresii obtained from lesions in horses are phenotypically similar to, but genetically different from, those in cattle and have been designated *Actinobacillus* genomospecies 1.

Diagnosis

- Induration of the tongue is characteristic of the disease and there may be a history of grazing rough pasture.
- Specimens for laboratory examination include pus, biopsy material and tissues from lesions at postmortem.
- Gram-negative rods are demonstrable in smears from exudates.
- Pyogranulomatous foci containing club colonies may be evident in tissue sections.
- Cultures on blood agar and MacConkey agar are incubated aerobically at 37°C for 24 to 72 hours.
- Identification criteria for isolates:
  – Small, sticky, non-haemolytic colonies on blood agar
  – Slow lactose fermentation on MacConkey agar
  – Biochemical profile (Table 26.1)
  – Definitive identification is based on analysis of 16S rRNA gene sequences.

Treatment and control

- Animals with discharging lesions should be isolated.
- Sodium iodide parenterally or potassium iodide orally is effective.
- Potentiated sulphonamides or a combination of penicillin and streptomycin are usually effective. Oral isoniazid for 30 days has been used in animals with refractory lesions.
- Rough feed or pasture which may damage the oral mucosa should be avoided.

Infections in other animals caused by *A. lignieresii*

Cutaneous actinobacillosis of sheep presents as granulomatous lesions mainly on the head without tongue involvement. Granulomatous mastitis in sows, bite wounds in dogs and glossitis in a horse have been attributed to infection with *A.*

lignieresii (Baum *et al*., 1984). However, it is likely that isolates obtained from other species of animals were misclassified as *A. lignieresii* and were in fact other species within the actinobacilli or *Pasteurellaceae*.

Pleuropneumonia of pigs

Actinobacillus pleuropneumoniae is the only organism within the genus considered to be a primary pathogen. Pleuropneumonia can affect susceptible pigs of all ages and occurs in major pig-rearing regions worldwide. This highly contagious disease occurs primarily in pigs under 6 months of age and appears to be increasing in prevalence as a consequence of intensive rearing practices.

Pathogenesis and pathogenicity

Acute infection presents as a necrotizing fibrinohaemorrhagic pneumonia with pleuritis and pericarditis. In the more chronic stages, abscesses associated with adhesive fibrinous pleuritis are common findings (Rycroft and Garside, 2000).

Colonization represents the first phase of infection and *A. pleuropneumoniae* initially colonizes the tonsils and also binds to the cells of the lower respiratory tract. The organism possesses fimbriae and other proteinaceous structures such as fibronectin which facilitate adherence to epithelial cells. Lipopolysaccharide (LPS) is also thought to play a role in adhesion. Multiplication following adherence is dependent on the ability of the organism to obtain adequate nutrients, especially iron. *Actinobacillus pleuropneumoniae* can obtain iron through the utilization of heme products released by haemolysis and is capable of obtaining iron from porcine transferrin also. Virulent strains possess capsules which are both antiphagocytic and immunogenic, whereas non-encapsulated strains are avirulent (Bertram, 1990). Thus, capsules are important in protection against host defence mechanisms. Both neutrophils and macrophages phagocytose *A. pleuropneumoniae* but only neutrophils are capable of effectively killing these organisms. *Actinobacillus pleuropneumoniae* can survive within macrophages, in part due to its thick capsule but also because of other factors such as LPS and possession of superoxide dismutase. This pathogen produces four related cytotoxins which belong to the RTX cytolysin family and are designated ApxI to IV. These toxins are the most significant virulence factors produced by the organism and produce pores in cell membranes. Different serovars possess different combinations of toxins although all possess ApxIV (vanden Bergh, 2008a). The potency of the four toxins differs, with ApxI and

ApxII of particular pathogenic significance. Thus, the virulence of different serovars varies. In addition to direct damage to a number of cell types, the toxins stimulate the release of inflammatory mediators (MacInnes, 2010). Neutrophils chemically attracted to infected pulmonary tissue are damaged and release lytic enzymes. Damage to endothelial cells and activation of factor XII by LPS initiates the coagulation and fibrinolysis systems, which leads to the formation of microthrombi, localized ischaemic necrosis and the characteristic lesions of acute pleuropneumonia.

Clinical signs and epidemiology

Subclinical carrier pigs, which are encountered in unaffected populations, harbour the organisms in the respiratory tract and tonsillar tissues. Poor ventilation and sudden drops in ambient temperature seem to precipitate disease outbreaks. Aerosol transmission occurs in confined groups. In outbreaks of acute disease, some pigs may be found dead and others show dyspnoea, pyrexia, anorexia and a disinclination to move. Blood-stained froth may be present around the nose and mouth and many pigs show cyanosis. Pregnant sows may abort. Morbidity rates can range from 30 to 50% and case fatality rates may reach 50%. Concurrent infections with *Pasteurella multocida* and mycoplasmas may exacerbate the condition. At post-mortem, areas of consolidation and necrosis are found in the lungs along with fibrinous pleurisy. Blood-stained froth may be found in the trachea and bronchi.

Diagnosis

- There may be a history of ventilation failure or a rapid drop in environmental temperatures prior to an outbreak of pulmonary disease.
- Specimens for laboratory examination should include tracheal washings or affected portions of lung tissue.
- Areas of haemorrhagic consolidation close to the main bronchi and severe fibrinous pleuritis may suggest this condition.
- Specimens, cultured on chocolate agar, specialized selective media and blood agar, are incubated in an atmosphere of 5 to 10% CO_2 at 37°C for 2 to 3 days.
- Identification criteria for isolates:
  – Small colonies surrounded by clear haemolysis

– No growth on MacConkey agar
– Positive CAMP test with *Staphylococcus aureus*
– Biochemical profile (Table 26.1).

- Fifteen serovars and two biotypes are recognized (vanden Bergh *et al*., 2008a). Isolates belonging to biotype 1 require V factor (NAD) for growth whereas those belonging to biotype 2 are NAD-independent. Serovars 1 and 5 are divided into two subtypes and serovars 13 and 14 are found only within biotype 2. The occurrence of serovars tends to be associated with particular geographical regions (vanden Bergh *et al*., 2008b) and those prevalent in a particular region should be identified prior to the implementation of vaccination programmes.
- Immunofluorescent or PCR-based techniques may be used to demonstrate the organism in tissues (Christensen and Bisgaard, 2004).
- Serological techniques, principally based on ELISA, can be used for diagnosis and epidemiological investigation.
- Strain typing techniques employing molecular methods, in particular PCR-restriction fragment length polymorphism, have been developed (vanden Bergh *et al*., 2008b).

Treatment

- As antibiotic resistance is encountered in some strains, chemotherapy should be based on the results of antibiotic susceptibility testing. However, the setting of specific clinical breakpoints for antibiotics used in porcine respiratory disease is not yet complete, and thus the criteria used to determine susceptibility and resistance are usually adopted from human medicine (Schwarz *et al*., 2008).
- Prophylactic administration of antibiotics to incontact pigs may limit the severity of clinical disease.

Control

- The original vaccines developed for *A. pleuropneumoniae* were polyvalent bacterins which induced protective immunity but failed to prevent transmission or the development of a carrier state. Research efforts have concentrated on finding antigens that are conserved among all serovars and could be used in subunit vaccines. Most commercially available subunit vaccines contain Apx toxins, frequently combined with other virulence

factors such as capsular antigen or outer membrane proteins (Ramjeet *et al*., 2008). Development of live and DIVA (differentiating infected from vaccinated animals) vaccines is under investigation also (Ramjeet *et al*., 2008).

- Predisposing factors such as poor ventilation, chilling and overcrowding should be avoided.

Sleepy foal disease

Sleepy foal disease is an acute, potentially fatal septicaemia of newborn foals caused by *Actinobacillus equuli*. The species is divided into two subspecies, *equuli* and *haemolyticus*. Both subspecies cause disease in horses but subspecies *equuli* has been isolated from septicaemia and abortion in pigs also (Christensen and Bisgaard, 2004). Although primarily a pathogen of foals, *A. equuli* occasionally produces disease conditions, such as abortion, septicaemia and peritonitis, in adult horses (Gay and Lording, 1980). The organism is found in the reproductive and intestinal tracts of mares. Foals can be infected *in utero* or after birth, via the umbilicus. Affected foals are febrile and recumbent. Death usually occurs in 1 to 2 days. Foals which recover from the acute septicaemic phase may develop polyarthritis, nephritis, enteritis or pneumonia.

Foals dying within 24 hours of birth have petechiation on serosal surfaces and enteritis. Meningoencephalitis can be detectable histologically. Foals which survive for 1 to 3 days have typical pin-point suppurative foci in the kidneys. *Actinobacillus equuli* subspecies *haemolyticus* shows RTX activity and the toxin is encoded by the *eqx* gene. This gene has not been demonstrated in subspecies *equuli*.

Diagnosis

- History of the disease occurring on the premises in previous seasons.
- The clinical signs in a neonatal foal may suggest the disease.
- Specimens should be cultured on blood agar and MacConkey agar and incubated aerobically at 37°C for 1 to 3 days.
- Identification criteria for isolates:
  – Sticky colonies with variable haemolysis on blood agar
  – Lactose-fermenting colonies on MacConkey agar
  – Biochemical profile (Table 26.1).

- PCR for the direct detection of the organism in clinical specimens is available (Pusterla *et al*., 2009).

Treatment and control

Unless the disease is detected early, antimicrobial therapy is of little benefit.

- The organism is usually susceptible to streptomycin, tetracyclines and ampicillin.
- Supportive treatment includes blood transfusion and bottle-feeding with colostrum.
- Mares that have had affected foals should be monitored closely at subsequent foalings.
- Good hygiene should be observed.
- Prophylactic antibiotic therapy may be considered for newborn foals.
- Commercial vaccines are not available.

Actinobacillus suis infection of piglets

Actinobacillus suis may be present in the upper respiratory tract of sows and piglets become infected by aerosols or possibly through skin abrasions. The infection occurs mainly in pigs under 3 months of age (Sanford *et al*., 1990) but disease may occur in animals of all ages if the organism is introduced into a naïve high-health herd. The organism may cause pleuropneumonia similar to *A. pleuropneumoniae* but appears to be more invasive and may cause a variety of disease conditions including septicaemia, meningitis, enteritis, metritis and abortion. Mortality may be up to 50% in affected litters. Clinical signs include fever, respiratory distress, prostration and paddling of the forelimbs. Petechial and ecchymotic haemorrhages occur in many organs and there may be evidence of interstitial pneumonia, pleuritis, meningoencephalitis, myocarditis and arthritis. An unusual form of the infection in mature pigs has been reported, with skin lesions resembling those of swine erysipelas (Miniats *et al*., 1989). Knowledge of virulence factors is incomplete but outer membrane protein A may play an important role in initial colonization (Ojha *et al*., 2010) and the organism is known to produce RTX toxins Apx I and ApxII (van Ostaaijen *et al*., 1997). *Actinobacillus suis* isolates recovered from animals other than pigs are now thought to have been identified incorrectly.

Diagnosis

- Specimens from tissues obtained at post mortem should be cultured on blood agar and MacConkey agar and incubated at 37°C for 1 to 3 days.
- Identification criteria for isolates:
  – Sticky, haemolytic colonies
  – Pink, lactose-fermenting colonies on MacConkey agar
  – Biochemical profile (Table 26.1)
  – Definitive identification can be made using analysis of 16S rRNA gene sequences.

Treatment and control

- Treatment should be based on antibiotic susceptibility testing of isolates. The organism is usually susceptible to ampicillin, carbenicillin, potentiated sulphonamides and tetracyclines.
- Contaminated pens should be disinfected.
- Commercial vaccines are not available.

Actinobacillus seminis infection in rams

Actinobacillus seminis is a common cause of epididymitis in young rams. The condition is endemic in New Zealand, Australia and South Africa and has also been reported in the USA and the UK (Sponenberg *et al*., 1982; Low *et al*., 1995). The organism is found in the prepuce, and epididymitis may follow an ascending opportunistic infection. However, detailed knowledge of the sources and modes of transmission of *A. seminis* is lacking and some studies suggest that the ewe may be involved as an intermediate carrier or through ewe- to - lamb transmission (Al - Katib and Dennis, 2009). Abscesses form in affected epididymides and there may be purulent discharge through fistulae on to the scrotal skin. Virgin rams between 4 and 8 months of age are most commonly affected.

Diagnosis

- Specimens for laboratory examination should include pus, biopsy material or tissue obtained at post-mortem.
- Specimens should be cultured on blood agar and incubated aerobically at

37°C for 24 to 72 hours.

- Identification criteria for isolates:
  – Small pin-point, non-haemolytic colonies
  – No growth on MacConkey agar
  – Catalase-positive
  – Unreactive in many biochemical tests.
- PCR-based methods may be used for differentiation from related organisms.
- Serological tests include the CFT and ELISA.

Treatment and control

A vaccine is not available. Recovered rams show much reduced fertility and, if a high incidence of disease is expected in a flock, prophylactic antibiotics in feed or water may be indicated.

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Chapter 27

Pasteurella species, Mannheimia haemolytica and Bibersteinia trehalosi

Pasteurella and *Mannheimia* species are small (0.2 × 1–2 μm), non-motile, Gram-negative rods or coccobacilli. They are oxidase-positive facultative anaerobes, and most species are catalase-positive. Although non-enriched media will support their growth, these organisms grow best on media supplemented with blood or serum. They usually remain viable for only a few days on culture plates. Some species, such as *Mannheimia haemolytica*, *Bibersteinia trehalosi* and P. *aerogenes*, can tolerate the bile salts in MacConkey agar. In smears from infected tissues stained by the Giemsa method, pasteurellae exhibit bipolar staining (Fig. 27.1).

The family *Pasteurellaceae* comprises 15 genera, seven of which contain organisms of veterinary importance: *Actinobacillus, Avibacterium, Haemophilus, Histophilus, Mannheimia, Pasteurella* and *Bibersteinia*. These genera share a number of common features, and some organisms have been reclassified within these genera following deoxyribonucleic acid hybridization studies and 16S rRNA gene sequencing. *Pasteurella trehalosi* (formerly *Pasteurella haemolytica* biotype T) has been reclassified as *Bibersteinia trehalosi* (Blackall *et al.*, 2007). Isolates of *P. haemolytica* previously known as biotype A have been allocated to a new genus and renamed *Mannheimia haemolytica* (Sneath and Stevens, 1990; Angen *et al.*, 1999). *Pasteurella multocida, B. trehalosi* and *M. haemolytica* are major animal pathogens (Table 27.1). The genera *Actinobacillus, Histophilus* and *Haemophilus* also contain major pathogens of domestic animals (see Chapters 26 and 29). Other *Pasteurella* and *Mannheimia* species which have been isolated from domestic animals and humans are presented in Table 27.2.

Key points

- Small Gram-negative rods
- Optimal growth on enriched media
- Non-motile, oxidase-positive, facultative anaerobes

- Most species are catalase-positive
- Some species grow on MacConkey agar
- Bipolar staining is prominent in smears from lesions using the Giemsa method
- Commensals in the upper respiratory tract
- Respiratory pathogens

Usual habitat

Most *Pasteurella*, *Bibersteinia* and *Mannheimia* species are commensals on the mucosae of the upper respiratory tract of animals. Their survival in the environment is relatively short.

Differentiation of *Pasteurella, Bibersteinia* and *Mannheimia* species

Pasteurellae and *Mannheimia* species can be distinguished by colonial and growth characteristics, and by biochemical reactions. Strains of *P. multocida* can be differentiated by serotyping and biotyping, whereas *M. haemolytica* and *B. trehalosi* strains are usually differentiated by serotyping. In addition, a number of molecular typing methods have been developed for differentiating these pathogens.

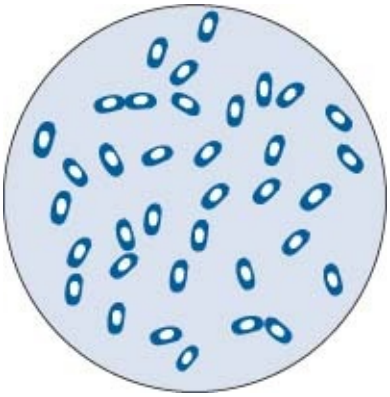
- Colonial characteristics:

  – *P. multocida* colonies are round, greyish, shiny and non-haemolytic. Colonies of some pathogenic strains are mucoid due to the production of thick hyaluronic acid capsules. The colonies have a subtle but characteristic sweetish odour.

  – *M. haemolytica*, *M. granulomatis* and *B. trehalosi* colonies are haemolytic and odourless.

  – Colonies of the other *Pasteurella* species are round, greyish and non-haemolytic except those of P. *testudinis*, which are haemolytic.

Figure 27.1 Bipolar staining of *Pasteurella* species. Bacteria in Giemsa-stained blood smears from lesions have this characteristic staining pattern.



- On MacConkey agar, *M. haemolytica* and *B. trehalosi* grow as pin-point, red colonies. Most pathogenic *Pasteurella* species do not grow on MacConkey agar.
- Biochemical methods for differentiating the main pathogenic *Pasteurella* and *Mannheimia* species are summarized in Table 27.3.
- *Pasteurella* and *Mannheimia* species are relatively active biochemically:

  – Reactions in conventional biochemical tests are indicated in Table 27.3.

  – Commercially available biochemical test strips such as API 20NE (BioMérieux) can also be used.

  – In TSI agar slopes, a yellow slant and yellow butt without H_2S production are typical.
- Serotyping of *Pasteurella* and *Mannheimia* species:

  – The types (or serogroups) of *P. multocida* are identified on the basis of differences in capsular polysaccharides (Carter, 1955) and are designated A, B, D, E and F (Table 27.1). The organisms are further subdivided into about 16 somatic types (serotypes) on the basis of serological differences in cell wall lipopolysaccharides (Namioka and Murata, 1961- Heddleston *et al*., 1972). Both capsular and somatic antigens are used to designate a specific serotype. Serological methods for establishing both the capsular and somatic types include agglutination and agar gel diffusion tests.

Table 27.1 The major pathogenic *Pasteurella* and *Mannheimia* species, their principal hosts and associated diseases.

Bacterial species	Hosts	Disease conditions
P. multocida		
type A	Cattle	Associated with bovine pneumonic pasteurellosis (shipping fever); associated with enzootic pneumonia complex of calves; mastitis (rare)
	Sheep	Pneumonia, mastitis
	Pigs	Pneumonia, atrophic rhinitis

	Poultry	Fowl cholera
	Rabbits	Snuffles
	Other animal species	Pneumonia following stress
type B	Cattle, buffaloes	Haemorrhagic septicaemia (Asia)
type D	Pigs	Atrophic rhinitis, pneumonia
type E	Cattle, buffaloes	Haemorrhagic septicaemia (Africa)
type F	Poultry, especially turkeys	Fowl cholera
	Calves	Rare cases of peritonitis
M. haemolytica	Cattle	Bovine pneumonic pasteurellosis (shipping fever)
	Sheep	Septicaemia (under 3 months of age), pneumonia, gangrenous mastitis
B. trehalosi	Sheep	Septicaemia (5 to 12 months of age)

Table 27.2 *Pasteurella* and *Mannheimia* species of minor veterinary significance.

Pasteurella species	Hosts	Comments
P. aerogenes	Pigs	Intestinal commensal; implicated in abortion (rare)
P. anatis	Ducks	Found in intestine
P. langaaensis	Chickens	Commensals in upper respiratory tract
(P. langaa)		
P. volantium		
P. caballi	Horses	Commensal in the upper respiratory tract; occasionally implicated in respiratory disease and peritonitis
P. canis	Dogs	Commensal in oral cavity; occasionally infects wounds
P. dagmatis	Dogs, cats	Commensal in oral cavity and nasopharynx; occasionally infects wounds
M. granulomatis	Cattle	Fibrogranulomatous panniculitis
P. lymphangitidis	Cattle	Lymphangitis (rare)
P. mairii	Pigs	Abortion (rare)
P. pneumotropica	Rodents	Commensal in the upper respiratory tract; sporadic cases of pneumonia and bite-wound abscess
P. stomatis	Dogs, cats	Found in respiratory tract
P. testudinis	Turtles, tortoises	: Abscessation (rare)

Table 27.3 Differentiation of the main pathogenic *Pasteurella* and *Mannheimia* species.

Feature	*M. haemolytica*	*Pasteurella* species		
		P. multocida	*B. trehalosi*	*P. pneumotropica*
Haemolysis on sheep blood agar	+	–	+	–
Growth on MacConkey agar	+	–	+	v
Distinctive odour from colonies	–	+	–	–
Indole production	–	+	–	+
Catalase activity	+	+	–	+
Urease activity	–	–	–	+
Ornithine decarboxylase activity	–	+	–	+
Acid from:				
lactose	+	–	–	v
sucrose	+	+	+	+
D-trehalose	–	v	+	+
L-arabinose	–	v	–	–
maltose	+	–	+	v
D-xylose	+	v	–	v

+, most strains positive.

−, most strains negative.

v, variable reactions.

– Seventeen serotypes of *M. haemolytica/B. trehalosi* are recognized on the basis of extractable surface antigens. An indirect haemagglutination test or a rapid plate agglutination test may be used to identify each serotype. Serotypes 3, 4, 10 and 15 are classified as *B. trehalosi*; the remaining serotypes are classified as *M. haemolytica* except serotype 11 which is reclassified as *M. glucosida*.

- Biotyping of *Pasteurella multocida* is occasionally carried out in epidemiological investigations but is not generally used for diagnosis. In addition, the frequent occurrence of unassigned biotypes limits the usefulness of biotyping (Dziva *et al*., 2008). Three subspecies of *P. multocida* are recognized, namely *P. multocida* subspecies *multocida*, *P. multocida* subspecies *septica* and *P. multocida* subspecies *gallicida*. An additional subspecies, *P. multocida subspecies tigris*, has been proposed (Capitini e t al., 2002).
- Molecular typing methods using techniques such as 16S rRNA gene sequencing, amplified fragment length polymorphism, pulsed- field gel electro-phoresis, multi-locus sequence typing and other PCR-based methods have been developed for investigating strains of *Pasteurella* and

Mannheimia species (Katsuda *et al*., 2003; Dziva *et al*., 2008). Unfortunately a definitive correlation between serotype and genotypic characteristics has not been established and thus complete characterization of strains requires both phenotypic and genotypic information.

Pathogenesis and pathogenicity

Many *P. multocida* infections are endogenous. The organisms, which are normally commensals of the upper respiratory tract, may invade the tissues of immunosuppressed animals. Exogenous transmission can also occur either by direct contact or through aerosols. Whether there are inherent differences between commensal and pathogenic strains is unresolved at present (Dabo *et al*., 2007). Factors of importance in the development of disease include adhesion of the pas-teurellae to the mucosa and avoidance of phagocytosis. A number of adhesins have been described including fimbriae, surface fibrils and filamentous haemagglutinins. The capsule, particularly in type A strains, has a major antiphagocytic role and may also function in adhesion in some strains. Differences in capsular composition occur in different serotypes. The capsule of types A and B is composed of hyaluronic acid; that of type D contains heparin and type F contains chondroitin. These compounds are similar to those found naturally in the host species colonized by the particular serotype and this molecular mimicry limits the development of a strong immune response to the organism. As with other bacteria, the availability of iron is important for colonization and multiplication of the organism and *P. multocida* has a number of iron acquisition systems. The major virulence factor of *P. multocida* strains of type D, which produce atrophic rhinitis in pigs, is PMT toxin, a cytotoxic protein that stimulates cytoskeletal rearrangements and growth of fibroblasts. In septicaemic pasteurello-sis, severe endotoxaemia and disseminated intravas-cular coagulation cause serious illness which can prove fatal.

Four main virulence factors have been identified in strains of *M. haemolytica* and *B. trehalosi* (Confer *et al*., 1990) : adhesins which may enhance colonization; a capsule that inhibits complemen^mediated destruction of the organisms in serum; endotoxin which can alter bovine leukocyte functions and is directly toxic for bovine endothelial cells; leukotoxin, a pore-forming cytolysin that affects leukocyte and platelet functions when present at low concentrations and causes cytolysis at high concentrations. The subsequent release of lysosomal enzymes from damaged cells together with inflammatory mediators, such as

tumour necrosis factor-α and eicosanoids, contributes to severe tissue damage in these infections.

The major adhesins of *M. haemolytica* appear to be two outer membrane proteins, OmpA and Lpp1 (Czuprynski, 2009) although the organism also has fimbriae and enzymes such as sialoglycoprotease and neuraminidase which may enhance colonization of the respiratory tract (Rice *et al*., 2008). Once established in the lung, the organism produces leukotoxin, which has potent effects on ruminant neutrophils whereas neutrophils of other species are resistant. The specificity is based on the ability of the leuko-toxin to bind to the transmembrane receptor CD18, a subunit of beta2-integrin. This receptor, when associated with CD11a, is expressed as the heterodimeric glycoprotein lymphocyte-function-associated antigen 1 (LFA) 1) which is responsible for the high)affinity binding of leukotoxin to ruminant leukocytes (Rice *et al*., 2008). Binding initiates a series of intracellular events which impair mitochondrial function and leads to the release of cytochrome c and ultimately cell death (Czuprynski, 2009). The importance of viral infections in predisposing to infection with *M. haemolytica* is well known and recent work has shown the possible mechanism behind this observation. *In vitro* studies have shown increased expression of LFA-1 on bovine peripheral blood mononuclear cells infected with BHV- 1, thus making them more susceptible to the effects of *M. haemolytica* leukotoxin (Leite *et al*., 2004).

Diagnostic procedures

- There may be a history of exposure to stress arising from transportation or overcrowding.
- Suitable specimens for laboratory examination from live animals include tracheobronchial aspirates, nasal swabs or mastitic milk.
- Tissue or blood smears from septicaemic cases, stained by Giemsa or Leishman methods, may reveal large numbers of bipolar-staining organisms.
- Specimens should be cultured on blood agar and MacConkey agar. Plates are incubated aerobically at 37°C for 24 to 48 hours. Blood agar, supplemented with neomycin, bacitracin and actidione, can be used for the isolation of *P. multocida* from heavily contaminated specimens.
- Identification criteria for isolates:

– Colonial characteristics

– Growth on MacConkey agar

– Positive oxidase test

– Biochemical profile

– PCR-based methods for the identification of colonies phenotypically suggestive of *Pasteurella* or *Mannheimia* species appear to be more reliable than biochemical methods (Alexander *et al*., 2008; Dziva *et al*., 2008).

- Serological tests are generally of little diagnostic value in the majority of the diseases caused by pas-teurellae and *Mannheimia* species.

Clinical infections

Clinical infections caused by pasteurellae and *Mannheimia* species in domestic animals are mainly attributable to *P. multocida, M. haemolytica and B. tre-halosi* (Table 27.1). *Pasteurella multocida* has a wide host range whereas *M. haemolytica* is largely restricted to ruminants and *B. trehalosi* to sheep. The diseases associated with *P. multocida* infection include haemor-rhagic septicaemia in ruminants and occasionally in other domestic species, porcine atrophic rhinitis, fowl cholera and bovine pneumonic pasteurellosis. However, the main aetiological agent of bovine pneumonic pasteurellosis is *M. haemolytica*, and this organism is also responsible for pneumonia in sheep and septicaemia in young lambs. Infection with *B. trehalosi* frequently results in septicaemia in older lambs.

Mannheimia haemolytica can cause a severe necrotiz-ing mastitis in ewes, and both *P. multocida* and M. *haemolytica* have been isolated occasionally from cases of bovine mastitis. Both organisms have also been implicated aetiologically in the enzootic pneumonia complex of calves.

Haemorrhagic septicaemia

Haemorrhagic septicaemia or barbone is an acute, potentially fatal septicaemia mainly affecting buffaloes and cattle. Predisposing factors such as overwork, poor body condition and monsoon rains are important in its development. *Pasteurella multocida* serotype B:2 causes the disease in Asia, the Middle East and some southern European countries, while serotype E:2 is the cause in Africa. However, the epidemiology of haem-orrhagic septicaemia appears to have

changed in recent years with a decline in serotype E strains isolated in Africa and increasing prevalence of type B strains (Dziva *et al*., 2008). Serotypes B and E are the only *P. multocida* serotypes with hyaluronidase activity. Haemorrhagic septicaemia, which is of considerable economic importance in endemic regions, is notifiable in some countries.

Buffaloes tend to be more susceptible to the disease than cattle. All ages can be affected but in endemic areas the disease is most common in animals between 6 and 24 months of age. Older animals may have a degree of immunity from previous exposure. Many older animals are latent carriers, with pasteurellae located in the tonsillar crypts. Periodically, these animals shed *P. multocida* in nasal secretions and in aerosols. Explosive outbreaks of disease can occur if an active carrier is introduced into a stressed, susceptible population.

Clinical signs

The incubation period of the disease is 2 to 4 days and the course ranges from 2 to 5 days. Death, without prior signs of illness, may occur within 24 hours of infection. Sudden onset of high fever, respiratory distress and a characteristic oedema of the laryngeal region are features of the disease. The oedema may extend to the throat and parotid regions and to the brisket. Recumbency is followed by death from endo-toxaemia. Mortality rates are usually over 50% and can approach 100% (De Alwis, 1992).

Diagnosis

- A history of acute disease with high mortality in areas where haemorrhagic septicaemia is endemic may suggest a presumptive diagnosis of the condition.
- Gross pathological changes may include widespread petechial haemorrhages, enlarged haemor-rhagic lymph nodes and blood-tinged fluid in the pleural cavity and the pericardial sac.
- Giemsa-stained blood smears from a recently dead animal often reveal large numbers of bipolar-staining organisms.
- Isolation, identification and serotyping the *P. multocida* isolate are confirmatory. Serotypes B:2 and E:2 are the specific strains associated with the disease. A multiplex PCR method for identification of serotypes described by Townsend *et al*. (2001) has replaced the traditional indirect haemagglutination assay in many laboratories.

- An antibody titre of 1:160 or above in an indirect haemagglutination test is indicative of recent exposure to the pathogen.

Treatment and control

- Antibiotic therapy in the early febrile stage is usually effective. Although the organism is susceptible to penicillin, tetracyclines are more often used.
- A slaughter policy for affected and in-contact animals is usually pursued in countries where the disease is exotic.
- Vaccines available for control of the disease include bacterins and a live heterotypic vaccine (Myint and Carter, 1989). Modified live deletion mutant vaccines have been developed also (Dalgleish *et al*., 2007).
- Latent carriers can be detected using immunohisto-chemical techniques on samples of tonsillar tissue.

Bovine respiratory disease

Both shipping fever and enzootic pneumonia of calves are associated with infection by *M. haemolytica* and *P. multocida*. either separately or jointly. Shipping fever, characterized by severe bronchopneumonia and pleurisy, occurs most commonly in young cattle within weeks of being subjected to severe stress, such as transportation, assembly in feedlots and close confinement. The condition is commonly associated with *M. haemolytica*, although *P. multocida* has also been isolated from lungs of affected cattle. The principal serotype of *M. haemolytica* associated with disease is A1 although recent surveys have demonstrated the increasing importance of serotype A6 in Europe and elsewhere (Donachie, 2000; Katsuda *et al*., 2008). The principal serotype of *P. multocida* isolated from cases of respiratory disease in cattle is A3. While *M. haemo-lytica* is known to cause serious disease acting as the sole infecting agent in cattle, there is debate as to whether *P. multocida* is a primary pathogen in bovine respiratory disease or whether it merely acts as an opportunistic invader with other pathogens (Dabo *et al*., 2007). Several respiratory viruses including parainfluenzavirus 3, bovine herpesvirus 1 and bovine respiratory syncytial virus may predispose to the bacterial invasion. At post-mortem, the cranial lobes of the lungs are red, swollen and consolidated. There is often an overlying fibrinous pleurisy.

Clinical signs

Clinical features of shipping fever include sudden onset of fever, depression, anorexia, tachypnoea and serous nasal discharge. In mixed infections, there is usually a marked cough and ocular discharge (Dalgleish, 1990). Morbidity rates can reach 50% and mortality rates range from 1 to 10%. Enzootic calf pneumonia is classically described as occurring in housed calves from 2 to 6 months of age with morbidity rates of up to 30% and mortality rates of between 5 and 10%.

Diagnosis

- There may be a history of exposure to stress factors and of sudden onset of respiratory disease.
- Gross pathological findings are of diagnostic value.
- Cytospin preparations from bronchoalveolar lavage usually reveal large numbers of neutrophils.
- Isolation of *M. haemolytica*, often in association with other pathogens, from transtracheal wash samples or affected lung tissue is confirmatory.

Treatment and control

- Affected animals must be isolated and treated early in the course of the disease. Treatment with oxy-tetracycline, potentiated sulphonamides and ampi-cillin is usually effective. However, increasing resistance to these antimicrobial agents in *M. haemolytica* has been reported in some countries (Hendriksen *et al.*, 2008).
- Stress factors must be kept to a minimum. Procedures such as castration, dehorning, branding and anthelminthic therapy should be carried out several weeks before young cattle are transported.
- Vaccination regimes for respiratory pathogens should be completed at least 3 weeks before transportation. Vaccines for *M. haemolytica* which incorporate modified leukotoxin and surface antigens may induce protection. A vaccine containing serotype- specific antigens of both *M. haemolytica* A1 and *M. haemolytica* A6 has been developed (Schreuer *et al.*, 2000) and recent research has concentrated on the development of vaccines for mucosal delivery (Rice *et al*, 2007).

Pasteurellosis in sheep

Outbreaks of ovine pneumonic pasteurellosis are usually caused by *M. haemolytica* whereas *P. multocida* tends to produce sporadic cases of the disease. *Mannheimia haemolytica* is a commensal of the upper respiratory tract in a proportion of healthy sheep. Factors that predispose to clinical disease are poorly understood and may include adverse climatic conditions or concurrent infections with respiratory viruses such as parainfluenzavirus 3. Flock outbreaks usually start with sudden deaths of some sheep and acute respiratory distress in others. Post- mortem findings include ventral consolidation in the cranial lobes of the lungs and fibrinous pleural and pericardial effusions. Laboratory confirmation relies on isolation of a heavy growth of *M. haemolytica* from lung lesions. Long-acting oxytetracycline is usually effective for treatment. Multivalent bacterins are available and may be of value in control programmes.

Septicaemic pasteurellosis in lambs less than 3 months of age is caused by *M. haemolytica*. In older animals between 5 and 12 months of age, septicaemic pasteurellosis is usually associated with *B. trehalosi* infection. *Bibersteinia trehalosi* is found in the tonsillar tissues of carrier sheep. As with most other pasteurella infections, clinical disease may be precipitated by a range of predisposing factors including transportation. Affected sheep which tend to be in good bodily condition may die suddenly and the mortality rate may approach 5%.

Atrophic rhinitis of pigs

Toxigenic strains of *P. multocida* type D or A cause a severe, progressive form of atrophic rhinitis. These toxigenic *P. multocida* isolates are designated AR+ (atrophic rhinitis-positive) strains. Infection with *Bordetella bronchiseptica* may cause mild, nonprogressive turbinate atrophy without significant distortion of the snout. However, the presence of this organism predisposes to infection with *P. multocida* AR+. Other factors that may predispose to infection include overstocking and poor ventilation. *Bordetella bronchiseptica* and non-toxigenic strains of *P. multocida* are widely distributed in pig herds. The introduction of a *P. multocida* AR+ carrier may initiate an outbreak of progressive atrophic rhinitis in a susceptible herd. Although young pigs are particularly vulnerable to infection, non-immune pigs of any age can be infected by these toxigenic strains.

Clinical signs

Early signs, usually encountered in pigs between 3 and 8 weeks of age, include

excessive lacrimation, sneezing and, occasionally, epistaxis. The snout gradually becomes shortened and wrinkled. As the disease progresses, a distinct lateral deviation of the snout may develop (Rutter, 1989). Atrophic rhinitis is rarely fatal. Affected pigs are usually underweight and damage to the turbinate bones may predispose to secondary bacterial infections of the lower respiratory tract.

Diagnosis

- In severely affected pigs, characteristic facial deformities are diagnostic.
- Visual assessment of the extent of turbinate atrophy can be made following slaughter by transverse section of snouts between the first and second premolar teeth.
- Isolation and identification of *P. multocida* should be followed by tests to confirm that the isolate is a toxigenic strain. Suitable tests include demonstration of toxicity for tissue culture cells (Rutter and Luther, 1984; Chanter *et al*., 1986), an ELISA test for toxin detection (Foged *et al.*, 1988) and the detection of the toxin gene by a polymerase chain reaction technique (Nagai *et al.*, 1994).
- A multiplex PCR for the detection of toxigenic and non-toxigenic strains of *P. multocida* and *Bordetella bronchoseptica* has been developed by Register and DeJong (2006).
- A multiplex PCR for the detection of toxigenic and non-toxigenic strains of *P. multocida* and *Bordetella bronchoseptica* has been developed by Register and DeJong (2006).

Control

- Chemoprophylaxis with sulphonamides, trimetho-prim, tylosin or tetracyclines in weaner, grower and sow rations could be considered. However, resistance to these agents is increasing worldwide (San Millan *et al.*, 2009 ; Sellyei *et al.*, 2009 ; Tang *et al.*, 2009) and antimicrobial susceptibility testing of isolates should be carried out before antimicrobial agents are administered for treatment or prophylaxis.
- Improvement in husbandry and management must be instituted to minimize the influence of predisposing factors and to reduce reliance on antimicrobial agents.
- Vaccination with a combined *B. bronchiseptica* bacterin and *P. multocida* toxoid may reduce the severity of the disease and improve growth rates

(Voets *et al.*, 1992). Sows should be vaccinated at 4 and 2 weeks before farrowing and young piglets at 1 week and 4 weeks of age.

Fowl cholera

Fowl cholera is a primary avian pasteurellosis caused by *P. multocida* capsular types A and F. It is highly contagious and affects both domestic and wild birds. The disease usually presents as an acute septicaemia which is often fatal. Turkeys tend to be more susceptible than chickens. Post-mortem lesions include haemorrhages on serous surfaces and accumulation of fluid in body cavities. In sporadic chronic cases of the disease, the signs and lesions are often related to localized infections. The wattles, sternal bursae and joints are usually swollen due to the accumulation of fibri-nopurulent exudates.

In the acute septicaemic form of the disease, numerous characteristic bipolar-staining organisms can be detected in blood smears and *P. multocida* can be isolated from blood, bone marrow, liver or spleen. The bacterium may be difficult to isolate from chronic lesions.

Medication of the feed or water early in an outbreak of acute disease may decrease the mortality rate. Polyvalent adjuvant bacterins are widely used and usually contain the most commonly isolated sero-types, 1, 3 and 4 (Anon., 2008). Autogenous vaccines may be required if the commercial vaccines are ineffective. Modified live vaccines are available in some regions of the world, including North America.

Snuffles in rabbits

Snuffles is a common, recurring, purulent rhinitis in rabbits which is caused by type A strains of *P. multoc-ida. Bordetella bronchiseptica* infection may sometimes cause similar clinical signs. *Pasteurella multocida* is a commensal in the upper respiratory tract of healthy carrier rabbits. Clinical disease is often precipitated by stress factors such as overcrowding, chilling, transportation, concurrent infections and poor ventilation, resulting in high levels of atmospheric ammonia. There is a purulent nasal discharge which cakes on the fore legs because affected rabbits paw their noses. Sneezing and coughing may be observed. Sequelae include conjunctivitis, otitis media and subcutaneous abscessation. Bronchopneumonia may develop in young rabbits. Treatment or prophylactic therapy with antibiotics may be of value. Predisposing stress factors must be eliminated. Commercial vaccines for this disease are not available.

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Chapter 28

Francisella tularensis

Francisella tularensis, originally classified as a *Pasteurella* species, is a tiny, poorly staining, Gram-negative rod (0.2 × 0.2-0.7 µm) which tends to have a coccobacillary appearance. It is an obligate aerobe, non-motile, oxidase-negative and weakly catalase-positive. This fastidious organism requires the addition of cysteine or cystine to blood agar for growth. It does not grow on MacConkey agar.

Francisella tularensis has a high lipid content and virulent isolates from infected animals produce capsules. *F. tularensis* subspecies *tularensis* (formerly subsp. *nearctica*), which includes highly virulent type A strains and were thought to occur only in North America, have been reported in Europe (Gurycova, 1998). Less virulent type B strains, *F. tularensis* subspecies *holarctica* (formerly subsp. *palaearctica*), which are assigned to two biogroups (Pearson, 1998), are found in both Eurasia and North America (Fig. 28.1). A third subspecies, *mediasiatica*, is occasionally associated with disease. Distinguishing features of the two pathogenic subspecies are presented in Table 28.1.

Key points

- Gram-negative coccobacillary rods
- Non-motile, obligate aerobes
- Fastidious; cysteine required for growth
- No growth on MacConkey agar
- Oxidase-negative, catalase-positive
- Facultative intracellular pathogen
- Survives in the environment for up to 4 months
- Wildlife reservoirs and arthropods important in epidemiology
- Causes tularaemia in animals and humans

Three other *Francisella* species, *F. noatunensis*, *F. novicida* and *F. philomiragia*, are pathogens of fish and may occasionally be associated with human infections (Hollis *et al.*, 1989). In addition, there may be other, as yet

uncharacterized, environmental species of *Francisella* (Berrada and Telford, 2010).

Figure 28.1 Geographical distribution and comparison of the subspecies of *Francisella tularensis*.

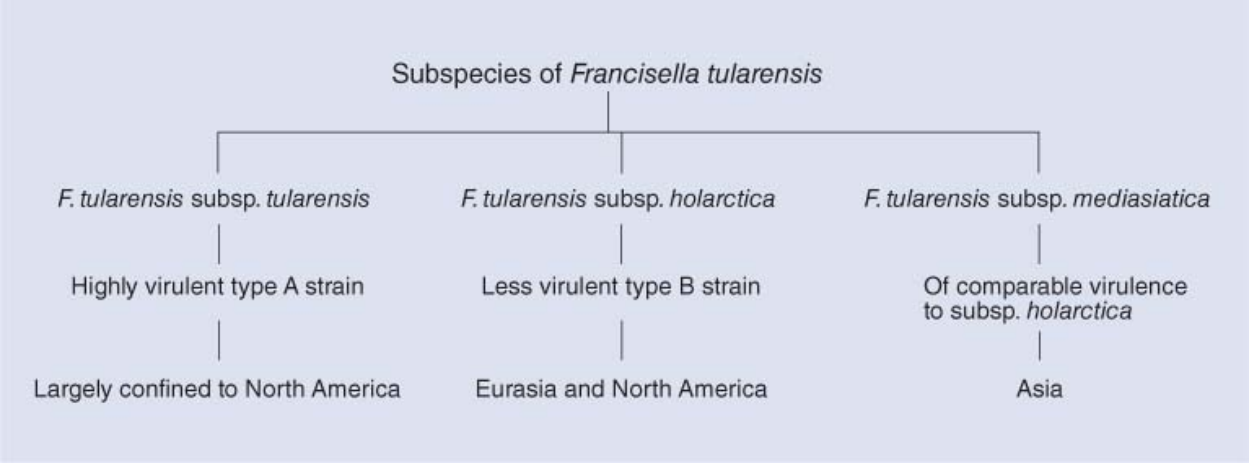

Table 28.1 Distinguishing features of *Francisella tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*.

Feature	*F. tularensis* subsp. *tularensis*	*F. tularensis* subsp. *holarctica*
Pathogenicity	Classical tularaemia in animals and humans	Less serious disease in animals and humans
Reservoirs	Lagomorphs, rodents, galliform birds, ticks	Water, mud, aquatic animals, protozoa
Capsule production	+	+
Cysteine required for growth	+	+
Citrulline ureidase activity	+	−
Acid from glucose	+	−

Usual habitat

Tularaemia has been reported in over 250 species of mammals, birds, reptiles, fish and invertebrates. The important reservoir hosts of *F. tularensis* for infection in humans and domestic animals are not entirely clear but probably include lagomorphs, rodents, galliform birds and deer. A more accurate picture may be that these are amplifying hosts and the true reservoir host is a tick (Foley and Nieto, 2010). *Francisella tularensis* may survive for 3 to 4 months in mud, water and contaminated carcasses (Rohrbach, 1988). *Francisella tularensis* subsp. *tularensis* is associated with terrestrial animals and subsp. *holarctica* is frequently linked to waterborne infections and to aquatic mammals such as beavers and muskrats. Unlike, subspecies *tularensis*, the true reservoir of subspecies *holarctica* is now considered to be a protozoan organism rather than a

mammal (Foley and Nieto, 2010).

Epidemiology

Ticks and the deerfly (*Chrysops discalis*) are important vectors in North America. The main tick species, in which *F. tularensis* can be passed transstadially and transovarially, include *Dermacentor variabilis*, *D. ander-soni* and *Amblyomma americanum*. At each stage of their life cycles, these ticks usually feed on vertebrate hosts different from and larger than the hosts parasitized at the previous stage. Direct transmission between domestic animals is uncommon.

Clinical infections

Francisella tularensis can infect wildlife species, domestic animals and humans. Fulminating disease can occur in immunosuppressed individuals. Chronic, granulomatous lesions or subclinical infections may develop. Subspecies *tularensis* strains probably account for the majority of clinical infections in domestic animals while subspecies *holarctica* strains tend to cause a comparatively mild illness that may not be evident.

Tularaemia in domestic animals

Although infection with *F. tularensis* is probably common in domestic animals in endemic areas, outbreaks of tularaemia are relatively rare. Outbreaks of disease have been reported in sheep (Frank and Meinershagen, 1961), horses (Claus *et al*., 1959) and young pigs.

Adult pigs and cattle appear to be comparatively resistant to infection. Dogs and cats may be infected and seroconvert without clinical signs of disease. In serological surveys, significant antibody titres to the pathogen were found in 6% of feral cats (McKeever *et al*, 1958) and 48% of dogs (Schmid *et al*.,1983). Cats are probably the domestic animal in which clinical disease has been most frequently documented; infection is presumably acquired through hunting. The forms of disease described in humans occur in cats also, including typhoidal, respiratory, ulceroglandular and oropharyngeal tularaemia.

Pathogenesis and pathogenicity

Infection with *F. tularensis* usually occurs through skin abrasions or by arthropod bites. Animals can also acquire infection through inhalation or by ingestion. The organism is a facultative, intracellular pathogen which can survive in macrophages but not in neu-trophils. The organism enters macrophages via a unique system of pseudopod loops (Clemens *et al*., 2005). Once within the cell, the organism inhibits phagosome/lysosome fusion and survives within the phagosome up to several hours by suppression of acidification (Barker and Klose, 2007). The organism then escapes from the phagosome, replicates in the cytoplasm and is released from the cell by induction of pyroptosis, a form of apoptosis (Foley and Nieto, 2010). Many putative virulence genes have been identified in the *F. tularensis* genome but the molecular pathogenesis of this organism is only partly known (Meibom and Charbit, 2010). However, a *Francisella* pathogenicity island (FPI) has been identified and encodes a putative Type IV secretion system and other virulence genes. Two copies of this FPI are found in virulent strains of *F. tularensis*; single copies are found in less virulent species.

Lymphadenitis, either local or generalized, is a constant finding and septicaemia is common. Pale necrotic foci are present in enlarged superficial lymph nodes, and miliary lesions may be evident in the liver and spleen. Areas of pulmonary consolidation may also be present. Primary pulmonary lesions due to aerosol inhalation have been described in affected cats.

Clinical signs

Outbreaks of tularaemia have been reported in sheep, and disease has been described in cats and other domestic animals. Transmission of infection often correlates with heavy tick infestation.

In most domestic species, the disease is characterized by fever, depression, inappetence, stiffness and other manifestations of septicaemia.

Diagnosis

- Although clinical signs are non-specific, heavy tick infestation in severely ill animals in endemic regions may indicate the presence of tularaemia.
- Suitable specimens for laboratory tests include blood for serology, scrapings from ulcers, lymph node aspirates and biopsy material or post-

mortem samples from affected tissues.

- Agglutination antibody titres of 1:80 or higher are presumptive evidence of infection with *F. tularensis*. A rising antibody titre is indicative of an active infection.
- A fluorescent antibody technique can be used for the identification of *F. tularensis* in tissues or exu-dates and from cultures.
- Isolation procedures for *F. tularensis* must be carried out in a biohazard cabinet. Special precautions should also be observed when handling suspect cases of tularaemia and during post-mortem examinations.
- Glucose-cysteine-blood agar is used for culture with the addition of antibiotics when samples are contaminated. Plates are incubated aerobically at 37°C for up to 7 days.
- Identification criteria for isolates:

  – Small, grey, mucoid colonies, surrounded by a narrow zone of incomplete haemolysis, appear after incubation for 3 to 4 days.

  – Immunofluorescence can be used to confirm the identity of the pathogen in smears from the colonies.

  – A slide agglutination test can be carried out on cultures using antiserum specific to *F. tularensis*. Biochemical tests for distinguishing the subspecies are carried out in reference laboratories (Table 28.1).

  – PCR procedures for identification and differentiation of strains are increasingly used (Forsman *et al*, 1995; Anon., 2008). Real-time PCR for rapid identification of isolates has been developed also (Tomaso *et al*., 2007).
- Detection of *F. tularensis* in blood and other tissues by polymerase chain reaction procedures has been reported (Long *et al*., 1993; Johansson *et al*., 2000).
- If samples contain few organisms, isolation in embryonated eggs or laboratory animals can be attempted.

Treatment

Effective antibiotics include amikacin, streptomycin, imipenem-cilastatin and the fluoroquinolones. A high relapse rate may occur if animals are treated with bacteriostatic antibiotics. *Francisella tularensis* produces a β-lactamase and thus is not susceptible to penicillins. Broth microdilution is the recommended method for testing of isolates; antimicrobial resistance was not detected when seven

agents were tested in a recent North American study (Urich and Petersen, 2008).

Control

Defined control measures are required in endemic areas as commercial vaccines are not available for use in animals.

- Ectoparasite control is essential. Daily removal of ticks from dogs and cats is advisable.
- Precautions should be taken to prevent contamination of food and water with infected carcasses or excreta of wildlife species.
- I n endemic regions dogs and cats should be prevented from hunting wildlife species.

Tularaemia in humans

Tularaemia in humans, a serious and potentially fatal infection, often presents as a slow-healing ulcer accompanied by lymphadenopathy. Individuals particularly at risk, such as hunters, trappers, veterinarians and laboratory workers, should take precautions when handling suspect animals or materials.

A modified live vaccine is available for personnel working with *F. tularensis* in specialized laboratories.

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Chapter 29

Histophilus somni, Haemophilus parasuis and Avibacterium paragallinarum

There have been a number of taxonomic changes to organisms within the genus *Haemophilus* in recent years. The species *Histophilus somni* now includes the bovine commensal and opportunistic pathogen '*Haemophilus somnus*' and the ovine organisms *Histophilus ovis* and *Haemophilus agni* (Angen *et al*., 2003). *Haemophilus paragallinarum* has been reclassified as *Avibacterium paragallinarum* (Blackall *et al*., 2005). These species are small (less than 1µm × 1 to 3µm), Gramenegative rods, which often appear coccobacil-lary and may occasionally form short filaments. These motile organisms, which are facultative anaerobes with variable reactions in catalase and oxidase tests, do not grow on MacConkey agar. They are fastidious bacteria; *H. parasuis* and *Avibacterium paragallinarum* require growth factor V (nicotinamide adenine dinu-cleotide, NAD). Optimal growth for all these species occurs in an atmosphere of 5 to 10% CO_2 on chocolate agar which supplies both X (haemin) and V factors although, in the case of *H. somni*, X and V factors are not absolute requirements for growth. Most isolates of these organisms form small, transparent, dewdrop-like colonies after incubation for 48 hours. Colonies of *H. somni* have a yellowish hue and some isolates are haemolytic on sheep blood agar.

The diseases caused by *H. somni, H. parasuis* and *A. paragallinarum* are summarized in Table 29.1. Other *Haemophilus* and *Avibacterium* species, which are commensals on the mucous membranes of animals, rarely cause disease (Table 29.2). The soluble antigens of these organisms exhibit heterogeneity. A total of 15 serotypes of *H. parasuis* have been identified and serotyping is also used for characterization of *H. somni* and *A. paragallinarum* strains.

Key points

- Small, motile, Gram-negative rods
- Fastidious: some species require X and V factors in chocolate agar

- Optimal growth in 5 to 10% CO_2
- Facultative anaerobes
- Commensals on mucous membranes of many animal species
- *Histophilus somni* is a pathogen of cattle and sheep, *Haemophilus parasuis* causes Glasser's disease in pigs and *Avibacterium paragallinarum* infects poultry

Usual habitat

These three species are commensals on the mucous membranes of the upper respiratory tract. They are susceptible to desiccation and do not survive for long periods away from their hosts.

Table 29.1 Disease conditions caused by *Histophilus somni*, *Haemophilus parasuis* and *Avibacterium paragallinarum*.

Organism	Hosts	Disease conditions
H. somni	Cattle	Septicaemia, thrombotic meningoencephalitis, bronchopneumonia (in association with other pathogens), sporadic reproductive tract infections
H. somni (ovine strains)	Sheep	Epididymitis in young rams; vulvitis, mastitis and reduced reproductive performance in ewes; septicaemia, arthritis, meningitis and pneumonia in lambs
H. parasuis	Pigs	Glasser's disease, secondary invader in respiratory disease
A. paragallinarum	Chickens Pheasants, turkeys, guinea fowl	Infectious coryza Respiratory disease

Table 29.2 *Haemophilus* and *Avibacterium* species which occur as commensals in domestic animals.

Species	Host	Comments
Avibacterium avium	Chickens	Commensal
A. gallinarum	Birds	Possible low - grade infections of upper respiratory tract
Haemophilus felis	Cats	Commensal of nasopharynx; occasionally involved in respiratory disease
H. haemoglobinophilus	Dogs	Commensal of the lower genital tract
H. paracuniculus	Rabbits	Isolated from intestines

Differentiation of *Histophilus somni*, *Haemophilus parasuis* and *Avibacterium paragallinarum*

These species are differentiated by requirements for X and V growth factors, by growth enhancement in an atmosphere of CO_2, by catalase and oxidase reactions and by carbohydrate utilization (Table 29.3).

- Isolation techniques:
  Both X and V factors are required in media for isolation of some *Haemophilus* and *Avibacterium* species. Although *Histophilus somni* does not have an absolute requirement for these factors, its growth is enhanced by their presence. The X factor is heat stable and is present in red blood cells. The heat-l abile V factor, which is also present in red blood cells, is susceptible to NADases in plasma. There are two common methods for ensuring the availability of both X and V factors in culture media:

  – Chocolate agar, which supplies both factors, is prepared by heating molten blood agar in a water bath at 80°C for about 10 minutes. The chocolate-brown colour of the medium is due to lysis of the red cells. The heat- stable X factor, released from the lysed cells, is unaffected by this procedure. The V factor, which is also released from the lysed cells, tolerates a temperature of 80°C for a short period whereas the plasma NADases which degrade V factor are destroyed.

  – *Staphylococcus aureus* growing on blood agar releases V factor into the medium. Colonies of *Haemophilus* species which require V factor grow close to the *S. aureus* colony, a phenomenon referred to as satellitism.
- Tests for X and V factor requirements:

  – The disc method for determining X and V factor requirements is illustrated and explained in Fig. 29.1. This test is particularly suitable for determining V factor requirement. The porphyrin test is a more accurate method for determining the growth requirement for X factor. The isolate is grown at 37°C for 4 hours in broth containing a porphyrin precursor. When the culture is exposed to UV light in the dark, porphyrin production is detected by a red fluorescence indicating that the isolate does not have a requirement for the X factor.
- Biochemical reactions:

  – Some biochemical tests (Table 29.3) canbe carried out using conventional media. For testing

  – Commercially available biochemical kits are used for testing isolates in a wider range of tests (Palladino *et al*., 1990).
- Molecular tests:

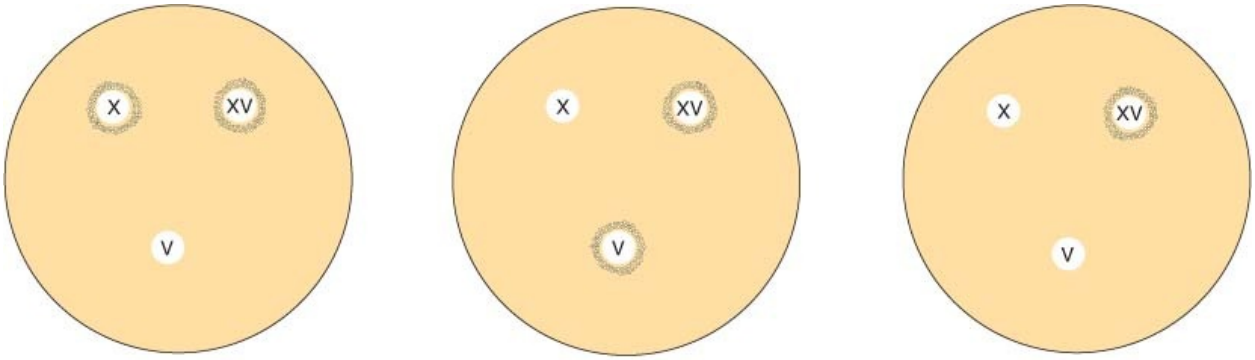
  – Although serotyping methods for characterization of all three species are used, molecular-based methods are employed with increasing frequency. Many of these are fingerprinting methods but, as with serotyping, clear

associations between virulence attributes and particular strains have not been established.

Table 29.3 Comparative features of *Histophilus somni*, *Haemophilus parasuis* and *Avibacterium paragallinarum*.

Organism	Growth factor required	Catalase production	Oxidase production	Carbohydrate utilization		
				Sucrose	Lactose	Mannitol
Histophilus somni	None	−	+	−	−	+
Haemophilis parasuis	Factor V	+	−	+	±	−
Avibacterium paragallinarum	Factor V	−	−	+	−	+

Figure 29.1 Disc method for determining the requirement for X and V growth factors. Isolates of *Haemophilus* species are spread over nutrient agar, and discs containing X, V, and X and V factors are placed on the inoculated media. After incubation in 10% CO_2 at 37°C for 3 days, colonies of *Haemophilus* species grow around the discs supplying the growth factor required by the particular isolate. carbohydrate utilization, a phenol red broth containing 1% of the sugar under test, filter-sterilized V and X factors and 1% serum is used.



Pathogenesis and pathogenicity

Histophilus somni, *H. parasuis* and *A. paragallinarum* are heterogeneous organisms and their virulence differs from strain to strain. Some isolates are apparently purely commensal in nature and are never isolated from pathological specimens. For example, many isolates of *H. parasuis* belonging to serotype 3 are found only in the upper respiratory tract and have not been isolated from systemic sites (Oliveira *et al*., 2003). An avirulent bovine preputial isolate of *H. somni* appeared to lack many putative virulence factors when compared with a

pathogenic isolate from a case of bovine pneumonia (Sandal and Inzana, 2010). This variability between strains can cause difficulties in diagnosis as isolation of the organism from some sites may not necessarily prove its involvement in the disease condition observed and further characterization may be required to prove pathogenicity. The natural habitat of all strains of *Histophilus somni, H. parasuis* and *A. para-gallinarum* is the host animal and disease occurs opportunistically due to predisposing factors. Young or previously unexposed animals are particularly susceptible to infections. Specific-pathogen-free pigs, which do not harbour *H. parasuis* as a commensal, often develop signs of disease on primary exposure to the pathogen. Environmental and other stress factors such as transportation, weaning and viral infections may contribute to the development of infections by this group of organisms.

Histophilus somni can adhere firmly to several host cell types, including endothelial and vaginal epithelial cells although the exact mechanisms of adherence are not clear. The organism has a number of virulence attributes, which include endotoxin production, phase variation of its lipo-oligosaccharide, induction of apoptosis in endothelial cells and production of trans-ferrin and immunoglobulin-binding proteins. The lipo-oligosaccharide (LOS) of *H. somni* is a major virulence factor, both because of its toxic lipid A component and because the organism can modify the structure of its LOS, resulting in phase variation and evasion of the host immune response. Vasculitis is a major feature of the lesions observed in *H. somni* infections and LOS is involved in induction of apoptosis in endothelial cells and in leukocytes (Czuprynski *et al*., 2004). Immunoglobulin-binding proteins bind the Fc portion of bovine IgG_2 and are responsible for resistance to complement-mediated killing in serum. In addition, *H. somni* produces an exopolysaccharide and filamentous haemagglutinin proteins, both of which may be involved in biofilm formation (Sandal *et al*., 2009; Sandal and Inzana, 2010).

The virulence attributes of *H. parasuis* are poorly characterized, and definitive proof of virulence requires reproduction of disease in animals. Olvera *et al*. (2006) suggest that *H. parasuis* strains may be classified into three groups: avirulent isolates from the upper respiratory tract, strains capable of producing bronchopneumonia and strains that produce systemic disease. However, although multilocus sequence typing results were partially consistent with this grouping of strains, to date no typing system capable of definitively identifying virulent strains has been developed. It is known that *H. parasuis* produces capsular polysaccharide, lipo-oligosaccharide and outer membrane proteins but detailed

information on their exact pathogenic role is not available.

Much remains to be learned about the pathogenic mechanisms of *Avibacterium paragallinarum* also. The organism produces a capsule which is associated with virulence and a number of haemagglutinins. Haemagglutination inhibition is the basis of the Kume serotyping scheme which divides the species into a number of different serotypes.

Diagnostic procedures

- Specimens for laboratory examination depend on the clinical condition and type of lesions. These bacterial species are fragile and neither refrigeration nor transport media maintain viability. Ideally, clinical specimens should be frozen in dry ice and delivered to a laboratory within 24 hours of collection.
- Either chocolate agar or blood agar inoculated with a streak of *S. aureus*, incubated in 5 to 10% CO_2 at 37°C for 2 to 3 days in a moist atmosphere, is used for isolation.
- Identification criteria for isolates:
  – Small, dewdrop-like colonies after 1to2 days
  – Enhancement of growth by CO_2
  – Requirement for X and V growth factors
  – Biochemical profile.
- Although serological tests have been developed for epidemiological purposes, these tests are of little diagnostic value because this group of organisms does not show a consistent relationship between serotype and virulence.
- PCR-based tests have been developed for detection and identification of these pathogens. Tegtmeier *et al*. (2000) found that PCR-based methods for the detection of *H. somni* were the most sensitive when compared with culture, immunohistochemical and *in situ* hybridization techniques. *Haemophilus parasuis* can be particularly difficult to isolate and a specific PCR method for this organism developed by Oliveira *et al*. (2001) may improve detection rates in clinical specimens, especially if these are not of optimal quality or diagnostic laboratories lack specific expertise in culturing this pathogen (Turni and Blackall, 2007). Chen *et al*. (1996) used PCR techniques for the identification of *A. paragallinarum* and also

developed a test for detection of the organism in clinical specimens (Chen *et al*., 1998). Realtime PCR for detection of *A. paragallinarum* was successfully developed by Corney *et al*. (2008).

Clinical infections

Histophilus and *Haemophilus* species which are pathogenic for animals tend to be host-specific (Table 29.1). Some *Haemophilus* species of uncertain pathogenicity which are occasionally isolated from domestic animals are listed in Table 29.2.

Infections caused by *Histophilus somni* in cattle

Histophilus somni is part of the normal bacterial flora of the male and female bovine genital tracts. The organism can also colonize the upper respiratory tract. Environmental stress factors contribute to the development of clinical disease. *Histophilus somni* is more resistant in the environment than many *Haemophilus* species. It can survive in nasal discharges and blood for up to 70 days at ambient temperatures and for up to 5 days in vaginal discharges. Transmission is by direct contact or by aerosols. Serological surveys indicate that at least 25% of cattle have antibodies to *H. somni* (Harris and Janzen, 1989).

Clinical signs

Because septicaemia is commonly associated with *H. somni* infection, many organ systems may be involved and the resulting clinical presentation is unpredictable. Thrombotic meningoencephalitis (TME), a common consequence of septicaemia, is encountered sporadically in young cattle recently introduced to feedlots. Some animals may be found dead and others may present with high fever and depression, sometimes accompanied by blindness, lameness and ataxia. Sudden death due to myocarditis has also been described. Arthritis often develops in animals which survive the acute phase of the disease.

Histophilus somni is one of the bacterial pathogens commonly isolated from the enzootic calf pneumonia complex. Sporadic cases of abortion, endometritis, otitis and mastitis caused by *H. somni* have been recorded.

Diagnosis

- Severe neurological signs in young feedlot cattle may be indicative of TME.
- Multiple foci of haemorrhagic necrosis, detectable grossly in affected brains at post-mortem, are consistent with TME. Vasculitis, thrombosis and haemorrhage are evident histologically in brain, heart and other parenchymatous organs.
- Confirmation is by isolation and identification of *H. somni* from cerebrospinal fluid, from lesions detected post mortem or from aborted foetuses.

Treatment and control

- Animals with clinical signs of septicaemia should be isolated and those at risk should be monitored closely to detect early signs of the disease.
- Although oxytetracycline is usually used for therapy, penicillin, erythromycin and potentiated sulphonamides are also effective.
- Commercially available bacterins may reduce morbidity and mortality rates but their efficacy is variable. The protective antigens in these bacterins are not known and work is continuing to characterize such antigens with a view to developing improved vaccines in the future (Siddaramppa and Inzana, 2004 ; Sandal and Inzana, 2010).

Infections caused by *Histophilus somni* in sheep

Healthy sheep may carry ovine strains of *H. somni* in the prepuce or vagina. Epididymitis in young rams caused by *H. somni* has been recorded (Lees *et al*., 1990). Vulvitis, mastitis and reduced reproductive performance in ewes have been attributed to infection with *H. somni*. The organism has also been associated with septicaemia, arthritis, meningitis and pneumonia in lambs.

Glasser’s disease

Glasser’s disease, caused by *Haemophilus parasuis*, manifests as polyserositis and leptomeningitis usually affecting pigs from weaning up to 12 weeks of age. Some cases present as polyarthritis.

Haemophilus parasuis is part of the normal flora of the upper respiratory tract of pigs. Piglets acquire the organism from sows shortly after birth either by direct contact or through aerosols. The presence of maternally derived antibodies

prevents the development of clinical signs. However, Glasser' s disease may occur sporadically in 2- to 4-week-old piglets subjected to stressful environmental conditions (Smart *et al*., 1989). Active immunity to *H. parasuis* is usually established by 7 to 8 weeks of age.

Clinical signs

The incubation period is 1 to 5 days. Clinical signs usually develop in conventionally reared pigs 2 to 7 days following exposure to stress factors such as weaning or transportation. Anorexia, pyrexia, lameness, recumbency and convulsions are features of the disease. Cyanosis and thickening of the pinnae are often encountered. Pigs may die suddenly without showing signs of illness.

Diagnosis

- Because organisms such as *Streptococcus suis* and *Mycoplasma hyorhinis* produce clinicopathological changes similar to those of Glasser's disease, diagnosis requires detection and identification of *H. parasuis*. Furthermore, detection of the organism from areas such as the upper respiratory tract or the lungs is not proof of involvement in observed lesions as non-pathogenic isolates of the organism can be found in these sites in normal pigs.
- Isolation and identification of *H. parasuis* from joint fluid, heart blood, cerebrospinal fluid or postmortem tissues of a recently dead pig are confirmatory.
- Post-mortem findings in Glasser's disease may include fibrinous polyserositis, polyarthritis and meningitis.

Treatment and control

- Antimicrobial drugs such as tetracyclines, penicillins or potentiated sulphonamides, administered early in the course of the disease, are usually effective. *Haemophilus parasuis* isolates remain susceptible to most drugs commonly used in pig production (Aarestrup *et al*., 2004).
- Predisposing stress factors should be identified and, where possible, eliminated.
- Commercially available bacterins or autogenous bacterins may stimulate protective immunity which is serotype specific.

Infectious coryza of chickens

Infectious coryza, caused by *A. paragallinarum*, affects the upper respiratory tract and paranasal sinuses of chickens. Its economic importance relates to loss of condition in broilers and reduced egg production in laying birds. Chronically ill and, occasionally, clinically normal carrier birds act as reservoirs of infection. Transmission occurs by direct contact, by aerosols or from contaminated drinking water. Chickens become susceptible at about 4 weeks after hatching and susceptibility increases with age.

Clinical signs

The mild form of disease manifests as depression, serous nasal discharge and slight facial swelling. In severe disease, swelling of one or both infraorbital sinuses is marked and oedema of the surrounding tissues may extend to the wattles. In laying birds, egg production may be severely affected.

A copious, tenacious exudate may be evident at post-mortem in the infraorbital sinuses. Tracheitis, bronchitis and airsacculitis may be present also.

Diagnosis

- Facial swelling is a characteristic finding.
- Isolation and identification of *A. paragallinarum* from the infraorbital sinuses of several affected birds is confirmatory.
- Immunoperoxidase staining can be used to demonstrate *A. paragallinarum* in the tissues of the nasal passages and sinuses (Nakamura *et al*., 1993).
- Serological tests such as agglutination tests, ELISA or agar gel immunodiffusion tests are used to demonstrate antibodies about 2 to 3 weeks after infection and to confirm the presence of *A. paragallinarum* in a flock.

Treatment and control

- Medication of water and feed with sulphonamides or oxytetracycline should be initiated early in an outbreak of disease.
- An all-in/all-out management policy should be implemented and replacement birds should be obtained from coryza-free stock. Good management of poultry units minimizes the risk of infection.
- Bacterins may be of value in units where the disease recurs. Vaccines

should be administered about 3 weeks before outbreaks of coryza are anticipated.

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Chapter 30

Taylorella species

Taylorella equigenitalis and the more recently described *Taylorella asinigenitalis* (Jang *et al*., 2001) are the only members of the genus. *Taylorella* species are short (0.7 × 0.7 to 1.8μm), non-motile, Gram-negative rods, which give positive reactions in catalase, oxidase and phosphatase tests. They are microaerophilic, slow-growing and highly fastidious, requiring chocolate agar and 5 to 10% CO_2 for optimal growth. Although the bacteria are not dependent on the X or V growth factors, availability of factor X stimulates growth. *Taylorella* species do not grow on MacConkey agar.

Usual habitat

The organism is found in the genital tracts of stallions, mares and foals. In stallions, *T. equigenitalis* is harboured in the urethral fossa and the pathogen localizes in the clitoral fossa of infected mares. *T. asinigenitalis* can be isolated from the genital tract of donkeys.

Clinical infections

Taylorella equigenitalis, the cause of contagious equine metritis, appears to infect only *Equidae* (Platt and Taylor, 1982). *Taylorella asinigenitalis* has not been associated with clinical disease in donkeys.

Contagious equine metritis

Contagious equine metritis (CEM) was first reported as a clinical entity in 1977 in thoroughbreds in Britain and Ireland (Crowhurst, 1977: O'Driscoll *et al*., 1977). Outbreaks of the disease were subsequently described in other European countries and in the USA, Australia and Japan. It is a highly contagious, localized, venereal disease characterized by mucopurulent vulval discharge and

temporary infertility in mares. The condition is economically important because it disrupts breeding programmes on thoroughbred stud farms.

Key points

- Short, non-motile Gram-negative rods
- Fastidious, optimal growth on chocolate agar
- Microaerophilic, 5 to 10% CO_2 required
- Positive oxidase, catalase and phosphatase tests but otherwise unreactive
- *T. equigenitalis* causes contagious equine metritis

Infected stallions and mares are the main reservoirs of infection. Transmission of the bacterium usually occurs during coitus although infection may also be introduced by contaminated instruments. It is considered that spontaneous ascending infection in mares is unlikely and that *T. equigenitalis* must be deposited in the uterus for infection to establish (Platt and Taylor, 1982). Foals born to infected dams may acquire infection *in utero* or during parturition. *Taylorella equigenitalis* has been isolated from more than 75% of the offspring of infected mares at 2 to 4 years of age (Timoney and Powell, 1982). These offspring and mares, which have recovered clinically, may act as sources of infection.

Pathogenesis

Pre-ejaculatory fluid and semen may be contaminated with *T. equigenitalis* from the urethral fossa. After introduction into the uterus, pathogenic organisms replicate and induce an acute endometritis. Initially, mononuclear cell and plasma cell infiltration predominates, a feature rarely observed in acute bacterial endometritis (Ricketts *et al*., 1978). Later, migration of neutrophils into the uterine lumen produces a profuse mucopurulent exudate. Although the pathogen may persist in the uterus, acute endometrial changes subside within a few days. There are few data available on the virulence mechanisms of the organism although its ability to invade equine cell lines has been investigated (Bleumink-Pluym *et al*., 1996). There is strong clinical and epidemiological evidence that strains differ in pathogenicity (Parlevliet *et al*., 1997) but the basis for the differences in pathogenicity is unknown.

Clinical signs

Infected stallions and a minority of infected mares remain asymptomatic. Most affected mares develop a copious, mucopurulent, vulval discharge without systemic disturbance within a few days of service by a carrier stallion. The discharge may continue for up to 2 weeks and affected mares remain infertile for several weeks. Some mares recover without treatment and up to 25% remain carriers (Platt and Taylor, 1982). Infection does not induce protective immunity and reinfection can occur.

Diagnostic procedures

The *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Anon., 2008) details the sample types and diagnostic requirements for this notifiable disease.

- A copious, mucopurulent, vulval discharge 2 to 7 days after service may indicate the presence of CEM.
- Specimens for bacteriology should be collected before and during the breeding season.
- Swabs from mares should be taken from the clitoral fossa and sinuses and from the endometrium at oestrus using a double-guarded swab. When taking swabs, disposable gloves should be changed between each animal.
- Foals of infected mares should be sampled before 3 months of age. Swabs should be taken from the clitoral fossa of fillies and from the penile sheath and tip of the penis in colts. Swabs from stallions and teaser stallions are taken from the urethra, ure-thral fossa and penile sheath in addition to pre-ejaculatory fluid.
- Swabs must be placed in Amies charcoal transport medium and reach the laboratory within 24 hours of collection. Samples should be submitted to laboratories which are officially certified by a regulatory authority.
- Chocolate agar-based media with the addition of amphotericin B, crystal violet and streptomycin are suitable for isolation of the organism. Plates with and without streptomycin should be inoculated as some isolates of *T. equigenitalis* are susceptible to this antibiotic. A medium incorporating trimetho-prim and clindamycin has been developed (Timoney *et al*., 1982). Inoculated plates are incubated under 5 to 10% CO_2 at 37°C for 4 to 7 days.
- Identification criteria for isolates:

– Colonies, which may be visible after 48 hours, are small, smooth and yellowish grey and have an entire edge.

– Reactions in the catalase, oxidase and phos-phatase tests are positive.

– A slide agglutination test, using high-titred *T. equigenitalis* antiserum, can be carried out on the culture.

– A fluorescent antibody technique using conjugated serum, rendered specific by absorption with *Mannheimia haemolytica*, may be used.

– A latex agglutination kit to identify the pathogen is available commercially.

- Polymerase chain reaction techniques for detecting *T. equigenitalis* in specimens have been described by a number of authors (Bleumink-Pluym *et al*., 1993; Chanter *et al*., 1998; Anzai *et al*., 1999; Moore *et al*., 2001; Duquesne *et al*, 2007). Wakeley *et al*. (2006) developed a real-time PCR method which can be used directly on genital swabs and, in addition, can differentiate between *T. equigenitalis* and *T. asinigenitalis*.
- Several molecular methods for strain typing of *T. equigenitalis* are available, most of them based on digestion with restriction enzymes followed by pulsed field gel electrophoresis (Matsuda and Moore, 2003).
- Serological tests including the agglutination, complement fixation and ELISA tests are useful for confirming active or recent infections but do not detect asymptomatic carriers.

Treatment

Asymptomatic carriers must be treated as well as clinically affected animals. Elimination of *T. equigenitalis* from both mares and stallions can usually be accomplished by washing the external genitalia with a 2% solution of chlorhexidine combined with local application of antimicrobial drugs such as nitrofurazone ointment on a daily basis (Watson, 1997). In addition, a daily intrauterine irrigation with antibiotic solution is carried out in mares for 5 to 7 days. Ablation of clitoral sinuses may be necessary in those mares in which *T. equigenitalis* persists after treatment.

Control

- Contagious equine metritis is a notifiable disease in many countries with an advanced thoroughbred industry.
- Control regimens are based on laboratory detection of asymptomatic and clinical infections with *T. equigenitalis* in animals used for breeding.
- Appropriate, routine, hygienic methods must be practised on stud farms to

prevent lateral spread of the pathogen.

- If CEM is diagnosed on stud farms, all breeding services should cease immediately.
- Animals which have been treated for CEM should be sampled to ensure bacteriological freedom from the pathogen.
- Test-mating a stallion to two maiden mares is a sensitive method for detecting infection. Samples from the mares are then examined bacteriologically.
- A vaccine for CEM is not available.

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Chapter 31

Bordetella species

The genus *Bordetella* contains eight species, including three which are pathogenic for animals, *B. bronchiseptica*, *B. avium* and *B. parapertussis*. *Bordetella pertussis*, the type species, is associated with whooping cough in children. The major animal pathogens are *Bordetella bronchiseptica*, which infects a wide range of animal species including humans, and *B. avium*, which is a pathogen of avian species (Table 31.1). Ovine strains of *Bordetella parapertussis* are associated with pneumonia in lambs while human strains of this species cause a mild form of whooping cough. It is suggested that some strains of *B. hinzii* may be capable of causing disease in turkeys although this organism is generally regarded as non-pathogenic (Register and Kunkle, 2009). The bordetellae are occasional pathogens which have an affinity for ciliated respiratory epithelium. *Bordetella bronchiseptica* and *B. avium* are small (0.2 to 0.5 × 0.5 to 1.5 μm), Gram-negative rods with a coc-cobacillary appearance. These species are catalase-positive, oxidase-positive aerobes and are motile peritrichous bacteria, which grow on MacConkey agar. Because they cannot utilize carbohydrates, they derive their energy mainly from the oxidation of amino acids and have no special growth requirements. *Bordetella parapertussis* is non-motile and some strains grow on MacConkey agar.

Usual habitat

Bordetella species are commensals on the mucous membranes of the upper respiratory tract and some species may be pathogenic for animals. In the environment, survival time is usually short although *B. avium* may survive for prolonged periods in water (Raffel *et al*., 2002).

Key points

- Small Gram-negative rods
- Growth on non-enriched media; major animal pathogens grow on MacConkey agar

- Strict aerobes
- Motile, catalase-positive, oxidase-positive
- Utilize amino acids for energy
- Toxigenic strains agglutinate mammalian red blood cells
- Commensals in upper respiratory tract; some species are opportunistic pathogens
- Cause respiratory disease in mammals and birds

Differentiation of Bordetella bronchiseptica and B. avium

These bacteria are usually identified by growth characteristics, by biochemical reactions and by their unique ability to agglutinate red blood cells (Table 31.2). *Bordetella avium* requires differentiation from *Alcaligenes faecalis*, which is non-pathogenic.

- On sheep blood agar, colonies of virulent strains, visible after incubation for 24 hours, are small, convex and smooth. Many isolates of *B. bronchiseptica* are haemolytic, unlike *B. avium*, which is non-haemolytic.
- On MacConkey agar, both *B. bronchiseptica* and *B. avium* produce pale, non-lactose-fermenting colonies.
- A selective indicator medium containing bromoth-ymol blue as pH indicator is used for the isolation and presumptive identification of bordetellae (Smith and Baskerville, 1979). Other selective media such as charcoal/cephalexin agar can be used also (Egberink *et al*., 2009).
- Miniaturized biochemical identification systems are available for these 'non-fermenting' bacteria, which do not metabolize carbohydrates.
- Haemagglutination, an attribute uncommon in bacteria, occurs with virulent isolates of both *B. bronchiseptica* and *B. avium*.
- Both conventional and real-time PCR methods for detection and differentiation of *Bordetella* species are available (Hozbor *et al*., 1999; Koidl *et al.*, 2007).
- Typing methods have been developed for epi-demiological and research purposes and include multilocus sequence typing, pulsed-field gel electrophoresis and randomly amplified polymorphic DNA analysis.

Table 31.1 *Bordetella* species of veterinary importance and disease conditions with which they are associated.

Bordetella species	Host	Disease conditions

B. bronchiseptica	Pigs	Atrophic rhinitis
	Dogs	Canine infectious tracheobronchitis
	Kittens	Pneumonia
	Horses	Respiratory infections
	Rabbits	Upper respiratory tract infection
	Laboratory rodents	Bronchopneumonia
B. avium	Turkeys	Coryza
B. parapertussis	Lambs	Pneumonia

Pathogenesis and pathogenicity

The bordetellae exhibit phase changes, which correlate with virulence and are identifiable by colonial appearance. Expression of virulence is regulated by a two-component signal transduction system encoded by the *BvgAS* locus. The avirulent phase occurs at 25°C whereas at 37°C BvgAS activates the expression of many virulence genes. Virulence is mediated by several factors, which can be broadly classified as adhesins or toxins. Adhesins include a filamentous haemagglutinin, pertactin and fimbriae which allow attachment to the cilia of the upper respiratory tract (Table 31.3). A tracheal cytotoxin inhibits ciliary motility and tracheobronchial clearance. In addition, *B. bronchiseptica* produces an adenylate cyclase-haemolysin which enters host cells through a type 1 secretion system. This toxin primarily targets phago-cytic cells (Gueirard and Guiso, 1993; Harvill *et al*., 1999) and has a unique structure as it has the features of a repeats-in-structural-toxin but with an extra domain for an adenylate cyclase enzyme (Table 31.3). Other toxins include dermonecrotic toxin which produces turbinate atrophy in pigs by interfering with differentiation of osteoblasts. A type III secretion system translocates various effector proteins into host cells, inducing cell death, and increased expression of this system appears to correlate with increased virulence (Buboltz *et al*., 2009). *Bordetella avium* lacks the adenylate cyclase toxin but the organism does produce a haemagglutinin, which specifically agglutinates guinea-pig red cells and correlates with pathogenicity for turkey poults (Gentry-Weeks *et al*., 1988; Sebaihia *et al*., 2006).Pertussis toxin is produced only by the human pathogen, *B. pertussis*.

Table 31.2 Differentiating features of *Bordetella bronchiseptica, B. avium* and *Alcaligenes faecalis*[a].

Feature	*B. bronchiseptica*	*B. avium*	*Alcaligenes faecalis*
Colonial characteristics on:			
Sheep blood agar	Haemolysis	No haemolysis	No haemolysis
MacConkey agar	Pale, pinkish hue	Pale, pinkish hue	Pale
Selective medium[b]	Small, blue	Small, blue	Large, greenish
Oxidase production	+	+	+
Catalase production	+	+	+
Urease production	+	–	–
Utilization of carbon exclusively from:			
Citrate	+	+	+
Malonate	–	–	+
Nitrate reduction	+	–	–
Motility	+	+	+
Haemagglutinating activity of virulent strains	Agglutination of ovine and bovine red blood cells[c]	Agglutination of guinea-pig red blood cells[c]	–

a, an organism which may require differentiation from bordetellae but not of veterinary significance.

b, Smith and Baskerville (1979).

c, suspension of 3% washed red blood cells using a slide test.

Table 31.3 Virulence factors of *Bordetella bronchiseptica* and *B. avium*.

Virulence factor	Activity	*Bordetella* species	
		B. bronchiseptica	*B. avium*
Filamentous haemagglutinin	Binds to cilia	+	–
Pertactin	Binds to cells	+	+
Fimbriae	Mediate attachment to cells	+	+
Adenylate cyclase-haemolysin	Interferes with phagocytic cell function	+	–
Tracheal cytotoxin	Inhibits ciliary action, kills ciliated cells	+	+
Dermonecrotoxic toxin	Induces skin necrosis, impairs osteogenesis	+	+
Osteotoxin	Toxic for osteoblasts	+	+
Type III secretion system and associated effector proteins	Disruption of cell signaling and induction of cell death	+	–
Lipopolysaccharide	Stimulates cytokine release, colonization of respiratory tract	+	+

Clearance of the bacteria is mediated by locally produced antibodies (IgA) which appear about 4 days after infection commences. Although these antibodies can block attachment of bordetellae to cilia, they are unable to remove attached bacteria. Clearance of bor-detellae from the respiratory tract may require several weeks. Carrier animals, including a percentage of adults which continue to shed the organisms, represent an important source of infection.

Diagnostic procedures

- Specimens for laboratory examination include nasal swabs, tracheal aspirates and exudates.
- Bordetellae are cultured on blood agar and MacConkey agar or on selective media. Plates are incubated aerobically at 37°C for 24 to 48 hours.
- Identification criteria for isolates:

– Colonial appearance on blood agar or selective media
– Growth on MacConkey agar
– Biochemical profile
– Slide haemagglutination tests correlating with the virulence of isolates.

- Serological tests which have been developed are of limited diagnostic value.
- PCR procedures have been developed for detection of bordetellae in clinical specimens, including realtime and multiplex assays.

Clinical infections

Clinical signs associated with bordetellae usually relate to upper respiratory tract infection. Young animals are most susceptible, and infections in adults are usually mild or subclinical. Predisposing factors such as stress or concurrent infections contribute to field outbreaks of disease. Although morbidity rates may be high, mortality rates are usually low. The diseases associated with *B. bronchiseptica* and *B. avium* are summarized in Table 31.1. *Bordetella parapertussis*, a recognized human pathogen, has been isolated from lambs with chronic non-progressive pneumonia (Cullinane *et al*., 1987). *Bordetella bronchiseptica* is implicated in a mild form of atrophic rhinitis in pigs and in canine infectious tracheobron-chitis (kennel cough). In addition, *B. bronchiseptica* may increase the severity of respiratory disease in pigs in association with other pathogens. Oropharyngeal swabs from healthy cats may yield *B. bronchiseptica* and severe bronchopneumonia associated with the organism has been reported in kittens (Willoughby *et al*., 1991). *Bordetella bronchiseptica* may occasionally cause outbreaks of respiratory disease in rabbits and in laboratory rodents. *Bordetella avium* causes turkey coryza and respiratory disease in quails (Blackall and Doheny, 1987).

Box 31.1 Microbial pathogens implicated in

canine infectious tracheobronchitis (kennel cough).

- *Bordetella bronchiseptica*
- Canine adenovirus 2
- Canine parainfluenzavirus 2
- Canine distemper virus
- Canine adenovirus 1
- Canine herpesvirus 1
- Reoviruses 1, 2 and 3
- *Mycoplasma* species

Canine infectious tracheobronchitis

Canine infectious tracheobronchitis, also known as kennel cough, is one of the most prevalent respiratory complexes of dogs. Although *Bordetella bronchiseptica*, canine parainfluenzavirus 2 (PI-2) and canine adenovirus 2 (CAV 2) are considered to be the most important aetiological agents, other microbial pathogens may also be involved (Box 31.1).

Transmission occurs through respiratory secretions either by direct contact or by aerosols. Mechanical transfer on footwear or clothing, on contaminated feeding utensils and on fomites can spread infection in kennels, pet shops and animal shelters. Although morbidity rates may reach 50%, mortality rates are usually low. Organisms may remain in the respiratory tract and be shed for several months after clinical recovery.

Clinical signs

Clinical signs of infection with *B. bronchiseptica* develop within 3 to 4 days of exposure and, without complications, persist for up to 14 days. They include coughing, gagging or retching and mild serous oculonasal discharge. Affected dogs usually remain active, alert and non-febrile. The disease is self-limiting unless complicated by bronchopneumonia which may develop in unvaccinated pups or in older immunosuppressed animals.

Diagnosis

- Diagnosis is based on a history of recent exposure to carrier dogs and characteristic clinical signs.

- The appropriate specimen for laboratory examination is tracheal fluid collected by transtracheal aspiration.
- Virulent isolates of *B. bronchiseptica* haemagglutinate ovine and bovine red cells.
- Serology, in association with vaccination history, may be of value for determining the involvement of respiratory viruses.

Treatment

- Dogs with mild clinical signs do not require specific therapy.
- If coughing persists for more than 2 weeks or if bronchopneumonia is present, antibiotic therapy may be required. Amoxicillin has proved effective in field trials (Thursfield *et al*., 1991). Emergence of resistance to penicillin, the cephalosporins and sulphonamides has been reported recently (Schwarz *et al*., 2007). Tetracyclines and fluoroquinolones may be effective (Bemis, 1992).

Control

- Affected dogs should be isolated immediately.
- If predisposing factors are identified, they should be corrected.
- Intranasal vaccines containing *B. bronchiseptica* and PI-2 antigens induce local protective immunity and are not affected by maternal antibodies. Some vaccines contain canine adenovirus type 2 also. Modified live *B. bronchiseptica* vaccines decrease the severity of clinical signs but may not prevent infection. Modified live vaccines are available for many of the viruses associated with respiratory disease in dogs.

Bordetella bronchiseptica and the development of atrophic rhinitis

Toxigenic strains of *B. bronchiseptica* are widely distributed in pig herds. They can cause turbinate hypopla-sia without distortion of the snout in piglets under 4 weeks of age. In uncomplicated infections, pigs reach slaughter age with relatively minor change in the tur-binate bones (Rutter, 1989). However, the infection with *B. bronchiseptica* may facilitate colonization by toxigenic *Pasteurella multocida* type D with the subsequent development of severe atrophic rhinitis and distortion of the snout. Factors such as overstocking and

poor ventilation can contribute to the development of atrophic rhinitis. The most severe form of the disease results from concurrent infection with *B. bronchiseptica* and *P. multocida* (Pedersen *et al*., 1988).

Turkey coryza

Turkey coryza, caused by *B. avium*, is a highly contagious upper respiratory tract disease of poults, with high morbidity and low mortality. Infection is spread through direct contact, by aerosols and from environmental sources. Mucus accumulates in the nares with swelling in the submaxillary sinuses. Beak-breathing, excessive lacrimation and sneezing may be evident. Infection with *B. avium* predisposes to secondary infections with bacteria such as *Escherichia coli*. Once *E. coli* becomes established, a more serious disease with high mortality can develop.

Diagnosis

- Clinical signs and gross pathological features may be indicative of the disease.
- Isolation and identification of *B. avium* from sinus and tracheal exudates is confirmatory.
- Virulent isolates agglutinate guinea-pig red blood cells.
- Microagglutination and ELISA techniques may be of diagnostic value.

Treatment and control

- Broad-spectrum antibiotics early in the course of disease may be beneficial.
- Commercially available bacterins and modified live vaccines may be used in susceptible flocks.
- Thorough cleaning and disinfection of turkey houses after an outbreak of disease are essential for the elimination of *B. avium*.

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Chapter 32

Moraxella species

The genus *Moraxella* has been divided into two subgenera, *Moraxella* and *Branhamella*. *Moraxella* species occur as short (1.0 to 1.5 × 1.5 to 2.5 µm), plump, Gram-negative rods or, occasionally, cocci which typically occur in pairs (Fig. 32.1). Moraxellae are non-motile, aerobic and usually catalase-positive and oxidase-positive. Most members of the genus associated with disease are proteolytic but are unable to utilize sugars. Growth, which is enhanced by the addition of blood or serum to media, does not occur on MacConkey agar. *Moraxella bovis* is the principal pathogen in the genus but *M. bovoculi*, *M. ovis* and *M. equi* are also associated with disease in animals. *Moraxella catarrhalis* can cause disease in humans. Other species, which are periodically isolated from clinical specimens, are generally regarded as non-pathogenic. Selected *Moraxella* species which may be isolated from animals and their clinical significance are presented in Table 32.1. When isolated from cases of infectious bovine keratoconjunctivitis, virulent strains of *M. bovis* are fimbriate and haemolytic and grow into the agar.

Moraxella bovis

Moraxella bovis is found on mucous membranes of carrier cattle. The organism is susceptible to desiccation and is short-lived in the environment. It can survive for up to 72 hours in the salivary organs and on the body surfaces of flies, which can act as vectors.

Key points

- Short Gram-negative rods, usually in pairs
- Optimal growth on enriched media
- Aerobic, non-motile
- Usually catalase-positive and oxidase-positive
- Proteolytic, unreactive with sugar substrates

- Virulent strains are fimbriated and haemolytic
- Susceptible to desiccation
- Found on mucous membranes
- *Moraxella bovis* is the principal pathogen in the genus and causes infectious bovine keratoconjunctivitis

Clinical infections

Moraxella bovis causes infectious bovine keratoconjunctivitis, an important ocular disease of cattle which occurs worldwide.

Infectious bovine keratoconjunctivitis

Infectious bovine keratoconjunctivitis (IBK), sometimes referred to as 'pink-eye' or New Forest disease, is a highly contagious condition affecting the superficial structures of the eyes, usually in animals under 2 years of age. The disease causes economic losses arising from decreased weight gain in beef breeds, loss of milk production, short-term disruption of breeding programmes and treatment costs.

Figure 32.1 Short, plump rods of *Moraxella bovis*, characteristically occurring in pairs.

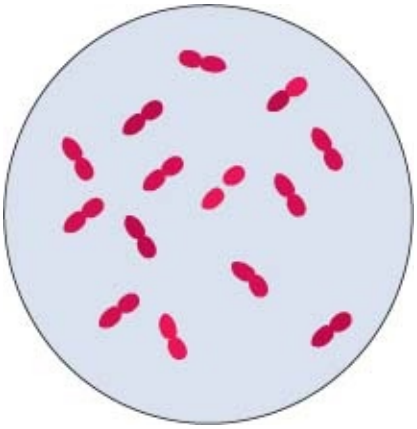

Table 32.1 Selected *Moraxella* species which may be isolated from animals, and their clinical significance.

***Moraxella* species**	**Clinical significance**
Moraxella boevrei	Isolated from the upper respiratory tract of healthy goats
M. bovis	Infectious bovine keratoconjunctivitis
M. bovoculi	Isolated from animals affected by infectious bovine keratoconjunctivitis. Possesses RTX toxin but pathogenicity unproven (Angelos *et al*., 2007b)
M. canis	Member of the oral flora of dogs. Has been isolated from dog bite wounds in humans

M. caprae	Isolated from the upper respiratory tract of healthy goats
M. cuniculi	Isolated from infectious bovine keratoconjunctivitis in association with other organisms
M. equi	Conjunctivitis in horses
M. lacunata	Isolated from a number of pathological conditions in animals including goats, dogs, pigs and aborted equine foetuses. Pathogenicity unknown
M. ovis	Isolated from healthy animals and those with keratoconjunctivitis
M. phenylpyruvica	Isolated from a number of sites in farm animals but pathogenicity uncertain

There appears to be an age-related immunity, probably as a result of previous exposure. Asymptomatic carrier animals harbour *M. bovis* in the nasolacrimal ducts, nasopharynx and vagina (Ruehl *et al*., 1993). Transmission can occur by direct contact, by aerosols and through fl ies acting as vectors. Factors that predispose to IBK are presented in Table 32.2.

Table 32.2 Factors which may exacerbate or predispose to outbreaks of infectious bovine keratoconjunctivitis.

Factor	**Comments**
Age	Young cattle less than 2 years of age are particularly susceptible to infection
Breed	*Bos taurus* breeds appear to be more susceptible than *Bos indicus* breeds
Fly activity	Flies can act as vectors of *Moraxella bovis*
Ocular irritants	Dust, tall grasses, grass seeds, wind, ultraviolet light and cold ambient temperatures may predispose to disease
Concurrent infections	Infection with bovine herpesvirus 1 or *Thelazia* species may exacerbate infectious bovine keratoconjunctivitis
Vitamin deficiency	A deficiency of vitamin A may predispose to disease

Pathogenesis and pathogenicity

The virulence of *M. bovis* is attributed to fimbriae, which allow adherence of the organisms to the cornea, circumventing the protective effects of lacrimal secretions and blinking. Two types of fimbriae are recognized, namely Q fimbriae (pili), which are specific for colonization, and I fimbriae, which allow local persistence of infection (Ruehl *et al*., 1993). Fimbrial antigens stimulate type- specific protective immunity. Filamentous-haemagglutinin-like proteins may be important for adherence also (Kakuda *et al*., 2006).

During bacterial replication, haemolysin and other lytic enzymes such as fibrolysin, phosphatase, hyaluronidase and aminopeptidase are produced. Lipopolysaccharides, associated with O antigens, also appear to play a role in virulence (DeBower and Thompson, 1997). The haemolysin is a calcium-dependent, pore-forming cytolysin which damages the cell membranes of neutrophils (Clinkenbeard and Thiessen, 1991). This toxin was subsequently shown to be an RTX (repeats in toxin) toxin with haemolytic and cytotoxic activity and designated Mbx A (Angelos *et al*., 2001). Release of hydrolytic

enzymes from neutrophils on the corneal surface contributes to breakdown of its collagen matrix.

Strains that lack either cytotoxin or fimbriae are avirulent. Isolates from carrier animals are often non-haemolytic and non-fimbriate but reversion to virulence can occur. It has been suggested that the deficiency of lysozyme in the lacrimal secretions of cattle may account for their susceptibility to *M. bovis* (Punch and Slatter, 1984).

Clinical signs

Infectious bovine keratoconjunctivitis initially manifests as blepharospasm, conjunctivitis and lacrima-tion. Progression of the condition through keratitis to corneal ulceration, opacity and abscessation may occasionally lead to panophthalmitis and permanent blindness (Punch and Slatter, 1984). Following ulceration, vascularization extends from the limbus and stromal oedema develops. There may be weakening of the cornea with the development of coning. In most mild cases, the cornea heals within a few weeks although there may be permanent scarring of the structure.

Some carrier animals may exhibit persistent lacrimation. Following infection with a virulent strain of *M. bovis*, neutralizing antibodies develop which are active against haemolysin produced by other strains. In contrast, antibodies that block fimbrial-mediated adherence are type-specific and exposure to *M. bovis* possessing a different fimbrial type may result in disease (Moore and Rutter, 1989).

Diagnostic procedures

- The disease characteristically affects a number of animals in a herd.
- Lacrimal secretion is the most suitable specimen for laboratory examination. Because *M. bovis* is extremely susceptible to desiccation, specimens must be processed promptly. For transportation, swabs of lacrimal secretions should be placed in 1 to 2 ml of sterile water. Ideally, specimens should be cultured within 2 hours of collection.
- A fluorescent antibody technique for demonstrating *M. bovis* in smears from lacrimal secretions is available.
- Specimens should be cultured on blood agar and MacConkey agar and incubated aerobically at 37°C for 48 to 72 hours.
- Identification criteria for isolates:

– Round, small, shiny, friable colonies appear after 48 hours. Colonies of virulent strains are surrounded by a zone of complete haemolysis and are embedded in the agar.

– No growth occurs on MacConkey agar.

– Cultures of virulent strains autoagglutinate in saline.

– Smears from colonies reveal short Gram-negative rods in pairs (Fig. 32.1).

– Reactions in the catalase and oxidase tests are positive. A Loeffler's serum slope may be pitted after 10 days.

- Differentiation of moraxellae isolated from the eyes of cattle can be carried out using PCR-based methods (Angelos and Ball, 2007).
- Fimbriate isolates can be assigned to seven serogroups (Moore and Lepper, 1991). Isolates may be investigated using molecular methods also, including randomly applied polymorphic DNA (RAPD) analysis (Conceicao *et al*., 2004) and PCR-DNA fingerprinting (Prieto *et al*., 1999).

Treatment

Antimicrobial therapy should be administered sub-conjunctivally or topically early in the disease (George, 1990; DeBower and Thompson, 1997).

Control

- Fimbriae-derived bacterins, which are available commercially in some countries, are of uncertain efficacy. Vaccines incorporating both cytotoxin and fimbriae have been developed and may provide enhanced protection (Angelos *et al*., 2007a).
- Management-related methods are important in the control of IBK. These include isolation of affected animals, reduction of exposure to mechanical irritants, the use of insecticidal ear tags and the control of concurrent diseases such as infectious bovine rhinotracheitis or *Thelazia* infestation.
- The prophylactic use of intramuscular oxytetracycline can be considered for animals at risk.
- Animals that are blind should be housed.
- Vitamin A supplementation may be beneficial.

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Chapter 33

Brucella species

Brucella species are small (0.6 × 0.6 to 1.5 µm), non-motile, coccobacillary, Gram-negative bacteria. As they are not decolorized by 0.5% acetic acid in the modified Ziehl-Neelsen (MZN) staining technique, they are referred to as MZN-positive. In MZN-stained smears of body fluids or tissues, they characteristically appear as clusters of red coccobacilli (Fig. 33.1). As all species of *Brucella* share a high degree of genetic similarity, there was a proposal that all described species be classified within the genus *Brucella melitensis* (Anon., 1988). However, this decision was reversed in 2005 and the six *Brucella* nomenspecies were re-approved (Osterman and Moriyón, 2006). In addition, a number of new species, including brucellae of sea mammals and voles, have been characterized in recent years (Table 33.1). *Brucella melitensis*, *B. abortus* and *B. suis* are subdivided into biovars based on cultural and serological properties. The *Brucella* genome is unusual among veterinary pathogenic bacteria as it is composed of two circular chromosomes, with the exception of *B. suis* biovar 3, which has a single chromosome. *Brucella* species are aerobic, capnophilic and catalase-positive. Apart from *B. ovis* and *B. neotomae*, they are oxidase-positive. All *Brucella* species are urease-positive except *B. ovis*. *Brucella ovis* and some biotypes of *B. abortus* require 5 to 10% CO_2 for primary isolation. Moreover, the growth of other *Brucella* species is enhanced in an atmosphere of CO_2. Media enriched with blood or serum are required for culturing *B. abortus* biotype 2 and *B. ovis*.

Usual habitat

As a general rule, brucellae have a predilection for both female and male reproductive organs in sexually mature animals and each *Brucella* species tends to infect a particular animal species. Infected animals serve as reservoirs of infection, which often persists indefinitely. Organisms shed by infected animals can remain viable in a moist environment for many months. However,

transmission is usually through direct contact with infected animals or fluids and tissues associated with abortion.

Key points

- Small Gram-negative coccobacilli
- Stain red using the modified Ziehl-Neelsen method
- Aerobic and capnophilic
- Non-motile, catalase-positive
- Most isolates are oxidase-positive
- Urease-positive
- Intracellular pathogens
- Target reproductive organs of certain species of animals
- Some species cause undulant fever in humans

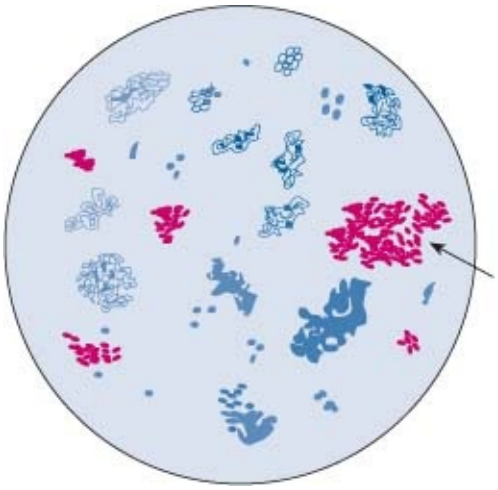
Differentiation of *Brucella* species

Brucella species are differentiated by colonial appearance, biochemical tests, specific cultural requirements and growth inhibition by dyes (Table 33.2). In addition, agglutination with monospecific sera, susceptibility to bacteriophages and molecular methods are employed for definitive identification.

- On primary isolation, colonies of *B. abortus*, *B. melitensis* and *B. suis* occur in smooth forms and are small, glistening, bluish and translucent after incubation for 3 to 5 days. Colonies become opaque with age. In contrast, primary isolates of *B. ovis* and *B. canis* always occur in rough forms. These rough colonies are dull, yellowish, opaque and friable. Brucellae are non-haemolytic on blood agar.
- Slide agglutination tests with monospecific antisera are used to detect the presence of important surface antigens, *abortus* antigen A and *melitensis* antigen M. The R antigen, a feature of the rough brucellae *B. ovis* and *B. canis*, can be detected by anti-R serum.
- Isolates of *B. abortus* are lysed by a specific bacteri-ophage (Tbilisi phage) at routine test dilution.
- If other tests give equivocal results, oxidative metabolic rates on selective substrates can be conducted in reference laboratories.

- PCR, PCR restriction fragment length polymorphism, pulsed-field gel electrophoresis methods and other molecular methods have been developed for identification and differentiation of *Brucella* isolates. One of the first PCR methods developed was the AMOS-PCR (Bricker and Halling, 1994), so named because it could differentiate between *B. abortus* biovars, 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis*. This method has been refined over time and can now differentiate other biovars and species in addition to *B. abortus* vaccine strains. A new multiplex PCR assay (Bruce-ladder) described in the OIE Terrestrial Manual (Anon., 2009a) identifies most *Brucella* species in a single step, including the newly described species from marine mammals and *B. abortus* and *B. melitensis* vaccine strains.

Figure 33.1 Clusters of *Brucella abortus* as they appear in a smear from a cotyledon of a cow with brucellosis. Using the modified Ziehl-Neelsen method, the small coccobacillary cells, present in clumps, stain red (arrow). Cellular debris and other bacterial cells stain blue.



Pathogenesis and pathogenicity

The establishment and outcome of infection with bru-cellae depend on the number of infecting organisms and their virulence and also on host susceptibility, including age of the host (Price *et al*., 1990). Brucellae which lack the major outer-membrane lipopolysac-charide, produce rough colonies and are less virulent than those derived from smooth colonies (Roop *et al*., 1991). Although smooth and rough organisms can enter host cells, rough forms are usually eliminated unlike smooth forms which may persist and multiply. Brucellae persist within macrophages but not within neutrophils. Non-opsonized

brucellae are taken up through interaction of the O side-chains of LPS with cholesterol-rich regions of the phagocyte plasma membrane, termed lipid rafts. Once engulfed, brucel-lae persist within the acidified phagosome, or 'Brucella-containing vacuole'. Acidification of the phagosome is important as it induces changes in gene expression of the brucella organism, which favour intracellular survival. Cyclic beta-1,2-glucans, constituents of the outer membrane, help in the prevention of phagolysosome fusion. Inhibition of phagosome–lysosome function is a major mechanism for intracellular survival and an important determinant of bacterial virulence. While survival within the phagosome occurs, replication of brucellae only takes place once the 'brucellosome' is formed. This structure is formed through the fusion of the *Brucella*-containing vacuole with the rough endoplasmic reticulum of the host cell. Effectors secreted by a type IV secretion system encoded by the *virB* operon appear to be important in maturation of the vacuole and in its transport to, and fusion with, the rough endoplasmic reticulum (Celli *et al*., 2006; Carvalho Neta *et al*., 2010). In the next phase of infection, virulent brucellae are transported to regional lymph nodes. Intermittent bacteraemia results in spread and localization in the reproductive organs and associated glands in sexually mature animals. Erythritol, a polyhydric alcohol which acts as a growth factor for brucellae, is present in high concentrations in the placentae of cattle, sheep, goats and pigs. This growth factor is also found in other organs such as the mammary gland and epididymis, which are targets for brucellae. Intracellular replication in trophoblastic cells is strongly influenced by the stage of gestation and increases in late gestation, when the cells actively secrete steroid hormones. In chronic brucellosis, organisms may localize in joints or intervertebral discs. It is suggested that brucellae may inhibit or delay the host immune response and this may be responsible in part for the persistent infections seen with this pathogen (Carvalho Neta *et al*., 2010).

Table 33.1 *Brucella* species, their host range and the clinical significance of infection.

***Brucella* species**	**Usual host / Clinical significance**	**Species occasionally infected / Clinical significance**
B. abortus	Cattle / Abortion, orchitis	Sheep, goats, pigs / Sporadic abortion Horses / Bursitis Humans / Intermittent fever, systemic disease
B. melitensis	Goats, sheep / Abortion, orchitis, arthritis	Cattle / Sporadic abortion, brucellae in milk Humans / Malta fever, severe systemic disease
B. suis	Pigs / Abortion, orchitis, arthritis,spondylitis, infertility	Humans / Intermittent fever, systemic disease
B. ovis	Sheep / Epididymitis in rams, sporadic abortion in ewes	

B. canis	Dogs / Abortion, epididymitis, discospondylitis, sterility in male dogs	Humans / Mild systemic disease
B. neotomae	Desert wood rat / Not isolated from domestic animals	
B. ceti	Cetaceans	Dolphins / May cause abortion, neurological disease has been described Humans / Little evidence of disease
B. pinnipedialis	Pinnipeds	Humans / Little evidence of disease

Table 33.2 Characteristics of *Brucella* species of veterinary importance.

Brucella species	Number of biotypes	Requirement for CO_2	Production of H_2S	Urease activity	Growth in media containing	
					Thionin (20 μg/ml)	Basic fuchsin (20 μg/ml)
B. abortus	7	v	v	+	v	v
B. melitensis	3	–	–	v	+	+
B. suis	5	–	v	+	+	v
B. ovis	1	+	–	–	+	–
B. canis	1	–	–	+	+	–

v, variable reactions related to different biotypes.

Diagnostic procedures

The diagnosis of brucellosis depends on serological testing and on the isolation and identification of the infecting *Brucella* species. Care should be taken during collection and transportation of specimens, which should be processed in a biohazard cabinet.

- Specimens for laboratory examination should relate to the specific clinical condition encountered.
- MZN-stained smears from specimens, particularly cotyledons, foetal abomasal contents and uterine discharges, often reveal characteristic MZN-positive coccobacilli. In specimens containing cells, the organisms may appear in clusters (Fig. 33.1).
- The polymerase chain reaction can be used to detect brucellae in clinical specimens; a number of different procedures have been described (Bricker, 2002). An advantage of this procedure is its high sensitivity and it can be used for testing samples with low numbers of organisms.
- A nutritious medium such as Columbia agar, supplemented with 5% serum and appropriate antimicrobial agents, is used for isolation. Plates are incubated at 37°C in 5 to 10% CO_2 for up to 5 days. Although CO_2 is a specific requirement for individual species, the majority of brucellae are

capnophilic.

- Serological testing is used for international trade and for identifying infected herds or flocks and individual animals in national eradication schemes (Table 33.3). Brucellae share antigens with some other Gram-negative bacteria such as *Yersinia enterocolitica* serotype O:9 (Hilbink *et al*., 1995), and consequently cross-reactions can occur in agglutination tests.

Table 33.3 Tests used for the diagnosis of bovine brucellosis using milk or serum.

Test	Comments
Brucella milk ring test	Conducted on bulk milk samples for monitoring infections in dairy herds. Sensitive but may not be reliable in large herds
Rose-Bengal plate test	Useful screening test. Antigen suspension is adjusted to pH 3.6, allowing agglutination by lgG1 antibodies. Qualitative test only, positive results require confirmation by CFT or ELISA
Complement-fixation test (CFT)	Widely accepted confirmatory test for individual animals
Indirect ELISA	Reliable screening and confirmatory test
Competitive ELISA (using monoclonal antibodies)	Recently developed test with high specificity; capable of detecting all immunoglobulin classes and can be used to differentiate infected animals from S19-vaccinated cattle
Serum agglutination test (SAT)	A tube agglutination test which lacks specificity and sensitivity; IgG1 antibodies may not be detected, leading to false-negative results
Antiglobulin test	Sensitive test for detecting non-agglutinating antibodies not detected by the SAT

Clinical infections

Although each *Brucella* species has its own natural host, *B. abortus*, *B. melitensis* and biotypes of *B. suis* can infect animals other than their preferred hosts (Table 33.1).

Bovine brucellosis

Bovine brucellosis, caused by *B. abortus* and formerly worldwide in distribution, has been eradicated or reduced to a low prevalence in many countries through national eradication programmes. In some countries, including the USA, wildlife species appear to have acquired infection from domestic cattle and infection has become established in wildlife. Endemic infection of wildlife such as bison and elk makes eradication of brucellosis in cattle extremely difficult in some parts of North America. Although acquired most often by ingestion of contaminated foetal tissues and fluids, infection can occasionally follow venereal contact, penetration through skin abrasions, inhalation or transplacental transmission

(Fig. 33.2). The number of organisms shed in foetal fluids post abortion can be in the order of 10^9 or 10^{10} cfu/g. As the infectious dose is estimated to be approximately 10^4 organisms, large numbers of animals may be infected by an aborting cow especially when animals are in close contact indoors. Abortion storms may be encountered in herds with a high percentage of susceptible pregnant cows. Abortion usually occurs after the fifth month of gestation and subsequent pregnancies are usually carried to term. Large numbers of brucellae are excreted in uterine discharges for about 2 to 4 weeks following an abortion and at subsequent parturitions, although infected calves appear normal. Infection in calves is of limited duration in contrast to cows in which infection of the mammary glands and associated lymph nodes persists for many years. Brucellae may be excreted intermittently in milk for a number of years. In bulls, the structures targeted include seminal vesicles, ampullae, testicles and epididymides. In tropical countries, hygromas involving the limb joints are often observed when the disease is endemic in a herd.

In affected herds, brucellosis can result in decreased fertility, reduced milk production, abortions in susceptible replacement animals and testicular degeneration in bulls. Abortion is a consequence of placentitis involving both cotyledons and intercotyledonary tissues. In the bull, necrotizing orchitis occasionally results in localized fibrotic lesions.

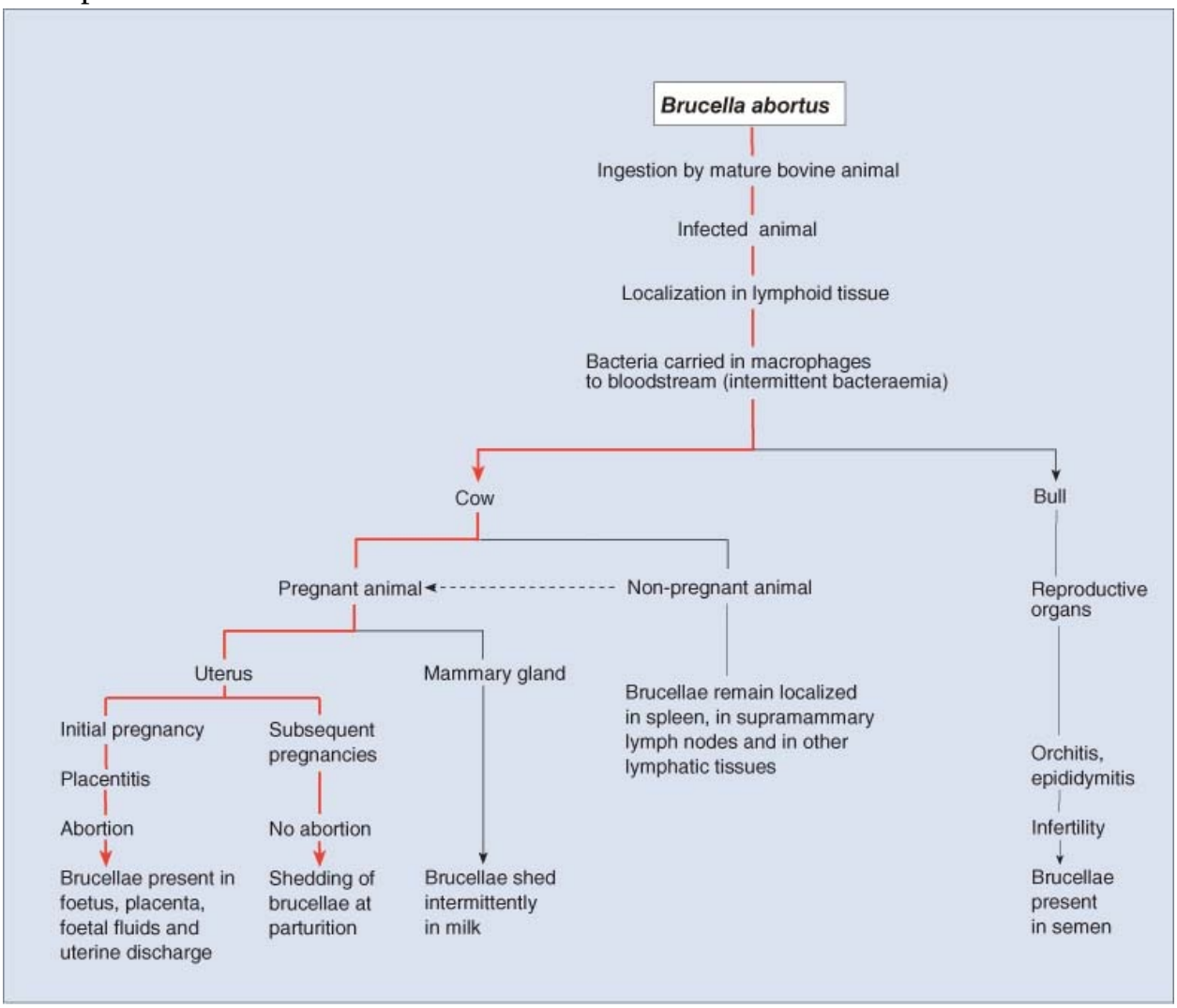
Diagnosis

- Clinical signs are not specific although abortions in first-calf heifers and replacement animals may suggest the presence of the disease.
- Clusters of MZN-positive coccobacilli may be evident in smears of cotyledons, and MZN-positive organisms may also be detected in foetal abomasal contents and uterine discharges.
- Isolation and identification of *B. abortus* is confirmatory.
- Identification criteria for isolates:
  – Colonial appearance
  – MZN-positive organisms
  – Bacterial cell agglutination with a high-titred antiserum
  – Rapid urease activity
  – Biotyping using tests and other features indicated in Table 33.2.
- A range of serological tests, varying in sensitivity and specificity, is

available for the identification of infected animals (Table 33.3).

- Brucellin, an extract of *B. abortus*, has been used for intradermal testing (Worthington *et al*., 1993).
- Molecular methods, including PCR-based techniques for the detection of brucellae in tissues and fluids, have been developed.

Figure 33.2 The progression of infection with *Brucella abortus* in mature susceptible cattle.



Treatment and control

- Treatment of cattle with brucellosis is ineffective.
- National eradication schemes are based on the detection and slaughter of infected cattle. Detection is usually based on serological methods (Table 33.3).

- Vaccination of young heifers, a strategic measure during the early years of eradication schemes, is discontinued when the prevalence of brucellosis reaches low levels. Immunity in brucellosis is predominantly cell-mediated. Two vaccines are currently used in cattle, attenuated strain 19 (S19) vaccine, and the more recent RB51 vaccine:

  – S19 vaccine is administered to female calves up to 5 months of age. Vaccination of mature animals leads to persistent antibody titres.

  – RB51 strain is a stable, rough mutant which induces good protection against abortion and does not result in serological responses detectable in tests used in conventional brucellosis surveillance programmes. It is now the official vaccine for the prevention of brucellosis in several countries.

  – An adjuvanted 45/20 bacterin, although less effective, has been used in some national eradication schemes in the past. Even when administered to adult animals, this vaccine did not induce persistent antibody titres.

  – More specific information relating to mechanisms of protective immunity against *B. abortus* would promote the development of more effective vaccines.

Caprine and ovine brucellosis

Caprine and ovine brucellosis, caused by *B. melitensis*, is most commonly encountered in countries around the Mediterranean littoral and in the Middle East, central Asia and parts of South America. Goats, in which the disease is more severe and protracted, tend to be more susceptible to infection than sheep. The clinical disease resembles brucellosis in cattle in many respects. Clinical features include high abortion rates in susceptible populations, orchitis in male animals, arthritis and hygromas. Infection resulting in abortion may not induce a protective immunity.

Diagnosis is based on clinical signs, direct examination of MZN-stained smears of fluids or tissues, isolation and identification of *B. melitensis* and serological testing. Intradermal brucellin tests are used for surveillance of unvaccinated flocks and herds. In countries where the disease is exotic, a test and slaughter policy is usually implemented. Test and slaughter policies can also reduce the prevalence of disease in endemic areas. The Rose-Bengal agglutination test and the complement fixation test are the most widely used methods for detecting infection with *B. melitensis* and are approved for the purposes of international trade. Indirect enzyme-linked immunosorbent assays

have been developed and are also approved tests for the purposes of international trade (Anon., 2009b). The modified live *B. melitensis* Rev. 1 strain, administered by the subcutaneous or conjunctival routes, is used for vaccination of kids and lambs up to 6 months of age.

Ovine epididymitis caused by *B. ovis*

Brucella ovis produces an infection in sheep which is characterized by epididymitis in rams and placentitis in ewes. The infection was first recorded in New Zealand and Australia and is now established in many other sheep-rearing regions, including some European countries. The consequences of infection include reduced fertility in rams, sporadic abortion in ewes and increased perinatal mortality. Both ram-to-ram and ram-to-ewe venereal transmission occurs. Few of the ewes served by an infected ram develop disease. There is a relatively long latent period in rams following infection. *Brucella ovis* may be present in semen about 5 weeks after infection and epididymal lesions can be detected by palpation at about 9 weeks. In countries where the disease is endemic, premating checks on rams include serological testing and scrotal palpation. Chronically affected rams often have unilateral or bilateral testicular atrophy with swelling and hardening of the epididymis. The most efficient and widely used serological tests for *B. ovis* are the agar gel immunodiffusion test, the complement fixation test and the indirect ELISA. An immunoblotting technique can also be used as a confirmatory diagnostic test (Kittelberger *et al*., 1997). *Brucella ovis* can be isolated from semen and a species-specific PCR test for detection of *B. ovis* in a range of clinical samples including semen, preputial washes and urine has been developed (Xavier *et al*., 2010). A multiplex PCR that will identify all three major causes of epididymitis in rams, *Actinobacillus seminis*, *Histophilus somni* and *B. ovis*, has been described (Saunders *et al*., 2007). Young rams may be vaccinated with the *B. melitensis* Rev. 1 vaccine or with *B. ovis* bacterin.

Porcine brucellosis

Porcine brucellosis, caused by *B. suis*, occurs occasionally in the USA but is more prevalent in Latin America and Asia. There is a prolonged bacteraemia and the disease is manifest as chronic inflammatory lesions in the reproductive organs of sows and boars. Lesions may also be found in bones and joints. Infection is acquired by ingestion or by coitus and may be self-limiting in some

animals. Clinical signs in sows include abortion, stillbirths, neonatal mortality and temporary sterility. Boars excreting brucellae in semen may either be clinically normal or present with testicular abnormalities. Associated sterility may be temporary or permanent. Lameness, incoordination and posterior paralysis are manifestations of joint or bone involvement. Biovars 1 to 3 of *B. suis* infect pigs; biovar 2 differs from the other biovars in its host range and in the lesions produced. It occurs in wild boar throughout continental Europe and the European hare is considered a reservoir host also. Wild boars have been implicated as the source of biovar 2 infections in domestic pigs reared outdoors. This biovar produces miliary lesions, particularly in the reproductive tract.

The Rose-Bengal plate agglutination test and the indirect ELISA are the most reliable serological methods for the diagnosis of porcine brucellosis. A test and slaughter policy is the main control measure in countries where the disease is exotic. A modified live *B. suis* vaccine is used for the vaccination of pigs in south China. *Brucella suis* biovar 4 infects reindeer and caribou in Northern Canada, Alaska and Siberia and biovar 5 infects wild rodents.

Canine brucellosis

Canine brucellosis, caused by *B. canis*, has been recorded in the USA, Japan and Central and South America. However, the distribution of the disease may be more extensive than currently recognized because of difficulties with diagnosis. As *Brucella canis* is permanently in the rough form, it is of comparatively low virulence causing relatively mild and asymptomatic infections. In breeding establishments infection may manifest clinically as abortions, decreased fertility, reduced litter sizes and neonatal mortality. Most bitches that have aborted subsequently have normal gestations. In male dogs the main clinical feature of the disease is infertility often associated with orchitis and epididymitis. Infertility may be permanent and dogs with chronic infections are often aspermic. Rarely, discospondylitis may result in lameness, paresis or paralysis, and uveitis. Generalized lymphadenitis has been reported also. A rapid slide agglutination test kit containing 2-mercaptoethanol is used as a screening test but lacks specificity. Confirmatory tests include a tube agglutination test, ELISA and an agar gel immunodiffusion test. PCR-based tests have been developed for detection of *B. canis* in blood and tissues and appear to be as sensitive as cultural techniques. Treatment, which should be confined to animals not intended for breeding, may be successful early in the course of the disease. A combination of

a tetracycline and an aminoglycoside may be effective (Nicoletti and Chase, 1987) but long-term resolution is difficult to achieve (Pretzer, 2008). Neutering infected animals reduces the risk of transmission. A commercial vaccine is not available and control is based on routine serological testing and removal of infected animals from breeding programmes.

Brucellosis in humans

Humans are susceptible to infection with *B. abortus, B. suis, B. melitensis* and, rarely, with *B. canis* and the brucellae of sea mammals. Transmission to humans occurs through contact with secretions or excretions of infected animals. Routes of entry include skin abrasions, inhalation and ingestion. Raw milk and dairy produce made with unpasteurized milk are important sources of infection. Laboratory accidents account for some human infections. Brucellosis in humans, known as undulant fever, presents as fluctuating pyrexia, malaise, fatigue and muscle and joint pains. Abortion is not a feature of human infection. Osteomyelitis is the most common complication. Severe infections occur with *B. melitensis* (Malta fever) and *B. suis* biovars 1 and 2. Human infections due to *B. abortus* are moderately severe whereas those caused by *B. canis* are usually mild. Antimicrobial therapy should be administered early in an infection. Humans can develop a severe hypersensitivity reaction following infection or after accidental inoculation with attenuated vaccinal strains.

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Chapter 34

Campylobacter and Helicobacter species

Campylobacter species are slender, curved, motile, Gram-negative rods (0.2 to 0.5 °m wide) with polar flagella. Daughter cells which remain joined have a characteristic gull-winged appearance, and long spirals formed by joined cells also occur (Fig. 34.1). These microaerophilic organisms grow best on enriched media in an atmosphere of increased CO_2 and decreased oxygen tension. Many *Campylobacter* species grow on MacConkey agar. They are non-fermentative and oxidase-positive and have variable catalase reactions.

Campylobacter species are found in the intestinal and genital tracts of domestic animals and are widely distributed geographically. The principal disease conditions associated with infection are either intestinal, presenting as diarrhoea, or genital, causing infertility or abortion. *Campylobacter* species were previously classified in the genus *Vibrio*, and the term ‘vibriosis’ has been retained for some of the disease conditions which they cause. Three species, namely *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus* and *C. jejuni* subsp. *jejuni* (hereafter referred to as *C. jejuni*), are recognized pathogens of veterinary importance (Fig. 34.2).

Key points

- Slender, curved, Gram-negative rods in gull-winged shapes and spiral forms
- Motile, microaerophilic
- Most species grow on MacConkey agar
- Enhanced growth on enriched media
- Non-fermentative, oxidase-positive with variable catalase reactions
- Commensals of the intestinal tract and sometimes of the reproductive tract
- Pathogens in the reproductive and intestinal tracts

A number of other species, some of which have been assigned to the genus *Arcobacter*, have been isolated from domestic animals and from humans (Table 34.1). The pathogenicity of these species has not been clearly determined.

The genus *Helicobacter* belongs to the order *Campylobacterales* and the species *H. pylori* is an established human pathogen. There are more than 30

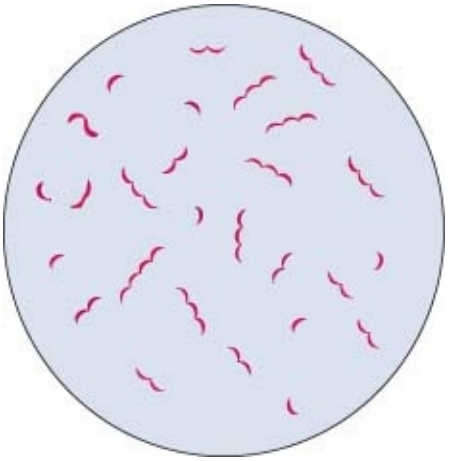
species in the genus described to date, many of which have been isolated from animals, sometimes in association with disease. In addition, these organisms may have zoonotic potential.

Campylobacter species

Usual habitat

Many *Campylobacter* species are commensals in the intestinal tracts of warm-blooded animals. *Campylobacter jejuni* and *C. lari* colonize the intestines of birds, which can result in faecal contamination of water-courses and stored food. A number of *Campylobacter* species are excreted in the faeces of pigs. *Campylobacter fetus* subspecies *venerealis* appears to be adapted principally to bovine preputial mucosa.

Figure 34.1 Slender curved rods of *Campylobacter* species. Characteristic gull-winged and spiral forms are shown.



Differentiation of *Campylobacter* species

Campylobacter species are strictly microaerophilic, requiring an atmosphere of 5 to 10% oxygen and 1 to 10% CO_2 for growth. A selective enriched medium such as Skirrow agar is usually used for primary isolation (Terzolo *et al*, 1991). Differentiation of isolates is based on colonial morphology and certain cultural, biochemical and antibiotic-susceptibility characteristics. In addition to traditional phenotyping methods, molecular techniques are increasingly used for differentiation.

- Colonial morphology:

  – *Campylobacter fetus* subspecies *venerealis* and *C. fetus* subspecies *fetus* have small, round, smooth, translucent colonies with a dewdrop appearance.

  – *Campylobacter jejuni* produces small, flat, grey colonies with a spreading, watery appearance.

  – Colonies of some *Campylobacter* species, which may contaminate clinical specimens, can be slightly pigmented.
- Because *Campylobacter* species do not ferment carbohydrates, other metabolic activities of these organisms must be used for identification. Differentiating characteristics of the main animal pathogens and some commonly isolated commensals are presented in Table 34.2.
- Many PCR-based methods have been developed for differentiation of *Campylobacter* species including multiplex and quantitative real-time PCR procedures. Chaban *et al*. (2009) report the development of real-time PCR assays for the detection of as many as 14 different *Campylobacter* species in dogs. Differentiation of the subspecies of *Campylobacter fetus* can be difficult and PCR may be a valuable technique for confirmation of phenotypic results (Schulze *et al*., 2006). PCR procedures developed in one geographical region may not detect clones of *C. fetus* from another region (Willoughby *et al*., 2005).
- Molecular typing methodologies are widely used for investigation of disease caused by *Campylobacter* species in humans (Foley *et al*., 2009) and to a lesser extent in animals (Sahin *et al*., 2008). Techniques employing pulsed-field gel electrophoresis (PFGE) are frequently used. *Campylobacter* species are included in the PulseNet International system whereby PFGE patterns of isolates from outbreaks of foodborne illnesses are transmitted around the world, enabling rapid comparison of isolates and investigation of infection sources. Other methods which have been applied to *Campylobacter* species include restriction fragment length polymorphism analysis, amplified fragment length polymorphism analysis and multilocus sequence typing (Foley *et al*., 2009).

Figure 34.2 Pathogenic *Campylobacter* species, their usual habitats and possible consequences of infection.

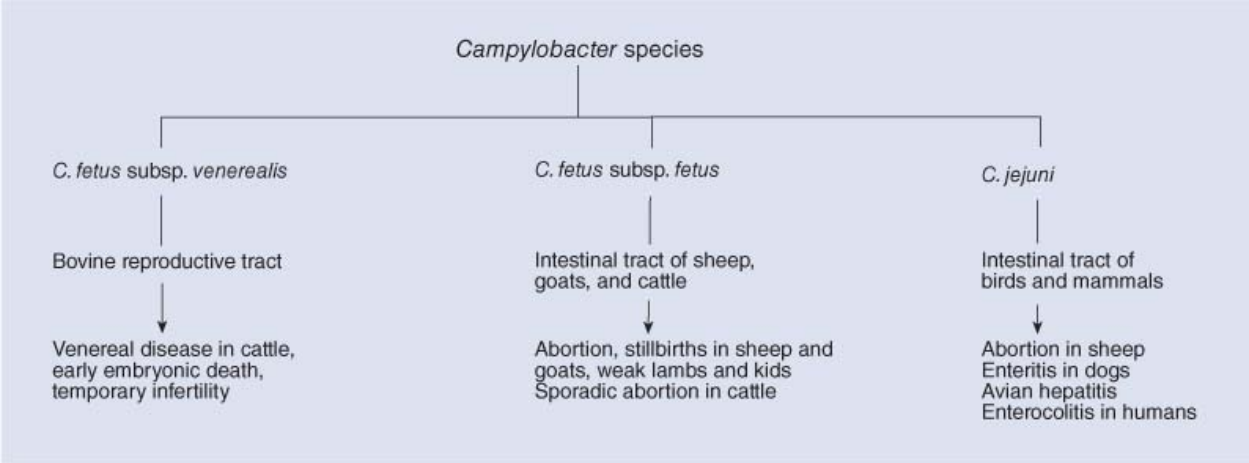


Table 34.1 *Campylobacter* and *Arcobacter* species of uncertain pathogenicity.

Microorganism	Host	Comments
Campylobacter coli	Pigs	Present in intestine
	Humans	Causes enterocolitis
C. helveticus	Dogs, cats	Present in faeces
C. hyoilei	Pigs	Present in faeces
C. hyointestinalis	Pigs	Present in faeces
C. lari	Dogs, birds, other animals	Present in faeces
	Humans	May cause enteritis
C. jejuni subsp. *doylei*	Humans	Isolated from clinical specimens
C. mucosalis	Pigs	Present in faeces
C. sputorum biovar Sputorum	Cattle, sheep	Present in genital tract
	Humans	Isolated from faeces and gingivae
C. sputorum biovar Faecalis	Sheep, cattle	Present in intestinal and genital tracts
	Cattle	Isolated from cases of bovine digital dermatitis
C. upsaliensis	Dogs	Present in faeces and associated with diarrhoea
	Humans	May cause diarrhoea in children
Arcobacter butzleri	Humans	May cause diarrhoea
	Cattle, pigs	Implicated in abortion
A. cryaerophilus	Many species	Isolated from faeces
	Sheep, horses	Isolated from normal and aborted foetuses
	Cattle	Mastitis (rare)
A. skirrowii	Cattle	Present in prepuce
	Cattle, sheep, pigs	Isolated from aborted foetuses

Table 34.2 Differentiating characteristics of *Campylobacter* species.

Campylobacter species	Catalase production	Growth at		Growth in 1% glycine	Growth in 3.5% NaCl	Production of H_2S[a]	Susceptibility to	
		25°C	42°C				Nalidixic acid[b]	Cephalothin[b]
C. fetus subsp. *venerealis*	+	+	–	–[c]	–	–	R	S
C. fetus subsp. *fetus*	+	+	–	+	–	+	V	S
C. jejuni subsp. *jejuni*	+	–	+	+	–	+	S	R
C. lari	+	–	+	+	–	+	R	R
C. coli	+	–	+	+	–	+	S	R
C. hyointestinalis	+	+	+	+	–	+	R	S
C. mucosalis	–	–	+	+	–	+	R	S
C. sputorum biovar Sputorum	–	–	+	+	+	+	R	S

a, lead acetate method of detection.

b, 30 μg discs.

c, some strains are glycine tolerant and are designated *C. fetus* subsp. *venerealis* biovar Intermedius.

+, most strains positive; −, most strains negative; R, resistant; S, susceptible; V, variable.

Pathogenesis and pathogenicity

Campylobacter fetus subsp. *venerealis* and *C. fetus* subsp. *fetus* are structurally unusual in that they possess a microcapsule or S layer, which consists of high-molecular-weight proteins arranged in a lattice formation. This S layer confers resistance to serum-mediated destruction and phagocytosis (Blaser and Pei, 1993) and enhances survival in the genital tract. In addition, because eight different antigenic variants of the protein can be expressed by the organism, the immune response of the host may be subverted (Grogono-Thomas *et al*., 2003).

Much recent research on the pathogenicity of *Campylobacter* species has concentrated on genomic analysis for the identification of putative virulence genes. A major outer membrane protein found in *C. jejuni* and *C. fetus* subsp. *fetus* and subsp. *venerealis* acts as an adhesin. Other genes encoding adhesion proteins such as *cadF* (*Campylobacter* adhesion to fibronectin) have been identified also. Flagellar genes have been identified in many species. Highly virulent species contain a wide repertoire of virulence genes. (Fouts *et al*., 2005; Moolhuijzen *et al*., 2009). The intestinal pathogen *C. jejuni* is internalized into epithelial cells following adhesion and may replicate within the membrane-bound compartment. It is then released by exocytosis from the basolateral

surface. Genes encoding cytolethal distending toxin have been identified in *C. jejuni* and *C. fetus*, and the pathogenic role of this toxin has been confirmed in *C. jejuni*. *Campylobacter fetus* subsp. *venerealis* has a particular tropism for the bovine host and it has recently been shown to contain a unique genomic island which contains genes encoding the components of a Type IV secretion system (Gorkiewicz *et al*., 2010). It appears that this island was originally acquired as a mobile genetic element and this acquisition may have been important in the development of the host tropism of subsp. *venerealis*. The role of heat- stable endotoxin in the pathogenesis of campylobacteriosis is uncertain.

Diagnostic procedures

Details of the diagnostic methods for individual clinical conditions are presented in relevant sections.

- Irrespective of the source of specimens for bacterial isolation, certain general principles relating to culture techniques apply. *Campylobacter* species require microaerophilic conditions for growth, usually supplied by commercially available generator envelopes which deliver 6% oxygen, 10% carbon dioxide and 84% nitrogen. Although most pathogenic species grow optimally at 37°C, *C. jejuni* requires up to 5 days at 42°C for optimum growth.
- Smears from cultures and from clinical specimens should be stained with dilute carbol fuchsin (DCF) for 4 minutes. This method stains the organisms more intensely than the Gram method.
- Identification criteria for isolates:
  – Growth only under microaerophilic conditions
  – Colonial morphology
  – Cell morphology in smears stained with DCF or by immunofluorescence
  – Metabolic characteristics and antibiotic susceptibility pattern
  – PCR-based methods for detection and specific identification of *Campylobacter* organisms are available.

Clinical infections

The most important consequences of infections with organisms in this group are infertility in cattle due to *C. fetus* subsp. *venerealis*, and abortion in ewes caused either by *C. fetus* subsp. *fetus* or by *C. jejuni* (Fig. 34.2).

Bovine genital campylobacteriosis

Campylobacter fetus subsp. *venerealis*, the principal cause of bovine genital campylobacteriosis, is transmitted during coitus to susceptible cows by asymptomatic carrier bulls. The bacteria survive in the glandular crypts of the prepuce and bulls may remain infected indefinitely. The disease is characterized by temporary infertility associated with early embryonic death, return to oestrus at irregular periods (Fig. 34.3) and, occasionally, by sporadic abortion. About one-third of infected cows become carriers. *Campylobacter fetus* subsp. *venerealis* persists in the vagina of carrier cows, a feature attributed to antigenic shifts in the immunodominant antigens of the S layer proteins. Extension of infection to the uterus with the development of endometritis and salpingitis can occur during the progestational phase of the oestrous cycle when both the numbers and the activity of neutrophils decline. The infertile period following uterine invasion can last for 3 to 5 months, after which natural immunity may develop. IgA antibodies, which predominate in the vagina, limit spread of the infection. IgG antibodies produced in the uterus opsonize the pathogens, facilitating phagocytosis by neutrophils and mononuclear cells (Fig. 34.3). This natural immunity may last for up to 4 years.

Campylobacter fetus subsp. *fetus*, an enteric organism acquired by ingestion, can cause sporadic abortions in cows.

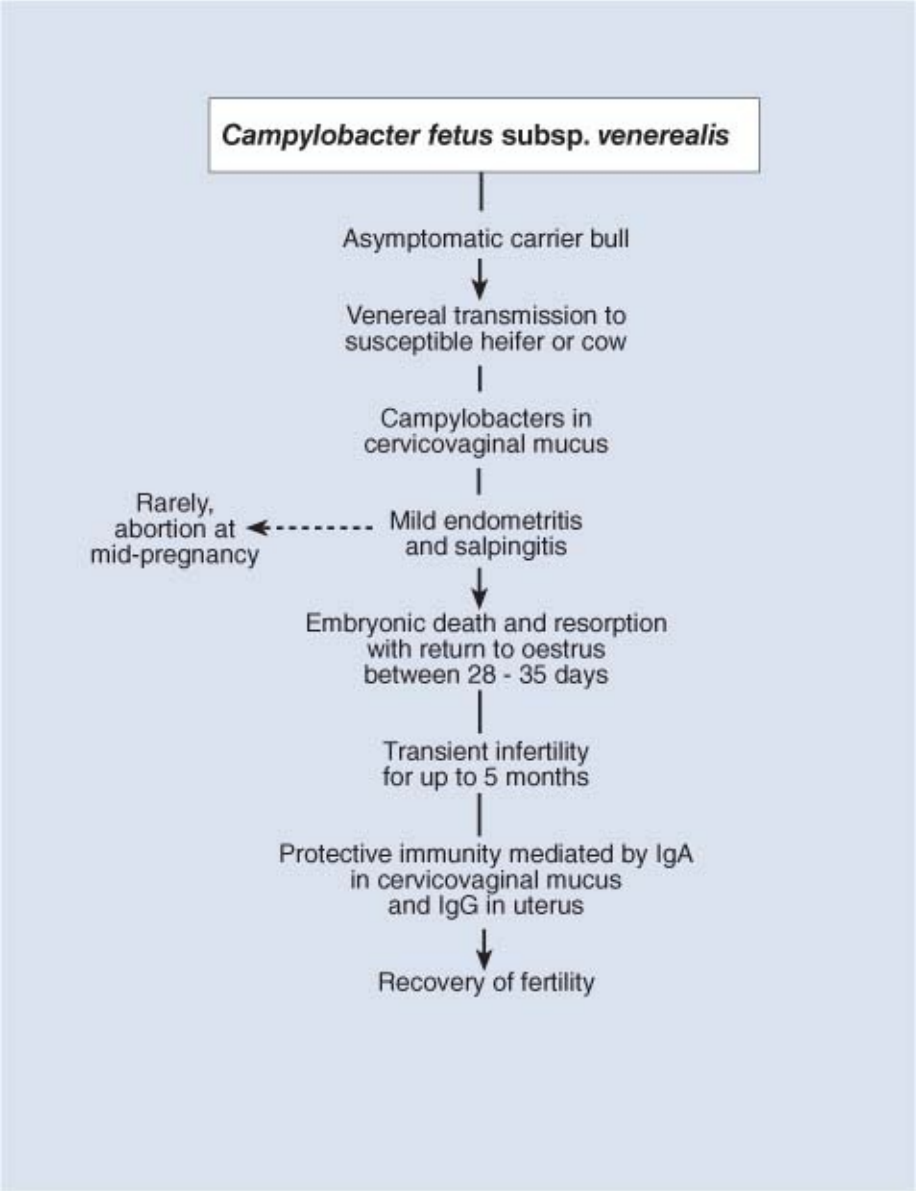
Diagnosis

- Investigation of the breeding records and vaccination history of an affected herd may suggest campylobacteriosis.
- *Campylobacter* species can be detected by the fluorescent antibody technique in sheath washings from bulls or cervicovaginal mucus from cows.
- Isolation and identification of *C.fetus* subsp. *venerealis* from preputial or vaginal mucus is confirmatory. Specimens of mucus should be placed in special transport medium (Lander, 1990). A more recently developed transport medium was described by Harwood *et al*. (2009) which increased isolation rates and was more suitable for PCR-based detection than other transport media tested.
- Vaginal mucus agglutination test detects about 50% of infected, infertile cows on a herd basis.
- An ELISA can be used to demonstrate IgA antibodies in vaginal mucus

after an abortion (Hum *et al*., 1991).

- PCR-based assays have been developed as rapid screening tests for the detection of *C. fetus* subsp. *venerealis* in clinical specimens (Eaglesome *et al*., 1995; McMillen *et al*, 2006; Abril *et al*, 2007).
- Infertility due to *C.fetus* subsp. *venerealis* must be differentiated from other causes of infertility in cows.
- *Campylobacter sputorum* biovar Sputorum, a commensal which is sometimes isolated from preputial washings, is not of clinical significance in cattle.

Figure 34.3 The role of *Campylobacter fetus* subsp. *venerealis* in infertility in cattle.



Treatment and control

- Dihydrostreptomycin, administered either systemically or topically into the prepuce, is used for treating bulls.
- Intrauterine administration of dihydrostreptomy-cin can be used therapeutically but does not eliminate the organism from the vagina and cervix.
- Vaccination with bacterins in an oil emulsion adjuvant is used therapeutically and prophylactically in problem herds. Vaccines are not available worldwide.

Ovine genital campylobacteriosis

Campylobacteriosis in ewes may be caused by either *C. fetus* subsp. *fetus* or *C. jejuni*. The disease is worldwide in distribution and is one of the most common causes of ovine abortion in some countries (Agerholm *et al*., 2006; Mannering *et al*., 2006). *Campylobacterfetus* subsp. *fetus* is found in the faeces of cattle and sheep and *C. jejuni* may be present in the faeces of a wide range of birds and mammals. Previously, *C. fetus* subsp. *fetus* was the most common *Campylobacter* species associated with ovine abortion but *C. jejuni* is becoming increasingly prevalent in many countries. Sahin *et al*. (2008) reported the emergence of a single tetracycline-resistant clone of *C. jejuni* associated with abortion, which now appears to be widespread in the United States. Transmission of both of these organisms is by the faecal–oral route. During pregnancy, localization in the uterus of susceptible ewes may occur following bacteraemia. The subsequent necrotic placentitis may result in abortion late in pregnancy or in stillborn or weak lambs. Round, necrotic lesions up to 2 cm in diameter with pale raised rims and dark depressed centres are evident on the liver surface in some aborted lambs. Aborting ewes are major sources of infection for susceptible animals in a flock. Up to 20% of ewes in a susceptible flock may abort. Recovered ewes are immune for at least 3 years and flock fertility in subsequent breeding seasons is usually good. Although no effect on male fertility attributable to *C.fetus* has been reported, recently Zan Bar *et al*. (2008) demonstrated decreased ram sperm quality and viability when incubated with *C. fetus in vitro*.

Diagnosis

- Typical hepatic lesions in aborted lambs are pathognomonic although such lesions are only occasionally observed.
- A presumptive diagnosis is made by demonstrating the organisms in foetal abomasal contents or birth fluids.
- Isolation and identification of *C.fetus* subsp. *fetus* or *C. jejuni* are confirmatory.
- These pathogens should be differentiated from other causes of abortion in ewes (see Chapter 86).

Treatment and control

- Aborting ewes should be isolated and placentae and aborted foetuses promptly removed. The remainder of the flock should be moved to clean pasture.
- After confirmation of the disease in a flock, vaccination of ewes with a *C. fetus* subsp. *fetus* bacterin is reported to reduce the number of abortions (Gumbrell *et al*., 1996).
- Routine vaccination of ewes with a bacterin is usually carried out immediately before or after mating, with a booster after the second month of gestation and annually thereafter. There is no cross-protection between *C. fetus* subspecies *fetus* and *C. jejuni*. In addition, variation between strains within species may result in insufficient cross-protection between vaccinal and field strains.
- Chlortetracycline administered daily in feed has been used to control outbreaks of abortion. Because of the emergence of tetracycline-resistant clones of *Campylobacter* as reported by Sahin *et al*. (2008), the resistance pattern of the responsible organism should be determined to assist in the selection of appropriate antimicrobial agents.

Intestinal campylobacteriosis in dogs

Diarrhoea in dogs and other domestic animals has been attributed to infection with *Campylobacter* species although confirmation is difficult because healthy animals may shed *Campylobacter* species in their faeces. However, the presence of large numbers of campylobacter- like organisms in DCF-stained faecal smears or rectal scrapings from dogs with diarrhoea may be indicative of infection. A limitation of direct examination is the inability to differentiate *Campylobacter* from other organisms with a similar appearance such as *Helicobacter* or *Anaerospirillium* species. A recent study using PCR-based techniques to detect and quantify *Campylobacter* species in the faeces of dogs found that 58% of healthy dogs and 97% of diarrhoeic dogs shed *Campylobacter* in faeces. A greater variety of species was detected in faeces of diarrhoeic dogs than in normal dogs (Chaban *et al*., 2010). *Campylobacter* species may contribute to the severity of enteric disease in dogs infected with other enter-opathogens such as enteric viruses, *Giardia* species and helminths. Young, debilitated or immunosup-pressed animals are particularly at risk. Enrofloxacin is usually effective in eliminating faecal shedding of *Campylobacter* species. Because of the development of resistance to the quinolones in *Campylobacter* species, this

class of drug should be reserved for the treatment of campylobacteriosis in humans. Accordingly, erythromycin is the preferred drug for treatment of dogs infected with *Campylobacter* species. As infection in dogs is usually self-limiting, treatment is not generally required except when there is a risk of zoonotic transmission.

Avian vibrionic hepatitis

Birds commonly harbour *C. jejuni* in their intestinal tracts and shed the organisms in their faeces. Chicks acquire infection from feed, water and litter when they are first introduced into contaminated premises. Infection in chickens and turkeys is usually asymptomatic and its principal importance is as a source of infection for humans following carcass contamination at slaughter. Outbreaks of disease, which are uncommon, are characterized by a substantial drop in egg production in the flock. Severely affected birds are listless and lose condition. There may be haemorrhage and multifocal necrosis in the liver. A presumptive diagnosis is made by demonstrating curved rods with darting motility in bile, using phase contrast microscopy. Dihydrostreptomycin sulphate should be administered in the food early in an outbreak of disease. Vibrionic hepatitis has been described in ostriches in association with *C. jejuni* and *C. coli* infection (Stephens *et al*., 1998).

Intestinal campylobacteriosis in humans

Campylobacter jejuni is the main cause of human intestinal campylobacteriosis, and campylobacter infection is the most frequent cause of food poisoning in many countries. *Campylobacter coli* and *C. lari* are sometimes implicated. These zoonotic infections are usually food-borne although infection by direct contact with animals such as dogs is now thought to be of importance. Poultry meat is a major source of human infection. Fever, abdominal pain and diarrhoea, sometimes with blood, are the most common manifestations of this enteric infection. In addition, antimicrobial resistance in campylobacters, particularly to fluoroquinolones, is a major public health concern.

Helicobacter species

These organisms are helical, S-shaped or curved, Gram-negative rods (3.0 × 0.5 to 0.9 μm). They are related to *Campylobacter* species and *Arcobacter* species.

Helicobacter species require enriched media; some grow on Skirrow's agar. They are microaerophilic, non-saccharolytic, oxidase-positive and, with the exception of *H. canis*, catalase-positive. Some helico-bacters are found in the gastric mucosa and others are found in the intestine of humans and animals, including both domestic and wild animals and birds. A strong urease reaction is characteristic of the helico-bacters which colonize the gastric mucosa. The morphology of *H. pylori*, the human pathogen associated with gastric ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma, is slightly curved. Non-*H. pylori* helicobacters are long and spiral-shaped. Many helicobacter organisms which are found in animals are difficult to culture, and detection and identification frequently rely on molecular methods. Their role in production of disease in animals is uncertain but they appear to be associated with gastritis in some species such as the pig, cat and dog (Haesebrouck *et al*., 2009). In addition, the organism known as '*Flexispira rappini*', associated with abortion in sheep, is now thought to comprise several *Helicobacter* species (Dewhirst *et al*., 2000). The morphology of these organisms is distinctive as they are spindle-shaped Gram-negative bacteria with spiral periplasmic fibres and bipolar tufts of sheathed flagella. Aborted lambs have multifocal hepatic necrosis resembling the hepatic lesions caused by *Campylobacter* species.

The principal importance of *Helicobacter* species in animals may be as a source of infection for humans. Contact with cats, dogs, cattle and pigs has been associated with infection in humans by non-*H. pylori* helicobacters (Harbour and Sutton, 2008).

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Chapter 35

Lawsonia intracellularis

This slender, curved, Gram-negative rod, *Lawsonia intracellularis*, has not been grown in cell-ree media. Its morphology is campylobacter-like and it was formerly referred to as ‘ileal symbiont intracellularis’. It is classified in the delta subdivision of the *Proteobacteria* and is a microaerophilic obligate intracellular pathogen which is aetiologically implicated in proliferative enteropathy of pigs and foals (McOrist *et al*.,1995; Lavoie *et al*., 2000). It has been cultured in enterocyte cell lines (Lawson *et al*., 1993).

Usual habitat

Lawsonia intracellularis grows intracellularly in pig enterocytes and infected animals excrete small numbers in their faeces (Smith and McOrist, 1997). It has been recovered from the tissues of foals and other animals affected with proliferative enteropathy, including hamsters, deer and ostriches (Cooper *et al*., 1997). Pigs and foals which are not clinically affected, but live on endemically-i nfected premises, may also shed the organism in their faeces, as may wildlife on such farms (Friedman *et al*., 2008; Pusterla *et al*., 2008).

Pathogenesis and pathogenicity

Lawsonia intracellularis has an affinity for enterocytes, its site of replication. Infection induces enterocyte proliferation with the development of adenomatous and inflammatory lesions in the terminal ileum, caecum and colon. This proliferative enteropathy can be reproduced experimentally by oral dosing of conventional specific-pathogen-free pigs with the pathogen. Gnotobiotic pigs, which are devoid of intestinal flora, do not develop the disease when dosed with *L. intracellularis* unless they are pre-dosed with porcine intestinal flora. There appears to be a synergistic interaction between *L. intracellularis* and common intestinal organisms such as *Escherichia coli, Clostridium* species and

Bacteroides species. These organisms probably provide appropriate microenvironmental conditions necessary for the colonization and proliferation of *L. intracellularis* (McOrist *et al*., 1994). In addition, active proliferation and differentiation of crypt cells, as occurs at weaning, appear to be a prerequisite for lesion production (McOrist *et al*., 2006). The exact mechanism of infection has not been established but the organism apparently attaches and enters epithelial cells followed by release from the endosome and multiplication free in the cytoplasm of the cells. Following infection, crypt cells proliferate and migrate to populate the surface of the epithelial lining of the terminal ileum, caecum and colon.

Key points

- Curved, Gram-negative rods
- Obligate intracellular pathogens
- Microaerophilic
- Does not grow on inert media
- Growth in tissue culture prepared from enterocytes
- Implicated in proliferative enteropathy of pigs and foals

Clinical signs

Porcine proliferative enteropathy, which occurs in weaned pigs 6 to 12 weeks of age, is characterized by proliferative and inflammatory changes in the terminal small intestine and large intestine. Under field conditions, infection usually occurs approximately 6 weeks post weaning with shedding persisting for 2 to 6 weeks (Stege *et al*., 2004). Clinical signs range from chronic intermittent diarrhoea with reduction in weight gain to acute haemorrhagic enteropathy. Although sudden deaths may occur in severely affected pigs, most animals with the milder form of the disease recover without treatment.

Lesions in the ileum, caecum and colon include thickening of the wall, mucosal necrosis and, in severe cases, clotted blood in the lumen. Enlargement of the mesenteric lymph nodes is a feature of the disease.

In foals, clinical signs are seen in weanlings and include rapid weight loss with diarrhoea and colic, depression, fever and subcutaneous ventral oedema.

Diagnosis

- Clinical signs and gross pathological findings may be sufficient for a presumptive diagnosis.
- *Lawsonia intracellularis* can be demonstrated in faeces or ileal mucosa by immunofluorescence or by the polymerase chain reaction technique. Multiplex PCR techniques for the detection of the common porcine intestinal pathogens *Brachyspira hyodysenteriae, Salmonella* serovars and *L. intracellularis* have been developed (Elder *et al*., 1997; Suh and Song, 2005).
- The organism can be demonstrated in sections from lesions by silver-impregnation stains or by immunostaining.
- *Lawsonia intracellularis* can be cultured only in enterocyte cell lines.
- Serological tests include IFA, ELISA and immu-noperoxidase monolayer assays (Guedes *et al*., 2002).

Treatment and control

- Antimicrobial agents such as tylosin or tiamulin may be used prophylactically or therapeutically in feed or water.
- Evaluation of antimicrobial susceptibility of the organism is difficult because it can be cultured only in cell lines. Wattanaphansak *et al*. (2008) evaluated antimicrobial activity in both the intracellular and extracellular environments and suggested that tiamulin and valnemulin were most effective when intracellular activity was evaluated and valnemulin was most effective extracellularly.
- Zinc bacitracin incorporated into feed has been reported to be effective in reducing the prevalence of intestinal lesions (Kyriakis *et al*., 1996).
- Thorough cleaning and disinfection of contaminated premises should be carried out at the end of each production cycle.
- A live attenuated vaccine for use in pigs is now available in many countries and is effective in reducing clinical signs of disease.

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Chapter 36

Spirochaetes

The order *Spirochaetales* contains five families, three of which, *Leptospiraceae, Spirochaetaceae* and *Brachyspiraceae*, contain pathogens of veterinary importance (Fig. 36.1) (Ludwig *et al*., 2008). It comprises spiral or helical bacteria (spirochaetes) which share some unique morphological and functional features. Members of the order are motile by means of endoflagella which are located within the periplasm (Fig. 36.2). The genus *Leptospira* within the family *Leptospiraceae* contains pathogens of veterinary and human medical importance. The genera *Borrelia, Brachyspira* and *Treponema* also contain significant animal and human pathogens. There are some nonpathogenic genera in each family.

Pathogenic spirochaetes are difficult to culture; many require specialized media and some require liquid media. Organisms in the group are classified on the basis of genetic relatedness. Serological methods are used for epidemiological investigations and clinical diagnosis.

Leptospira species

Members of this species (leptospires) are motile helical bacteria (0.1 × 6 to 12 µm) with hook-shaped ends (Fig. 36.3). They have two circular chromosomes, both of which contain genes essential to survival. Although cytochemically Gram-negative, they do not stain well with conventional bacteriological dyes and are usually visualized using dark- field microscopy. Silver impregnation and immunological staining techniques are used to demonstrate leptospires in tissues. Leptospirosis, which can affect all domestic animals and humans, ranges in severity from mild infections of the urinary or genital systems to serious systemic disease (Table 36.1).

Key points

- Spiral motile bacteria with endoflagella

- Labile in the environment and sensitive to desiccation
- Although Gram-negative, many stain poorly using conventional methods
- Some grow only in liquid media; most require specialized media
- Many produce zoonotic infections
- *Leptospira* species
  - – Found in aquatic environments
  - – Produce systemic infections in many species
  - – Shed in urine of affected species
  - – Cultured in liquid media aerobically at 30°C
  - – Dark-field microscopy, silver staining, immunofluorescence and molecular techniques used for recognition
- *Borrelia* species
  - – Transmission by arthropod vectors
  - – Cause systemic infections in many species
  - – Grow slowly in specialized culture media at 30 to 35°C in microaerophilic conditions
  - – Culture of borreliae from infected animals is confirmatory
- *Brachyspira* species
  - – Intestinal spirochaetes; some are important enteropathogens of pigs
  - – Can be demonstrated in stained faecal smears or in silver-stained histopathological sections
  - – Diagnosis confirmed by culture on selective blood agar anaerobically at 42°C

Figure 36.1 Classification of spirochaetes of veterinary importance.

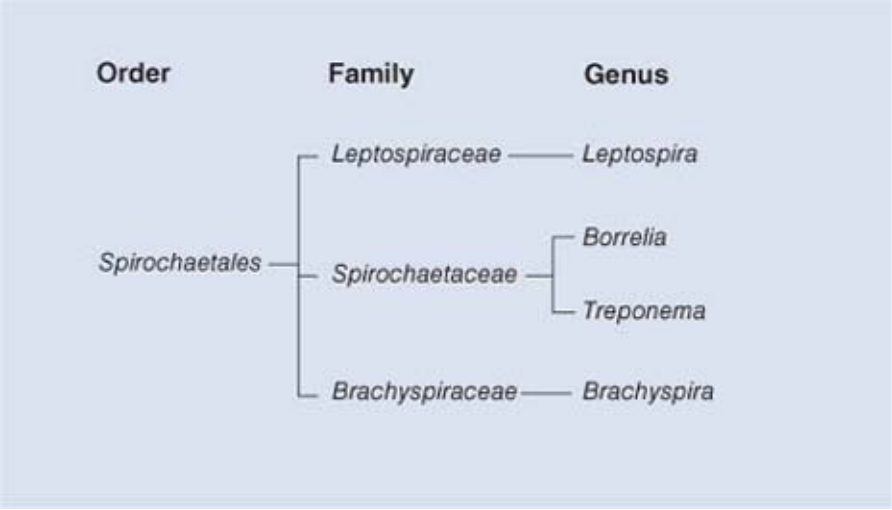
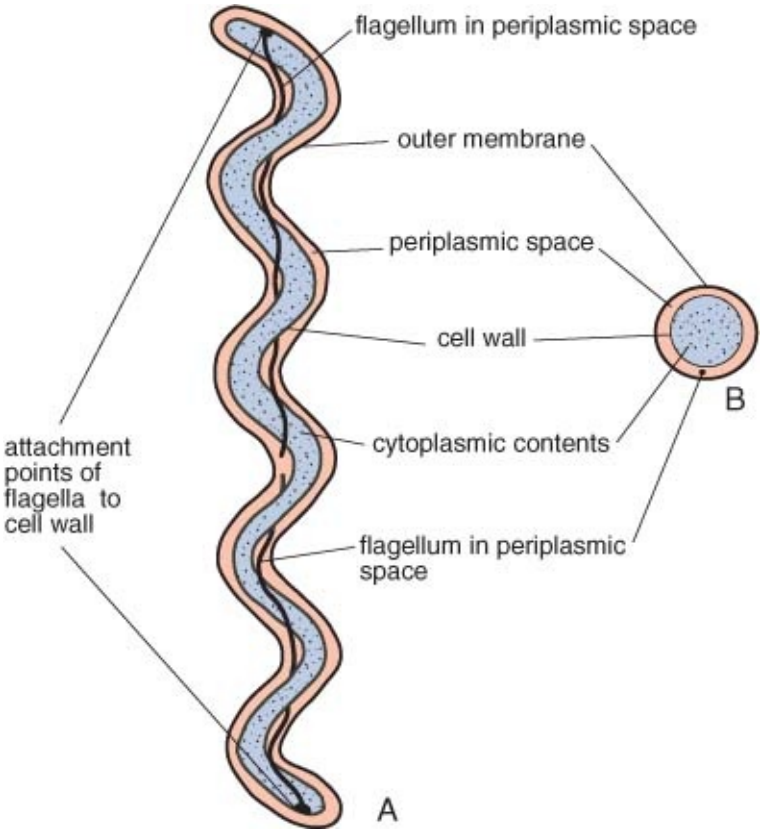


Figure 36.2 Diagrammatic illustration of a typical spirochaete indicating important structural features (A) and their relationships in cross section (B). The flagella, which are attached to the cell wall at each end of the organism, do not

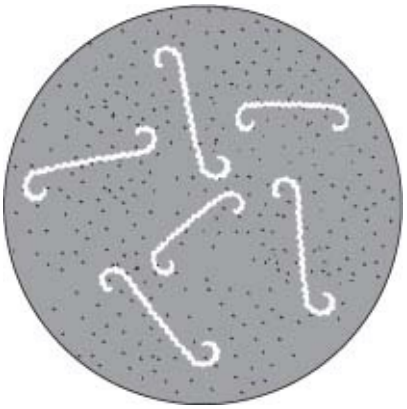
usually overlap.



Usual habitat

Leptospires can survive in ponds, rivers, surface waters, moist soil and mud when environmental temperatures are moderate. Pathogenic leptospires can persist in the renal tubules or in the genital tract of carrier animals. Although indirect transmission can occur when environmental conditions are favourable, these fragile organisms are transmitted most effectively by direct contact.

Figure 36.3 The appearance of leptospires when examined by dark-field microscopy. Their helical structure, which imparts a cord-like appearance, and their hooked ends differentiate these slender motile bacteria from most pathogenic microorganisms.



Differentiation of *Leptospira* species

Formerly, leptospires were differentiated by serological reactions and two species were recognized, *L. interrogans* containing pathogens and *L. biflexa* containing saprophytes. Leptospiral species (genospecies) are now classified by DNA homology and, within each species, various serovars are recognized on the basis of serological reactions (Ellis, 1995). Currently, there are 20 recognized leptospiral species which can be classified into three groups, pathogens, non-pathogens and organisms of uncertain pathogenicity (International Committee on Systematics of Prokaryotes, 2008; Cerqueira and Picardeau, 2009). Pathogenic leptospiral} species are listed in Box 36.1. More than 250 pathogenic serovars in 24 serogroups are defined (Yasuda *et al*., 1987; Perolat *et al*., 1998; Cerqueira and Picardeau, 2009). Cross - absorption of rabbit antisera against defined serovars is used to determine the serovar of an isolate. Serovars with antigens in common belong to the same serogroup. The taxonomic classification of leptospires based on genetic make-up has resulted in the classification of some serologically similar leptospires in different species. Serovar Hardjo, for example, belongs to two species, *L. borgpetersenii* and *L. interrogans*, because common surface antigens are shared by these two genetically distinct organisms. Serological classification remains clinically important because particular serovars tend to be associated with specific host animals and crossimmunity between serovars is minimal. Thus, identification and understanding of the infecting serovar is essential for understanding and controlling leptospiral infections.

Table 36.1 Serovars of *Leptospira* which cause leptospirosis in domestic animals.

Serovar	Hosts	Clinical conditions
Leptospira borgpetersenii serovar Hardjo	Cattle, sheep	Abortions, stillbirths, agalactia

L. interrogans serovar Hardjo	Humans	Influenza-like illness; occasionally liver or kidney disease
L. borgpetersenii serovar Tarassovi	Pigs	Reproductive failure, abortions, stillbirths
L. interrogans serovar Bratislava	Pigs, horses, dogs	Reproductive failure, abortions, stillbirths
L. interrogans serovar Canicola	Dogs	Acute nephritis in pups. Chronic renal disease in adult animals
	Pigs	Abortions and stillbirths. Renal disease in young pigs
L. interrogans serovar Grippotyphosa	Cattle, pigs, dogs	Septicaemic disease in young animals; abortion
L. interrogans serovar Icterohaemorrhagiae	Cattle, sheep, pigs	Acute septicaemic disease in calves, piglets and lambs; abortions
	Dogs, humans	Peracute haemorrhagic disease; acute hepatitis with jaundice
L. interrogans serovar Copenhageni	Domestic animals and humans	Peracute and acute disease; abortion in animals
L. interrogans serovar Pomona	Cattle, sheep	Acute haemolytic disease in calves and lambs; abortions
	Pigs	Reproductive failure; septicaemia in piglets
	Horses	Abortions, periodic ophthalmia

Box 36.1 Leptospiral species containing pathogenic serovars.

- *L. alexanderi*
- *L. alstonii*
- *L. borgpetersenii*
- *L. interrogans*
- *L. kirschneri*
- *L. noguchii*
- *L. santarosai*
- *L. weilii*

Epidemiology

Although leptospires are found worldwide, some serovars appear to have a limited geographical distribution. In addition, most serovars are associated with a particular host species, their maintenance host. These maintenance hosts readily acquire infection, and disease is frequently mild or subclinical and is often followed by prolonged excretion of leptospires in urine. Maintenance hosts are the main source of environmental contamination and of natural transmission to other animal species which are termed incidental hosts. Incidental host species usually exhibit low susceptibility to infection, develop severe disease and are inefficient transmitters of leptospires to other animals. Recent genomic studies have shown that some pathogenic leptospires have extremely limited survival in the environment. Serovars in the species *L. borgpetersenii* do not survive in the

environment in contrast to *L. interrogans* which shows prolonged survival in suitable habitats such as surface waters (Bulach *et al*., 2006; Xue *et al*., 2009). The maintenance hosts and the commonly infected incidental hosts of some serovars of *L. interrogans* are presented in Table 36.2. Many factors, including virulence of the infecting serovar and immune status of the host, influence the outcome of infection in different host species.

Table 36.2 Maintenance and incidental hosts for important serovars of *Leptospira interrogans*.

Serovar	Maintenance hosts	Incidental hosts
Bratislava	Pigs, hedgehogs, horses	Dogs
Canicola	Dogs	Pigs, cattle
Grippotyphosa	Rodents	Cattle, pigs, horses, dogs
Hardjo	Cattle, (sheep occasionally), deer	Humans
Icterohaemorrhagiae	Rats	Domestic animals, humans
Pomona	Pigs, cattle	Sheep, horses, dogs

Pathogenesis and pathogenicity

The pathogenicity of leptospires relates to the virulence of the infecting serovar and the susceptibility of the host species. Although disease may be severe in immature maintenance hosts, serious disease occurs most commonly in incidental hosts. There is limited information on virulence factors and mechanisms of disease production. Leptospires invade tissues through moist, softened skin or through mucous membranes; motility may aid tissue invasion. They spread throughout the body via the bloodstream but, following the appearance of antibodies at about 10 days after infection, they are cleared from the circulation. Some organisms may evade the immune response and persist in the body, principally in the renal tubules but also in the uterus, eye or meninges. The exact mechanisms whereby the organisms persist in these sites despite a specific immune response by the host are unclear. In maintenance hosts, such as the rat colonized by serovar Copenhageni, it appears that initially the leptospires produce little damage to the kidney and do not evoke a significant local cellular immune response, despite the occurrence of leptospiruria from day 7 onwards (Monahan *et al*., 2009). Although mild to severe interstitial nephritis develops from 1 month after infection, leptospiruria may persist for the life of the rat. Downregulation of expression of antigenic proteins on the surface of the leptospires or differential expression of proteins during chronic infection have been suggested as possible mechanisms allowing the persistence of leptospires in

the face of a specific immune response (Monahan *et al*, 2009). Binding of complement regulatory proteins such as plasma factor H by pathogenic leptospires may also play a role in immune evasion. Virulence factors of leptospires have not been fully characterized but the organisms do not appear to produce specific secreted toxins. Toxic components appear to be closely cell-associated and include outer membrane proteins. The LPS of leptospires has much less endotoxic activity than endotoxin of other Gramnegative organisms and activates the host immune response via TLR-2 rather than through the TLR-4 pathway. Initial adherence to host cells may be mediated through binding of leptospiral surface proteins such as a fibronectin-binding protein to host cell extracellular matrix proteins (Merien *et al*., 2000). There is evidence that chemotaxis of leptospires to haemoglobin may be involved in the initiation of infection (Yuri *et al*., 1993). Leptospires can evade phagocytosis in the bloodstream, possibly by inducing macrophage apoptosis (Merien *et al*., 1997). It has been suggested that, following attachment to host cells, the organisms gain entry by receptor-mediated endocytosis (Merien *et al*., 1997). In susceptible animals, damage to red cell membranes and to endothelial cells along with hepatocellular injury produces haemolytic anaemia, jaundice, haemoglobinuria and haemorrhage, associated with acute leptospirosis. Pulmonary haemorrhage is a significant lesion in peracute cases of disease in humans (Dolhnikoff *et al*., 2007). Pathogenic leptospires contain many haemolysins including sphingomyelinases which may be responsible in part for the lesions described.

Diagnostic procedures

- Diagnosis of leptospirosis in maintenance hosts usually requires screening of a defined population.
- Clinical signs, together with a history suggestive of exposure to contaminated urine, may suggest acute leptospirosis.
- Organisms may be detected in fresh urine by darkfield microscopy, but this technique is relatively insensitive.
- Leptospires may be isolated from the blood during the early days of infection and from urine approximately 2 weeks after initial infection either by culture in liquid medium or by animal inoculation. Slow-growing serovars such as Hardjo may require incubation for 6 months in liquid media at 30°C. Commonly, EMJH (Ellinghausen, McCullough, Johnson and Harris) medium, based on 1% bovine serum albumin and Tween 80, is

used for isolation. Tween provides the long-chain fatty acids required as nutrients and albumin adsorbs these compounds and releases them slowly as they are toxic to the leptospires if present in high concentrations.

- Isolates should be identified using DNA profiles and serology. Many different methods for typing leptospiral isolates have been investigated with genomic macrorestriction with rare cutting endonucleases followed by pulsed field gel electrophoresis currently regarded as the gold standard (Cerqueira and Picardeau, 2009). Agreement between PFGE results and serotyping is frequently good although discrepancies have been reported.
- Fluorescent antibody procedures are often used for the demonstration of leptospires in tissues. Suitable tissues include kidney and liver. Silver impregnation techniques can also be used for demonstration of leptospires in tissues.
- DNA hybridization, PCR, magnetic immunocapture PCR and immunomagnetic antigen capture systems have also been developed for the demonstration of leptospiral infection in tissues and urine. In addition, quantitative real-time PCR assays are now available and are useful for the evaluation of newly developed vaccines, for the study of pathogenesis and transmission of leptospirosis, and for diagnosis (Fearnley *et al*., 2008; Lourdault *et al*., 2009).
- The standard serological reference test, the microscopic agglutination test, is potentially hazardous because it involves mixing live culture growing in liquid medium with equal volumes of doubling dilutions of test serum. Titres in excess of 1:400 or a fourfold rise in the titre in paired samples are diagnostically significant when accompanied by clinical signs consistent with leptospirosis. Serological diagnosis of host-adapted leptospirosis is difficult as titres may be decreasing or absent when clinical signs are observed. Some hostadapted serovars, notably Hardjo in cattle, may elicit a poor immune response with the result that infection and prolonged urinary excretion occur without significant titres developing.
- A number of ELISA tests, developed in particular countries, are based on the predominant serovars occurring in those countries.

Clinical infections

The disease conditions associated with leptospiral infections in domestic animals are presented in Table 36.1.

Leptospirosis in cattle and sheep

Cattle are maintenance hosts for L. *borgpetersenii* serovar Hardjo and there is increasing evidence that this serovar is also host-adapted for sheep and deer (Cousins *et al*., 1989; Ayanegui-Alcerreca *et al*., 2007). *Leptospira interrogans* serovar Hardjo is also hostadapted for cattle. Although *L. interrogans* serovar Hardjo appears to cause only sporadic cases of disease in cattle, it may be more virulent than *L. borgpetersenii* serovar Hardjo (Ellis *et al*., 1988). Susceptible replacement heifers, reared separately and introduced into an infected dairy herd for the first time at calving, may develop acute disease with pyrexia and agalactia affecting all quarters. Infection may also result in abortions and stillbirths. If management practices allow exposure to infection and the subsequent development of immunity before breeding age, reproductive problems may not develop. Agalactia caused by leptospiral infection can be confirmed by demonstrating a rising antibody titre in paired serum samples. Infection with serovar Hardjo in sheep, particularly in intensively managed lowland flocks, can cause abortions and agalactia. Antimicrobial treatment with agents such as dihydrostreptomycin or amoxicillin can be used for reducing or eliminating urinary excretion of the organisms. Both monovalent and multivalent inactivated vaccines, which are commercially available, may not always be effective. The serovars incorporated into vaccines should be those that are associated with disease in a particular region. Infection with serovars Pomona, Grippotyphosa and Icterohaemorrhagiae can cause serious disease, particularly in calves and lambs. Infection is usually accompanied by pyrexia, haemoglobinuria, jaundice and anorexia. Extensive renal damage with resultant uraemia often precedes death. Vaccination is used for control of serovar Pomona which is an important cause of bovine abortion in some countries.

Leptospirosis in horses

Although serological evidence of leptospiral infection is common in horses, clinical disease is infrequent. Infection with serovar Bratislava, which has been associated with abortions and stillbirths in horses, may be maintained in the equine species. Clinical disease most often results from incidental infection with serovar Pomona, although other serovars have been implicated. Signs include abortion in mares and renal disease in young horses. Equine recurrent uveitis (periodic ophthalmia, ‘moon blindness’) may be a manifestation of chronic leptospirosis in horses. This condition may be associated with persistent

infection of the eye with leptospires (Wollanke *et al*., 2001). In addition, cross-reactions between leptospiral antigens and proteins from the cornea and lens suggest that autoimmune mechanisms may be involved (Parma *et al*., 1992). Leptospiral vaccines are not currently licensed for use in horses.

Leptospirosis in pigs

Acute leptospirosis in pigs is usually caused by rodentadapted serovars such as Icterohaemorrhagiae and Copenhageni. These serovars cause serious, sometimes fatal disease in young pigs with signs similar to those of acute leptospirosis in other species. In many parts of the world, the principal host-adapted serovar is Pomona. Pigs subclinically infected with Pomona may shed leptospires in their urine for extended periods. Infection can result in reproductive failure including abortions and stillbirths. Pigs also serve as maintenance hosts for serovars Tarassovi and Bratislava, which may also cause reproductive failure.

Leptospirosis in dogs and cats

The serovars associated with leptospirosis in dogs are Canicola and Icterohaemorrhagiae. The widespread use of vaccines incorporating these serovars has resulted in the emergence of serovars Grippotyphosa, Bratislava and Pomona as important pathogens for dogs (Rentko *et al*., 1992). Infection is most common in male dogs aged 4 to 7 years, with hunting dogs more at risk (Stokes and Forrester, 2004). In addition, infection levels are linked to season (late summer and early autumn) and to increased rainfall. Serovar Canicola, which is host-adapted for dogs, causes severe renal disease in pups. In animals that survive the acute phase, a chronic uraemic syndrome may subsequently develop. Incidental canine infections caused by Icterohaemorrhagiae, Copenhageni or Pomona are characterized by acute haemorrhagic disease or subacute hepatic and renal failure. In incidental canine infections due to serovars other than Icterohaemorrhagiae or Copenhageni, signs of renal involvement usually predominate. It is considered that serovar Bratislava, which has been associated with abortion and infertility, is becoming adapted to dogs which may act as maintenance hosts. Bacterins which contain only serovars Icterohaemorrhagiae and Canicola do not induce immunity against other serovars. Although clinical leptospirosis is uncommon in cats, infections with a number of serovars have been reported (Agunloye and Nash, 1996).

Control

Prevention of leptospirosis in domestic animals relies primarily on the use of vaccination. As immunity is serovar specific, vaccines should contain the prevalent leptospiral serovars present in the geographical region in question. Current vaccines are usually bacterins, and immunity must be boosted at least annually. Immunity following natural infection is almost entirely antibody-based but some vaccines appear to induce a T_H1 cell-mediated immune response also (Naiman *et al*., 2001). Recent studies on vaccine development have concentrated on the development of a subunit vaccine. Ideally, such a vaccine would consist of a recombinant protein which conferred cross protection against a number of pathogenic serovars.

Public health aspects

Leptospirosis is an occupational disease of abattoir workers, dairy and pig farmers, veterinary surgeons and those engaged in manual work related to sewage and drainage. It is a zoonosis of increasing importance in those who partake in water sports, in particular those sports that may result in bruising or scratching of the skin, such as whitewater rafting, as injury to the skin facilitates invasion by leptospires.

Borrelia species

Borreliae, which are longer and wider than other spirochaetes, have a similar helical shape (Fig. 36.4). In addition to a linear chromosome, which is unique among bacteria, borreliae possess linear and circular plasmids, some of which appear to be essential for growth and survival of the organism. Although these spirochaetes can cause disease in animals and humans, subclinical infections are also common. Borreliae are transmitted by arthropod vectors. *Borrelia* species of importance in animals, their arthropod vectors, and the diseases which they cause are summarized in Table 36.3.

Usual habitat

Borreliae are obligate parasites in a variety of vertebrate hosts. Although these organisms persist in the environment for short periods, they depend on vertebrate reservoir hosts and arthropod vectors for longterm survival. Associations of

certain *Borrelia* species with particular arthropod vectors and reservoir hosts are important in determining the epidemiology of infections with *Borrelia* species.

Figure 36.4 Spirochaetes of veterinary importance illustrating differences in size and shape: A, *Leptospira*; B, *Brachyspira*; C, *Borrelia*.

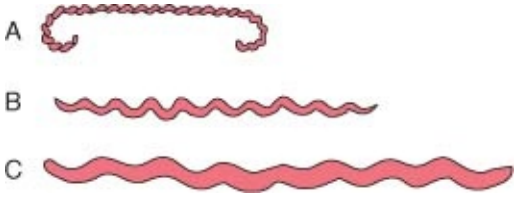


Table 36.3 Tick vectors and natural hosts of *Borrelia* species and associated clinical conditions.

Species	Vector	Reservoir hosts	Clinical conditions
B. burgdorferi sensu lato	*Ixodes* species	Rodents, birds, lizards (some Lyme disease strains)	Arthritic, neurological and cardiac disease in dogs and humans and occasionally in horses, cattle and sheep
B. anserina	*Argas* species	Birds	Fever, weight loss and anaemia in domestic poultry
B. theileri	Many species of ticks	Cattle, sheep, horses	Mild febrile disease with anaemia
B. coriaceae	*Ornithodoros* species	Cattle, deer	Associated with epizootic bovine abortion in USA

Differentiation of *Borrelia* species

Borreliae can be differentiated from other spirochaetes by their morphology, by the low guanine and cytosine content of their genomic DNA and by ecological, cultural and biochemical characteristics. Identification of *Borrelia* species depends mainly on genetic analysis. At least 13 genospecies or genomic groups of *B. burgdorferi* sensu lato have been identified using DNA– DNA hybridization, 16S rRNA sequencing and other molecular techniques. A limited number of these species are pathogenic (Rudenko *et al*., 2009).

Clinical infections

The species of particular veterinary importance are *B. burgdorferi* sensu lato, the cause of Lyme disease in animals and humans, and *B. anserina* which causes avian borreliosis. The significance of two other species, *B. theileri* and *B. coriaceae*, as animal pathogens is uncertain.

Lyme disease

This condition, also known as Lyme borreliosis, was first identified in 1975 following investigation of a cluster of arthritis cases in children near the town of

Old Lyme, Connecticut. The causative agent, a spirochaete, was named *Borrelia burgdorferi*. Several genospecies of *B. burgdorferi* have subsequently been identified in the USA and Europe. Although *B. burgdorferi* sensu stricto is the principal genotype isolated in the USA, genetic diversity among isolates has been documented (Oliver, 1996; Rudenko *et al*., 2009). The recognized species of *B. burgdorferi* sensu lato are presented in Box 36.2.

Epidemiology

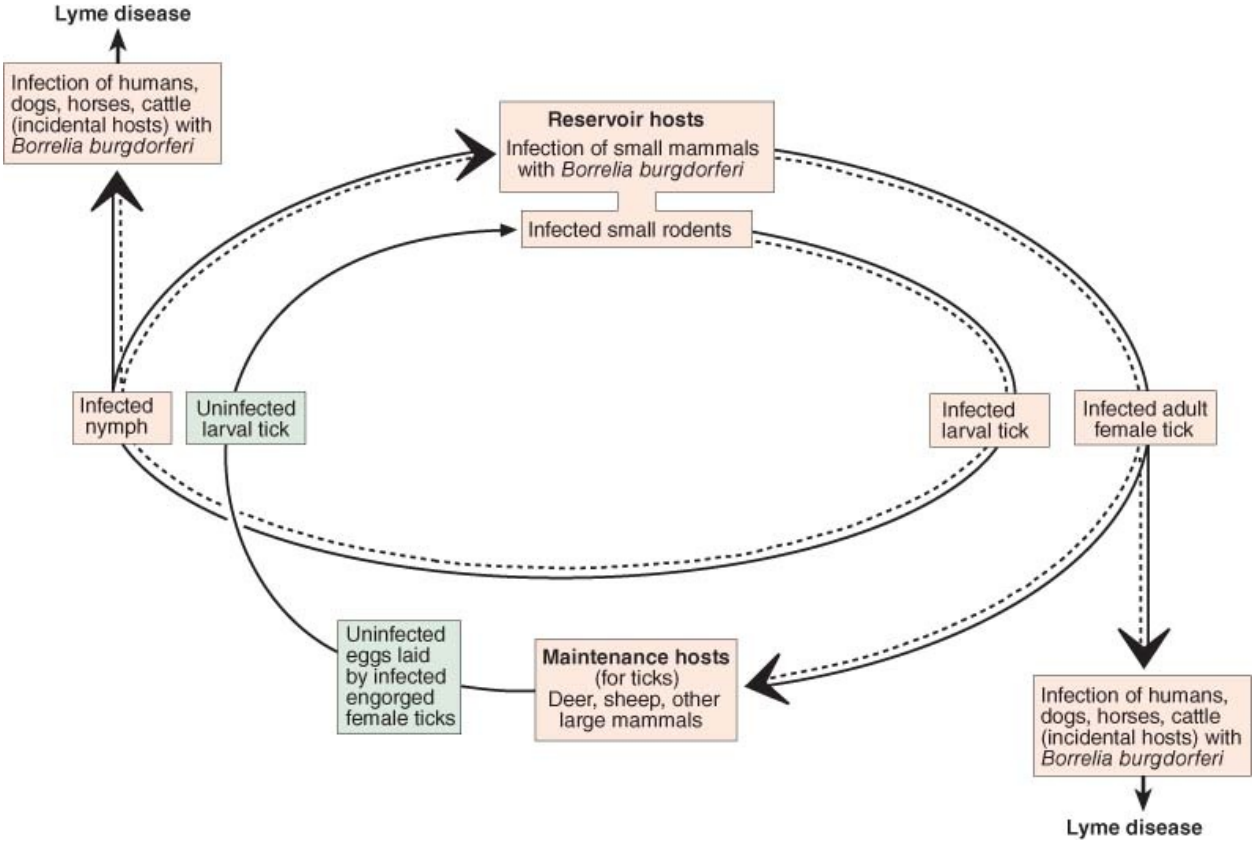
Lyme disease has been reported in humans, dogs, horses and cattle, and infection has been documented in sheep. Ticks are the only competent vectors of *B. burgdorferi* sensu lato. Infection is usually acquired by larval stages of ticks feeding on small rodents. A variety of small wild animals including mice, voles, hedgehogs, lizards and birds can act as reservoir hosts. The spirochaetes persist through nymphal and adult stages of ticks which transmit infection while feeding. Adult ticks feed preferentially on large mammals such as deer and sheep, which are maintenance hosts for the tick population but are unsuitable reservoirs for *B. burgdorferi* sensu lato. The persistence of these pathogenic bacteria in a region is dependent on the presence of suitable reservoir hosts for borreliae and maintenance hosts for ticks. The most common tick vector for *B. burgdorferi* sensu lato in Europe is *Ixodes ricinus*; in central and eastern USA it is *I. scapularis*; on the west coast of the USA it is *I. pacificus*, and in Eurasia it is *I. persulcatus*. The relationships between *B. burgdorferi* sensu lato, its hosts and tick vectors are shown in Fig. 36.5. Transovarial transmission of the spirochaete in the tick, which may occur infrequently, is not of epidemiological importance. Occasional transmission of borreliae from infected incidental hosts to uninfected ticks may occur.

Box 36.2 Currently recognized species within *Borrelia burgdorferi* sensu lato.

- *B. afzelii*
- *B. andersonii*
- *B. bissettii,*
- *‘Borrelia californiensis’*
- *B. burgdorferi sensu stricto*
- *B. garinii*
- *B. japonica*
- *B. lusitaniae*

- *B. sinica*
- *B. spielmanii*
- *B. tanukii*
- *B. turdi*
- *B. valaisiana*

Figure 36.5 The transmission of *Borrelia burgdorferi* sensu lato (broken line) to humans and animals by different stages of *Ixodes* ticks (solid line). The occurrence of Lyme disease, which is often seasonal, relates to periods of tick activity.



Although *B. burgdorferi* sensu lato has been demonstrated in the urine of dogs and horses, infected urine is an unlikely source of infection.

Pathogenesis

Transmission of *B. burgdorferi* sensu lato occurs when an infected tick feeds on a susceptible animal. Prior to feeding, the spirochaetes are restricted to the midgut of the ticks and, following ingestion of blood, they are found in the salivary glands. Following ingestion of blood by the tick, a change occurs in the expression of the outer surface protein (Osp) of the borreliae. Osp A is expressed

in the midgut of the tick and is involved in adherence by the borreliae to the gut of the tick. However, during feeding, Osp A expression is rapidly down-regulated as it is a potent stimulator of the immune response of the mammalian host. In contrast, Osp C is up-regulated and helps to protect the borreliae by binding a complement-inactivating component in tick saliva. Complement-binding proteins are produced by the borreliae themselves also and help in resisting complement-mediated killing by the host.

After entering the bloodstream of a susceptible host, borreliae multiply and are disseminated throughout the body. Organisms may be demonstrated in joints, brain, nerves, eyes and heart. Whether disease is caused by active infection or by host immune responses to the organism is unclear. Persistent infection leading to the induction of cytokines may contribute to the development of lesions (Sprenger *et al*., 1997 ; Straubinger *et al*., 1997; Roberts *et al*., 1998). There may be an association between different genotypes of *B. burgdorferi* and particular clinical syndromes in humans; *B. burgdorferi sensu stricto* is frequently associated with arthritis, *B. garinii* with neurological disease and *B. afzelii* with skin disease (van Dam *et al*., 1993).

Clinical signs

Most infections are subclinical. Serological surveys demonstrate that exposure is common in both animal and human populations in endemic areas (Santino *et al*., 1997).

The clinical manifestations of Lyme disease relate mainly to the sites of localization of the organisms. Clinical disease is reported frequently in dogs. Signs include fever, lethargy, arthritis and evidence of cardiac, renal or neurological disturbance. In the USA, arthritis is a common finding whereas neurological disturbance is the most frequent clinical feature in Europe and Japan. The clinical signs in horses are similar to those in dogs and include lameness, uveitis, nephritis, hepatitis and encephalitis. However, some authors maintain that definitive evidence of clinical Lyme disease in horses is lacking (Butler *et al*., 2005). Lameness in cattle and sheep associated with *B. burgdorferi* sensu lato infection has been reported.

Diagnosis

Laboratory confirmation of Lyme disease may prove difficult because the spirochaetes may be present in low numbers in specimens from clinically

affected animals. In addition, the organism is fastidious in its cultural requirements.

- A history of exposure to tick infestation in an endemic area in association with characteristic clinical signs may suggest Lyme disease.
- Rising antibody titres to *B. burgdorferi* sensu lato along with typical clinical signs are indicative of disease. Because subclinical infections are common in endemic areas, high titres alone are not confirmatory. The ELISA is extensively used for antibody detection; western immunoblotting is sometimes used for confirmation of ELISA results. A quantitative ELISA based on the detection of antibodies to C6 peptide is used in humans and may be useful in dogs also (Littman *et al*., 2006). It has been shown that ELISA techniques based on this antigen may be able to differentiate naturally infected and vaccinated animals (O'Connor *et al*., 2004).
- Immunofluorescence assays may also be used but the results of these methods may be difficult to interpret.
- Culture of borreliae from clinically affected animals is confirmatory. Cultures in Barbour-Stoenner-Kelly medium should be incubated for 6 weeks under microaerophilic conditions and should be carried out in specialized laboratories.
- Low numbers of borreliae can be detected in samples by PCR techniques. These techniques are most useful in early cases of Lyme disease in humans in which 50 to 70% of skin biopsies of erythema migrans lesions may be positive (Wilske *et al*., 2007). Similar results have been obtained with synovial fluid samples. As domestic animals do not usually show lesions of erythema migrans, PCR-based tests may be less useful than in humans. PCR techniques can also be used for identifying genospecies and for epidemiological investigations (Kurtenbach *et al*., 1998).

Treatment and control

- Acute Lyme disease responds to treatment with amoxicillin and oxytetracycline. In chronic disease, prolonged or repeated courses of treatment may be required.
- Acaricidal sprays, baths or dips should be used to control tick infestation. Where feasible, tick habitats such as rough brush and scrub should be cleared.
- Prompt removal of ticks from companion animals may prevent infection.

However, because some tick species can transmit spirochaetes shortly after attachment, it cannot be assumed that daily removal of ticks will prevent infection (Korenberg and Moskvitina, 1996).

- A number of vaccines, including whole cell bacterins and recombinant subunit vaccines, are commercially available for use in dogs in some countries. An OspA recombinant vaccine stimulates the production of antibodies which are able to kill the borreliae in the gut of the tick and thus prevent infection of the host. However, the benefit of vaccinating dogs with currently available vaccines is disputed (Littman, 2003 ; Littman *et al*., 2006).

Public health aspects

Lyme disease is an important tick-borne infection of humans. Clinical signs include skin rash localized at the site of tick attachment, followed, in the absence of treatment, by arthritis, muscle pains, and cardiac and neurological abnormalities. Infection is often acquired by walking in endemic areas during periods of tick activity. Dogs, cats and farm animals may act as transport hosts for infected ticks thereby exposing humans to the risk of infection.

Avian spirochaetosis

This acute disease of birds, caused by *Borrelia anserina*, can result in significant economic loss in flocks in tropical and subtropical regions where the disease is endemic. Chickens, turkeys, pheasants, ducks and geese are susceptible to infection. Soft ticks of the genus *Argas* frequently transmit the disease. However, when there is contact between susceptible birds and infected material such as blood, tissues or excreta, transmission may occur. Because *B. anserina* survives poorly in the environment and for a limited time in infected birds, *Argas* ticks are important reservoirs of the organisms. The borreliae survive trans-stadial moulting in ticks and can be transmitted transovarially between tick generations. Outbreaks of avian spirochaetosis coincide with periods of peak tick activity during warm, humid seasons. Morbidity and mortality are low in flocks continually exposed to infection. The disease is characterized by fever, marked anaemia and weight loss. Paralysis may develop as the disease progresses. Immunity, which follows recovery, is serotype specific. Several serotypes may be present in a particular region.

Diagnosis can be confirmed by demonstration of the spirochaetes in buffy coat

smears using dark-field microscopy. Blood or tissue smears can also be examined using immunofluorescence. Giemsa - stained smears or silver impregnation techniques can be used to demonstrate the borreliae in tissues. The organisms are usually isolated by inoculating embryonated eggs or young chicks with infected blood or homogenized tissues. PCR techniques have been applied for the characterization of *B. anserina* strains (Ataliba *et al*., 2007). Treatment with antibiotics is effective. Inactivated vaccines and tick eradication are the main control measures.

Brachyspira and *Treponema* species

Five species of brachyspires have been isolated from pigs, namely *Brachyspira hyodysenteriae, B. pilosicoli, B. intermedia, B. innocens* and *B. murdochii*, the latter two considered to be non-pathogenic. *Brachyspira intermedia* has been associated with intestinal spirochaetosis in chickens and pigs. *Treponema paraluiscuniculi* is associated with vent disease in rabbits, and a number of *Treponema* species have been identified in lesions of bovine digital dermatitis and contagious ovine digital dermatitis. *Brachyspira* and *Treponema* species have six to fourteen spirals and are 0.1 to 0.5 µm in width (Fig. 36.4).

Usual habitat

Pathogenic *Brachyspira* species are found in the intestinal tract of both clinically affected and normal pigs. Carrier pigs can shed *B. hyodysenteriae* for up to 3 months and are the principal source of infection for healthy pigs. *Brachyspira pilosicoli* colonizes the intestinal tract of chickens, dogs, wild birds, rodents and non-human primates and is recognized as an important cause of colitis and typhilitis in pigs and poultry.

Differentiation of *Brachyspira* species

The differentiation of *B. hyodysenteriae* from other intestinal spirochaetes is based on its pattern of haemolysis on blood agar. Tests for detecting indole production or the hydrolysis of hippurate are also useful diagnostically (Table 36.4). Restriction endonuclease analysis, restriction fragment length polymorphism, ribotyping using 16S rRNA analysis, PCR-based assays and multilocus enzyme electrophoresis have been developed both for differentiating species and for distinguishing strains of organisms within species. A scheme

based on multilocus sequence typing has been developed to examine the diversity and genetic relatedness of *Brachyspira hyodysenteriae* isolates. *Brachyspira hyodysenteriae* strains can also be allocated to several serogroups and serotypes.

Table 36.4 Laboratory differentiation of *Brachyspira* species isolated from pigs.

Species	Laboratory tests		
	Haemolysis	Indole spot test	Hippurate hydrolysis
B. hyodysenteriae	Strong	+	–
B. pilosicoli	Weak	–	+
B. innocens	Weak	–	–

Pathogenesis

Most information on the pathogenesis of *Brachyspira* species derives from studies of *B. hyodysenteriae*. Motility in mucus is an essential virulence factor of this organism; mutant strains with altered motility are less capable of colonizing the pig intestine (Kennedy *et al*., 1997). Colonization may be enhanced by factors in mucus with chemotactic activity for the organisms. Factors with such chemotactic activities have been demonstrated *in vitro* (Kennedy and Yancey, 1996). Haemolytic activity, demonstrated *in vitro*, correlates with pathogenicity, and six genes encoding haemolytic and cytotoxic activity have been identified (Muir *et al*., 1992; ter Huurne *et al*., 1994; Hampson and Ahmed, 2009). In addition, proteases produced by *B. hyodysenteriae* may be involved in disruption of the colonic mucosa (Hampson and Ahmed, 2009). Dietary and immune factors are important in the development of disease (Jacobson *et al*., 2004; Jonasson *et al*., 2004; Thomsen *et al*., 2007).

The pathogenesis of infection with *B. pilosicoli* differs from that of *B. hyodysenteriae* in that attachment of the spirochaetes to the intestinal mucosa appears to be important. Attachment of *B. pilosicoli* to the epithelial cells of the colonic mucosa leads to disruption of function with resultant cell shedding and oedema.

Clinical infections

Infections with *Brachyspira* species are of importance in pigs. *Brachyspira*

hyodysenteriae, the cause of swine dysentery, and *B. pilosicoli*, the cause of porcine intestinal spirochaetosis, are recognized pathogens. There is evidence that *B. intermedia* may be associated with porcine spirochaetal colitis, but this has not been confirmed experimentally. Pigs acquire infection through exposure to contaminated faeces. The disease usually spreads slowly through a herd, affecting only one or two pens at a time. Dogs, rats, mice and flies may act as transport hosts for the spirochaetes. Mice populations can maintain *B. hyodysenteriae*. Although strains of *B. pilosicoli* have been found in many species including humans, dogs, chickens and pheasants, cross-infection between species has not been clearly demonstrated. Colonic spirochaetosis, which causes wet faeces and poor production in chicken flocks, may be underdiagnosed (Smith, 2005). *Brachyspira* species can survive in the environment for limited periods only if protected from desiccation. *Brachyspira hyodysenteriae* can persist for several weeks in moist faeces and for at least 3 days in slurry.

Clinical signs

The pathogenic *Brachyspira* species and the clinical conditions associated with infection are presented in Table 36.5. Infection with *B. hyodysenteriae* causes dysentery which is most often encountered in weaned pigs from 6 to 12 weeks of age. Affected pigs lose condition and become emaciated. Appetite is decreased and thirst may be evident. During recovery, there may be large amounts of mucus in the faeces. Although mortality is low, reduced weight gain due to poor food conversion causes major economic loss.

Brachyspira pilosicoli was identified in 1996 as the cause of porcine intestinal spirochaetosis (Trott *et al*., 1996). Previously, enteric disease had been produced experimentally by infecting pigs with a weakly haemolytic spirochaete (Taylor *et al*., 1980). The clinical signs in porcine intestinal spirochaetosis are similar to those of swine dysentery but are less severe. Diarrhoea contains mucus rather than blood. Reduced feed conversion efficiency with poor weight gains has a major effect on production.

Table 36.5 Clinical conditions associated with infection caused by *Brachyspira* species.

Species	Clinical conditions
B. hyodysenteriae	Swine dysentery
B. pilosicoli	Intestinal spirochaetosis of pigs, dogs, birds and humans
B. intermedia	Implicated in porcine spirochaetal colitis

Diagnosis

- History, clinical signs and gross lesions may indicate swine dysentery.
- Blood agar with added antibiotics is used for the culture of *Brachyspira* species. Cultures are incubated anaerobically at 42°C for at least 3 days. Complete haemolysis is present around colonies of *B. hyodysenteriae*; other enteric spirochaetes are weakly haemolytic (Table 36.4).
- Definitive identification can be made using immunofluorescence, DNA probes or biochemical tests (Table 36.4).
- Serological tests such as ELISA can be used to investigate infection in herds.
- PCR-based techniques have been developed and may be useful for direct detection of *B. hyodysenteriae* in clinical samples and for laboratory confirmation of isolates. Real-time PCR techniques have been developed also (Akase *et al*., 2009).

Treatment and control

Medication of drinking water is a useful method of treatment. Drugs commonly used include tiamulin, lincomycin and the nitroimidazoles. Improved hygiene, medication of feed and alteration of the diet may assist in controlling infection. Depopulation, thorough cleaning and disinfection of premises and strict rodent control are required for eradication of the disease. Whole cell bacterin vaccines are available for control of swine dysentery in some countries but their efficacy is uncertain.

Treponema species

A number of *Treponema* species have been isolated from cases of bovine digital dermatitis and from the recently described condition of contagious ovine digital dermatitis. Several species may be present within the same herd and even within the same individual animal (Evans *et al*., 2009). The pathogenic mechanisms leading to development of lesions are poorly understood but spirochaetes can be demonstrated deep in infected tissue. The involvement of *Treponema* species in digital lameness of cattle and sheep is described in greater detail in Chapter 91.

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Chapter 37

Pathogenic anaerobic non-spore-forming Gram-negative bacteria

Many non-spore-forming, anaerobic, Gram-negative bacteria cause opportunistic mixed infections, often in association with facultative anaerobes. Synergistic interactions between the organisms in these mixed infections are common. *Fusobacterium* species and bacteria from the genera *Prevotella*, *Dichelobacter* and *Porphyromonas* constitute more than 50% of the anaerobic organisms isolated from these infections.

Usual habitat

Non-spore-forming, Gram-negative anaerobes are often found on mucous membranes of animals and humans, particularly in the digestive and urinogenital tracts. They are excreted in the faeces and they can survive for short periods in the environment. *Dichelobacter nodosus*, a primary pathogen of the epidermal tissues of the hoof region of ruminants, survives for less than 7 days in the environment.

Diagnostic procedures

- In order to ensure that isolates of anaerobes are aetiologically significant, specimens for isolation procedures should be obtained by direct sampling from discharges or lesions and by suprapubic puncture in urinary infections.
- Specimens should be processed promptly after collection. Commercial kits and transport media are available for specimens from suspected anaerobic infections. In the core of a tissue specimen over 2 cm^3, an anaerobic microenvironment is usually maintained. A fluid sample, collected in a syringe, remains suitable for anaerobic culture if air is expelled from the syringe and the needle is plugged.

- Anaerobic jars with an atmosphere of hydrogen and 10% CO_2 are used for incubating cultures at 37°C for up to 7 days. Anaerobic bags or pouches are available also and are suitable for culturing small numbers of samples.
- Blood agar (5 to 10% ruminant red blood cells) supplemented with yeast extract, vitamin K and haemin, is used for the isolation of anaerobes. Selective media can be prepared by adding appropriate antimicrobial agents. Media must be pre-reduced by storing them in an anaerobic atmosphere for at least 6 hours before inoculation. Special media for the isolation of anaerobes are available commercially.
- Liquid media, such as cooked meat broth or thioglycollate medium supplemented with vitamin K and haemin, are useful for subculturing and may be used as a back-up source of culture material but are unsuitable for primary isolation.
- Special selective media are required for the isolation of *Dichelobacter nodosus* from ruminant footrot (Skerman, 1989). In some media formulations, powdered ovine hoof is added to promote enhanced growth.
- Molecular methods based on PCR amplification or nucleic acid probes have been developed for the detection of anaerobic organisms in clinical specimens, particularly for *Dichelobacter nodosus*, as footrot is a disease of major economic importance in sheep (La Fontaine *et al*., 1993; Zhou *et al*., 2001).

Key points

- Gram-negative, anaerobic bacteria
- Endospores not produced
- Enriched media required for growth
- Majority are commensals on mucosal surfaces, principally in the alimentary tract
- Opportunistic pathogens
- Synergism with other bacteria in mixed infections
- *Dichelobacter nodosus* produces foot rot in sheep in association with other pathogens

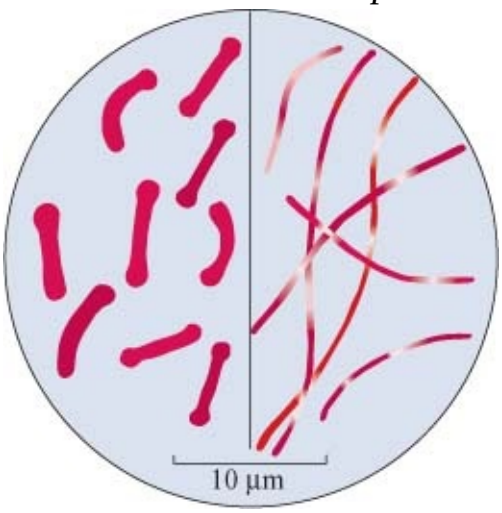
Differentiation of the non-spore-forming Gram-negative anaerobes

Non-spore-forming Gram-negative anaerobes are differentiated on the basis of

bacterial morphology, colonial appearance, antibiotic susceptibility testing and fatty acid production.

- Rods of *Dichelobacter nodosus* are thick, straight or slightly curved, up to 6 µm long and bulge at one or both ends. *Fusobacterium necrophorum* has irregularly staining, long, non-branching filamentous forms (Fig. 37.1).
- Colonies of Gram-negative anaerobes usually have a foetid or putrid odour due to volatile fatty acid production.
  – The appearance *of Dichelobacter nodosus* colonies is variable (Stewart *et al*., 1986). Colonies of virulent strains from lesions of ovine footrot usually have a dark central zone, a pale granular middle zone and a spreading irregular periphery with a ground glass appearance.

Figure 37.1 Straight or curved rods of *Dichelobacter nodosus* (left) showing characteristic swellings at one or both ends and slender, non-branching filaments of *Fusobacterium necrophorum* (right) which tend to stain irregularly.



– Colonies of *Fusobacterium necrophorum* are grey, round and shiny. Some isolates are haemolytic.

– Colonies of many *Prevotella* species and *Porphyromonas* species which become darkly pigmented after incubation for 5 days, may appear red under UV light.

- Antibiotic susceptibility testing, biochemical tests and gas liquid chromatography are used for more accurate identification of species.
- Methods for detecting virulent strains of *D. nodosus*, reviewed by Wani and Samanta (2006), include:
  – Elastin and gelatin gel tests for detection of protease activity.
  – ELISA, using monoclonal antibodies against *D. nodosus* proteases and

other antigens.

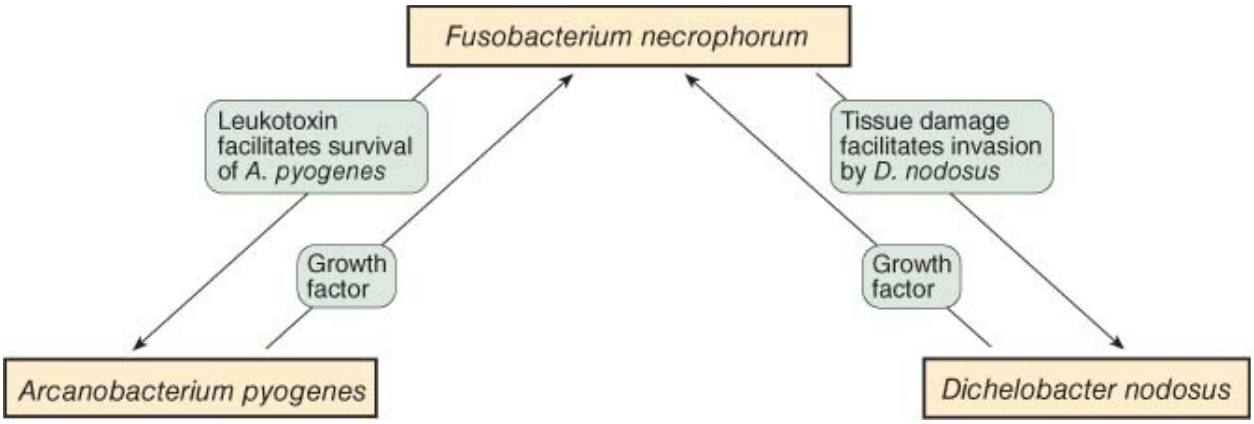
– Polymerase chain reaction techniques for detection of virulence-specific genes.

– Molecular tests are also used for grouping strains of *D. nodosus*. PCR techniques using serogroup-specific primers have largely replaced conventional serogrouping (Wani and Samanta, 2006).

Pathogenesis and pathogenicity

Non-spore-forming anaerobes usually exert pathogenic effects when anatomical barriers are breached allowing invasion of underlying tissues. They replicate only at low or negative reduction potentials (*Eh*). Most of the bacteria involved in opportunistic infections produce superoxide dismutase which allows them to survive in oxygenated tissues until the *Eh* reaches levels favouring their growth. Tissue trauma and necrosis followed by multiplication of facultatively anaerobic bacteria can lower *Eh* levels to a range suitable for the proliferation of non-spore-forming anaerobes. Most infections involving these organisms are mixed. Two or more bacterial species, interacting synergistically, may produce lesions which the individual organisms cannot produce. A relevant example of this type of synergism is the production of a heat-labile factor by *Arcanobacterium pyogenes* which stimulates *F. necrophorum* replication (Smith *et al*., 1989). In turn, *F. necrophorum* produces a leukotoxin which correlates with the strain virulence and aids survival of *A. pyogenes* (Emery *et al*., 1984). Synergism between *F. necrophorum* and *Dichelobacter nodosus* is important in the pathogenesis of ruminant pedal lesions (Fig. 37.2). In this instance, *F. necrophorum* facilitates tissue invasion by *D. nodosus* and is itself stimulated by a growth factor elaborated by *D. nodosus*.

Figure 37.2 The synergistic interaction of *Fusobacterium necrophorum* with *Arcanobacterium pyogenes* and with *Dichelobacter nodosus* in the development and progression of foot lesions in ruminants.



Two subspecies of *F. necrophorum* are recognized, designated *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme*. *Fusobacterium necrophorum* subsp. *necrophorum* has greater haemolytic activity and is more virulent than *F. necrophorum* subsp. *funduliforme*. Strains of *F. necrophorum* which produce oral, paraoral and necrotizing pneumonic infections in horses have been classified as a new species, *F. equinum* (Dorsch *et al*., 2001; Tadepalli *et al*., 2008). However, it is unclear at present whether all fusobacterial infections in horses are caused by this species or whether strains of *F. necrophorum* may be involved also.

Fusobacterium necrophorum produces a wide range of virulence factors in addition to leukotoxin, including haemagglutinins, haemolysins, dermotoxin, other extracellular enzymes and lipopolysaccharide (LPS) which contribute to the development of ruminal lesions. Of these, the most important virulence determinants are leukotoxin, haemagglutinins and LPS (Tadepalli *et al*., 2009). The haemagglutinins function as adhesins and promote adherence to, and invasion of, ruminal epithelial cells. The leukotoxin is active against ruminant neutrophils in particular but may also act against macrophages and hepatocytes. In contrast, it is only moderately toxic for equine neutrophils and has little or no activity against neutrophils of pigs and rabbits. Endotoxin from LPS is important in the genesis of hepatic abscesses and induces an intense neutrophilia.

Characteristics of *D. nodosus* which correlate with its ability to damage tissues include the production of thermostable proteases and elastase and the presence of agarolytic activity on agar-based media containing powdered hoof. The production of Type IV fimbriae, encoded by the *fimA* gene, is central to virulence and these highly immunogenic structures form the basis of classification of *D. nodosus* strains into 10 serogroups. Organisms belonging to several serogroups may be involved in disease production on any given farm.

Box 37.1 Gram-negative, non-spore-forming anaerobes which have been implicated in infections in domestic animals.

- *Bacteroides fragilis*
- Other *Bacteroides* species
- *Brachyspira hyodysenteriae*
- *Dichelobacter nodosus*
- *Fusobacterium equinum*
- *F. necrophorum*
- *F. nucleatum*
- *F. russii*
- Other *Fusobacterium* species
- *Porphyromonas asaccharolytica*
- *Porphyromonas levii*
- *Prevotella heparinolytica*
- *P. melaninogenica*
- Spirochaetes (unclassified)

Clinical infections

The non - spore - forming, Gram - negative anaerobic bacteria that have been implicated in infections in domestic animals are listed in Box 37.1. *Brachyspira hyodysenteriae* is discussed in Chapter 36.

Fusobacterium necrophorum is considered to be the primary pathogen in a number of disease conditions in farm animals (Table 37.1). Mixed bacterial infections are commonly implicated in foot lesions in domestic ruminants and pigs (Table 37.2). Pedal bacterial infections in farm animals, such as footrot and foot abscessation, are discussed in detail in Chapter 91. Mixed infections with non - spore - forming anaerobes are also present in aspiration pneumonias and in bovine traumatic reticuloperitonitis and pericarditis. In addition, many inflammatory conditions in domestic carnivores are caused by non-specific mixed anaerobic pathogens.

Table 37.1 Disease conditions of farm animals in which *Fusobacterium necrophorum* plays a primary role.

Cattle	Calf diphtheria	Rough feed producing mucosal damage
	Post-partum metritis	Dystocia
	Hepatic abscessation	Sudden dietary change leading to acidosis and rumenitis

	Black spot of teat	Trauma to region adjacent to teat sphincter
Horses	Thrush (hoof)	Poor hygiene and wet housing conditions
	Necrobacillosis of lower limbs	Poor hygiene
Pigs	Bull nose	Trauma to nasal mucosa

Table 37.2 Foot conditions in farm animals associated with mixed infections including anaerobic non-spore-forming bacteria[a].

Species	Disease condition	Bacteria implicated
Sheep	Interdigital dermatitis	*Fusobacterium necrophorum*, *Dichelobacter nodosus* (benign strains)
	Heel abscess and lamellar suppuration	Mixed anaerobic flora including *Arcanobacterium pyogenes*[b], *Fusobacterium necrophorum* and other bacteria
	Footrot	*Dichelobacter nodosus*, *Fusobacterium necrophorum*, *Arcanobacterium pyogenes*[b], unidentified spirochaetes
Cattle	Interdigital necrobacillosis (foul-in-the-foot)	*Fusobacterium necrophorum*, *Porphyromonas levii*
	Interdigital dermatitis	*Dichelobacter nodosus*, *Fusobacterium necrophorum*, *Prevotella* species, spirochaetes
	Digital dermatitis	*Treponema* species
Pigs	Foot abscess in young pigs and bush foot (lamellar suppuration) in older animals	Mixed anaerobes

a, bacterial and viral infections affecting the feet of cattle, sheep and pigs are reviewed in Chapter 91.

b, facultatively anaerobic.

Calf diphtheria

This condition usually presents as necrotic pharyngitis or laryngitis in calves under 3 months of age. The aetiological agent, *F. necrophorum*, can enter through abrasions in the mucosa of the pharynx or larynx often caused by ingestion of coarse feed. Clinical signs include fever, depression, anorexia, excessive salivation, respiratory distress and a foul smell from the mouth. Untreated calves may develop a fatal necrotizing pneumonia. Treatment with potentiated sulphonamides or tetracyclines early in the course of the disease is usually effective.

Bovine liver abscess

Hepatic abscessation in cattle, secondary to rumenitis, is encountered most commonly in feedlot animals. The feeding of rations high in carbohydrates and the resulting rapid intraruminal fermentation can lead to the development of rumenitis, ulcers and abscesses in the ruminal wall. High levels of lactate produced during ruminal acidosis may favour the growth of *F. necrophorum* as lactic acid is a major substrate for the organism. *Fusobacterium necrophorum* is

sensitive to pH, and the number of organisms in ruminal contents decreases markedly when the pH drops below pH 5.0. However, the pH of the ruminal wall microenvironment is likely to remain close to pH 7.0 even during ruminal acidosis, thus allowing survival and multiplication of the organism. *Fusobacterium necrophorum* together with other anaerobes and *Arcanobacterium pyogenes* invade the tissues of the ruminal wall, and occasionally emboli, which reach the liver via the portal vein, initiate abscess formation. Affected cattle rarely show clinical signs, and lesions are usually detected at slaughter; nevertheless, production losses in terms of reduced weight gain and feed conversion efficiency may be considerable. Management in feedlots should be aimed at reducing the incidence of rumenitis. Although chlortetracycline, tylosin or other antimicrobial agents administered in feed during the finishing period can reduce the prevalence of liver abscesses, this practice is no longer recommended owing to concerns about the development of antimicrobial resistance. Vaccines are available in some countries to protect against liver abscesses but their efficacy appears to be questionable (Fox *et al*., 2009).

Necrotic rhinitis of pigs

This sporadic condition, primarily affecting young pigs, is characterized by suppuration and necrosis of the snout as a result of infection with *F. necrophorum*, often in association with other anaerobes. These organisms enter through abrasions in the nasal mucosa. Signs include swelling of the face, sneezing and a foulsmelling nasal discharge. In chronic infections, involvement of the nasal and facial bones can result in permanent facial deformity (‘bull nose’). Potentiated sulphonamides administered early in the course of the infection may be beneficial.

Thrush of the hoof

This necrotic condition of the equine hoof is associated with poor hygiene, wet conditions and lack of regular cleaning of the hooves. Infection with *F. necrophorum*, secondary to hoof damage, results in a localized inflammatory response. Thrush, which commonly affects the hind feet, is characterized by a foul-smelling discharge in the sulci close to the frog. The aim of therapy is to encourage regeneration of the frog by providing dry, clean stabling, regular attention to the hooves and exercise.

Black spot of bovine teats

Black spot or black pox of the teat orifice and sphincter of dairy cows presents as a localized area of necrosis with black scab formation due to invasion by *F. necrophorum*. The condition can contribute to stenosis of the sphincter and may predispose to mastitis.

Other conditions

A number of other conditions have been reported associated with infection by non-spore-forming anaerobic bacteria. These include a specific syndrome of bovine necrotic vulvovaginitis reported in association with *Porphyromonas levii* in Israeli dairy herds (Elad *et al*., 2004; Friedgut and Stram, 2006).

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Chapter 38

Mycoplasmas

The mycoplasmas are microorganisms in the class *Mollicutes*. Of the nine genera in this class, five contain species of veterinary interest (Fig. 38.1). The genus *Mycoplasma*, in which there are more than 100 species, contains most of the animal pathogens. The first mycoplasma identified in 1890 was *Mycoplasma mycoides* subspecies *mycoides*, the cause of contagious bovine pleuropneumonia. Similar types of mycoplasmas which were subsequently identified were called pleuropneumonia-like organisms (PPLO).

Mycoplasmas, the smallest prokaryotic cells capable of self-replication, are pleomorphic organisms ranging from spherical (0.3 to 0.9 μm in diameter) to filamentous (up to 1.0 μm long). Because they cannot synthesize peptidoglycan or its precursors, they do not possess rigid cell walls but have flexible, triple-layered outer membranes. Their flexibility allows them to pass through bacterial membrane filters of pore sizes from 0.22 μm to 0.45 μm. Mycoplasmas are susceptible to desiccation, heat, detergents and disinfectants. However, they are resistant to antibiotics such as penicillin which interfere with the synthesis of bacterial cell walls. Based on 5S rRNA sequence analyses, the mycoplasmas have been shown to be linked phylogenetically to Gram-positive bacteria such as *Clostridium* species which have low guanine–cytosine content in their DNA. They require enriched media for growth, characteristically forming umbonate microcolonies when illuminated obliquely and microcolonies with a ‘fried egg’ appearance in transmitted light (Fig. 38.2). The dense central zone is due to extension of the microcolony into the agar (Fig. 38.3). Mycoplasmas have relatively small genomes (approximately 800 genes) as they have lost the genes required for many metabolic processes. They are dependent on the host cell for essential nutrients that they cannot produce and are fastidious in their growth requirements when cultured *in vitro*.

Key points

- Smallest free-living prokaryotic microorganisms

- Possess triple-layered limiting membranes but lack cell walls
- Do not stain by the Gram method
- Highly pleomorphic, filterable, plastic forms
- Susceptible to desiccation and disinfectants
- Microcolonies have a ‘fried-egg’ appearance
- Most are facultative anaerobes
- Do not replicate in the environment
- Most are host-specific
- *Mycoplasma* and *Ureaplasma* contain species of veterinary importance
- *Mycoplasma* species cause a wide range of diseases in animals, including contagious bovine pleuropneumonia

Most mycoplasmas are facultative anaerobes and some grow optimally in an atmosphere of 5 to 10% CO_2. Non-pathogenic anaerobic mycoplasmas are found in the rumens of sheep and cattle. The genera *Mycoplasma* and *Ureaplasma* contain animal pathogens, with the latter being associated principally with reproductive disorders. Recent changes in the classification of the mycoplasmas have resulted in some organisms previously classified in the rickettsial group being incorporated into the genus *Mycoplasma*. These organisms are known as the haemotropic mycoplasmas or by the trivial name ‘haemoplasmas’ as they parasitize red blood cells. They previously belonged to the genera *Haemobartonella* and *Eperythrozoon* in the family *Anaplasmataceae*. The major diseases associated with infection by *Mycoplasma* species are summarized in Table 38.1. Other clinical conditions of lesser economic significance are listed in Table 38.2.

Figure 38.1 Families and genera of veterinary interest in the class Mollicutes, members of which may be isolated from clinical specimens. *Mycoplasma* and *Ureaplasma* are the only genera of pathogenic significance in domestic animals and humans.

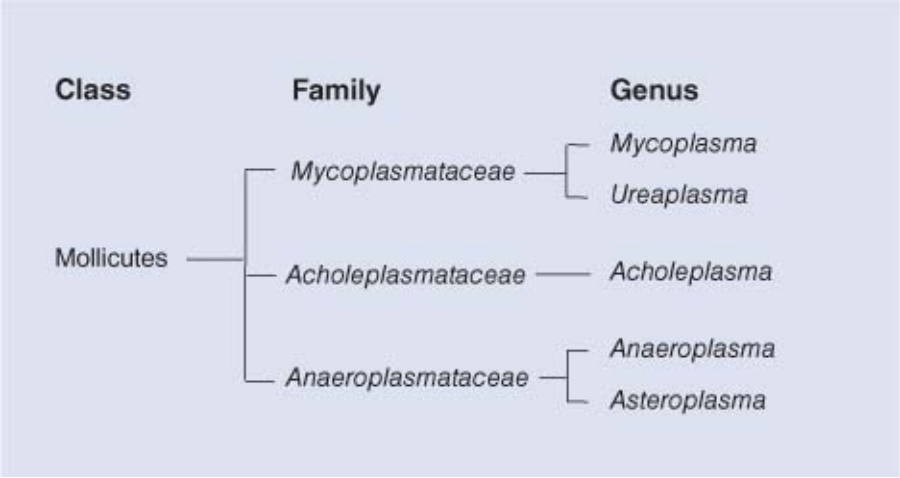


Figure 38.2 The appearance of mycoplasma microcolonies in oblique illumination (A) and in transmitted light (B). When illuminated obliquely, the microcolonies have an umbonate appearance. They have a 'fried-egg' appearance in transmitted light.

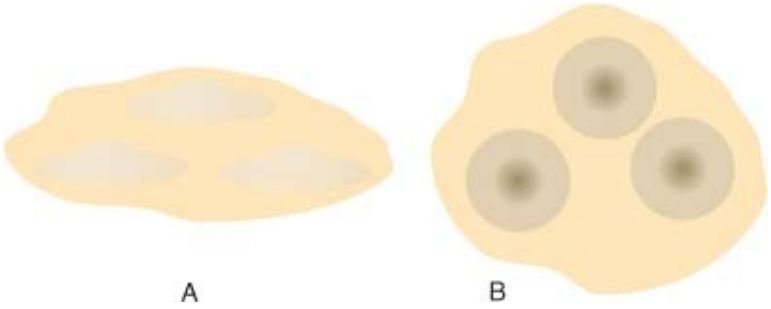
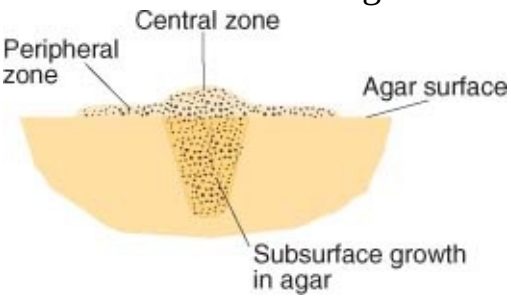


Figure 38.3 A section through a mycoplasma microcolony on agar showing surface and subsurface growth.



Usual habitat

Mycoplasmas are found on mucosal surfaces of the conjunctiva, nasal cavity, oropharynx and intestinal and genital tracts of animals and humans. Some species have tropisms for particular anatomical sites while others are found in many locations. The haemotropic mycoplasmas are found on the surface of red

blood cells. In general, they are host-specific although some species have a broad host range. It is suggested that these organisms may cross the species barrier more often than previously thought and this may be of significance in immunocompromized or stressed animals and humans (Pitcher and Nicholas, 2005). Many mycoplasmas are non-pathogenic and constitute part of the normal flora of their host. Mycoplasmas survive for short periods in the environment.

Differentiation of the mycoplasmas

Mycoplasmas are differentiated by their host specificity, colonial morphology, requirement for cholesterol and biochemical reactivity (Table 38.3). They can be identified by serological methods. As biochemical and serological confirmation of identity is difficult and these tests are usually performed in specialized laboratories, PCR techniques are now used as rapid, specific and relatively easy-to-use identification tests.

- *Mycoplasma* species and *Ureaplasma* species require enriched media containing animal protein, a sterol component and a source of DNA or adenine dinucleotide. Commercially available mycoplasma agar or broth media (often heart infusions) are supplemented with 20% horse serum and yeast extract providing amino acids and vitamins. In addition, penicillin is used to inhibit Gram-positive bacteria, and thallous acetate is incorporated to inhibit Gram-negative bacteria and fungi. Media are buffered at pH 7.3 to 7.8 for *Mycoplasma* species and at pH 6.0 to 6.5 for *Ureaplasma* species. For culturing ureaplasmas, urea is added to the medium and thallous acetate, which is toxic for these organisms, is omitted. *Acholeplasma* species occasionally grow as contaminants on mycoplasma media.
- Colonial morphology:

  – When examined microscopically at low magnification, unstained microcolonies of *Mycoplasma* species are 0.1 to 0.6 mm in diameter and have a ‘fried-egg’ appearance (Fig. 38.2). Some species produce colonies up to 1.5 mm in diameter which can be seen without magnification.

  – Colonies of *Ureaplasma* species are usually 0.02 to 0.06 mm in diameter and often lack a typical peripheral zone. Because their colonies are tiny, these organisms were formerly referred to as T- mycoplasmas.

  – Dienes stain facilitates recognition of microcolonies by staining the

central zone dark blue and the peripheral zone a lighter blue.

– Microcolonies of *Mycoplasma* species require differentiation from colonies of bacterial L-forms. Because bacterial L-forms lack a rigid cell wall, they assume shapes resembling mycoplasmas. However, L-forms can revert to their normal bacterial shape and produce cell walls and typical bacterial colonies when subcultured on non-inhibitory media.

- *Mycoplasma* species and *Ureaplasma* species require sterols for growth and this is reflected in their sensitivity to inhibition by digitonin. As *Acholeplasma* species are sterol-independent, they are resistant to inhibition by digitonin. In the digitonin sensitivity test, a filter paper disc impregnated with digitonin is placed on medium inoculated with the isolate. A zone of growth inhibition exceeding 5 mm around the disc indicates sensitivity to digitonin.
- Biochemical tests are carried out in liquid or solid media with the appropriate reagent added. Table 38.4 indicates the biochemical reactions of the main pathogenic mycoplasmas of sheep and goats. Unlike *Ureaplasma* species which produce urease, *Mycoplasma* species do not metabolize urea.
- Immunological tests, using specific antisera produced against each pathogenic species, are used for specific identification. Growth inhibition tests, in which filter paper discs containing specific antisera are placed on an agar surface seeded with the mycoplasma under test, are used for diagnosis. A zone of growth inhibition up 8 mm wide develops around the disc containing homologous antiserum. Fluorescent antibody staining of individual microcolonies can also be used for identification.
- PCR tests for differentiation of most mycoplasmas pathogenic for animals have now been described including differentiation of wild type and vaccinal strains in some instances, such as differentiation of the T1 vaccine strains for contagious bovine pleuropneumonia (Lorenzon *et al*., 2000).

Table 38.1 *Mycoplasma* species of veterinary significance, the disease conditions which they cause and their geographical distribution.

Mycoplasma species	Hosts	Disease conditions	Geographical distribution
M. mycoides subsp. *mycoides* (small colony type)	Cattle	Contagious bovine pleuropneumonia	Endemic in parts of Africa, Middle East, Asia; sporadic outbreaks in some European countries
M. bovis	Cattle	Mastitis, pneumonia, arthritis	Worldwide
M. agalactiae	Sheep, goats	Contagious agalactia	Parts of Europe, northern Africa, western Asia
M. capricolum subsp. *capripneumoniae*	Goats	Contagious caprine pleuropneumonia	Northern and eastern Africa, Turkey
M. capricolum subsp. *capricolum*	Sheep, goats	Septicaemia, mastitis, polyarthritis, pneumonia	Africa, Europe, Australia, USA
M. mycoides subsp. *capri* includes strains previously classified as *M. mycoides* subsp. *mycoides* (large colony type)	Goats, sheep	Septicaemia, pleuropneumonia, arthritis, mastitis	Parts of Asia, Africa, Europe, Australia, Middle East, North America, India
M. hyopneumoniae	Pigs	Enzootic pneumonia	Worldwide
M. hyorhinis	Pigs (3–10 weeks of age)	Polyserositis	Worldwide
M. hyosynoviae	Pigs (10–30 weeks of age)	Polyarthritis	Worldwide
M. gallisepticum	Chickens Turkeys	Chronic respiratory disease Infectious sinusitis	Worldwide
M. synoviae	Chickens, turkeys	Infectious synovitis	Worldwide
M. meleagridis	Turkeys	Airsacculitis, bone deformities, reduced hatchability and growth rate	Worldwide
M. haemofelis	Cats	Feline infectious anaemia	Worldwide

Table 38.2 Clinical conditions of minor economic importance in animals associated with *Mycoplasma* and *Ureaplasma* species.

Hosts	Pathogen	Clinical conditions
Cattle	*Mycoplasma alkalescens*	Mastitis
	M. bovigenitalium	Seminal vesiculitis, vaginitis, mastitis
	M. bovirhinis	Mastitis
	M. bovoculi	Role in keratoconjunctivitis
	M. californicum	Mastitis
	M. canadense	Mastitis
	M. dispar	Pneumonia in calves
	M. leachii	Mastitis, polyarthritis, pneumonia
	Ureaplasma diversum	Vulvitis, infertility, abortion
	M. wenyonii	Mild anaemia
Sheep, goats	*M. conjunctivae*	Keratoconjunctivitis
	M. ovipneumoniae	Pneumonia
	M. ovis	Haemolytic anaemia, varying in severity
Goats	*M. putrefaciens*	Mastitis, arthritis
Turkeys	*M. iowae*	Embryo mortality
Horses	*M. felis*	Pleuritis

	M. equigenitalium	Implicated in abortion
Cats	*M. felis*	Conjunctivitis
	M. gateae	Arthritis, tenosynovitis
Dogs	*M. cynos*	Implicated in the kennel cough complex
	M. haemocanis	Mild or subclinical anaemia; more severe signs in splenectomized animals
Pigs	*M. suis*	Mild anaemia, poor growth rates

Table 38.3 Differentiating features of *Mollicutes* isolated from domestic animals.

Isolate	Effect of digitonin	Requirement for cholesterol	Urease production	Colony size
Mycoplasma species	Growth inhibition	+	–	0.1–0.6 mm
Ureaplasma species	Growth inhibition	+	+	0.02–0.06 mm
Acholeplasma species	No growth inhibition	–	–	up to 1.5 mm

Table 38.4 Biochemical tests which aid differentiation of *Mycoplasma* species pathogenic for sheep and goats.

Test	*Mycoplasma agalactiae*	*M. capricolum* subsp. *capricolum*	*M. mycoides* subsp. *capri*
Glucose fermentation	–	+	+
Arginine hydrolysis	–	+	–
Phosphatase activity	+	+	–
Casein digestion	–	+	+

Pathogenesis and pathogenicity

Unlike many bacterial pathogens, mycoplasmas do not appear to produce specific toxins or invasins. However, some of the intrinsic metabolic functions of the organism appear to be important in its ability to produce disease, in addition to its ability to adhere to host cells and to evade the immune response. Production of H_2O_2 can induce toxic damage to host cells to which the organisms are adhering. Accordingly, adhesion to host cells is an essential attribute for pathogenicity but mycoplasmas do not invade the cells. Production of these soluble factors may be central to the pathogenicity of some strains as it has been shown that European strains of *Mycoplasma mycoides* subsp. *mycoides*

produce significantly less H_2O_2 than the more pathogenic African strains of this species (Pilo *et al*., 2007). Some pathogenic species possess structures composed of unique adhesion proteins which promote attachment to mammalian cells (Krause and Stevens, 1995). Mycoplasmas can adhere to neutrophils and macrophages and can also impair phagocytic functions.

Variation in surface proteins is an important virulence attribute of *Mycoplasma* species as it allows the organism to rapidly adapt to the host environment and to evade the developing immune response. Such alterations in surface proteins have been demonstrated in many of the important animal pathogens including *Mycoplasma mycoides* subsp. *mycoides* (Persson *et al*., 2002), *M. bovis* (Sachse *et al*., 2000) and the avian pathogens *M. gallisepticum* and *M. synoviae* (Noormohammadi, 2007). It is not known if the antigenic variation which occurs in these surface proteins is a regulated event or whether it is a random response due to selection pressure by the host immune response to surface antigens. An additional mechanism which may contribute to persistence of mycoplasmas in the host is the similarity between some mycoplasmal antigens and host tissue antigens. This may interfere with host recognition of mycoplasmal antigens during tissue invasion. In addition, it may predispose to the development of autoimmune disease if the host's immune response to mycoplasmal antigens damages tissues which share antigenic determinants with the invading pathogen.

Modulation or activation of host immune responses is critical in the pathogenesis of mycoplasmal diseases. Some pathogenic mycoplasmas, including those involved in pulmonary diseases, are mitogenic for B and T lymphocytes (Muhlradt and Schade, 1991). Activation of macrophages and monocytes leads to the release of proinflammatory cytokines including tumour necrosis factor and interleukins. Pneumonia-producing mycoplasmas, which adhere to ciliated respiratory epithelium, can induce ciliostasis, loss of cilia and cytopathic change.

Diagnostic procedures

Isolation of mycoplasmas from clinical samples does not necessarily confirm aetiological involvement because certain mycoplasmas of questionable clinical significance are widely distributed. In regions where mycoplasmal diseases are endemic, clinical findings may point to the involvement of a particular mycoplasmal pathogen.

- Specimens for laboratory examination, ideally collected early in the course of a disease, should be kept refrigerated and delivered to a laboratory within 48 hours. Suitable samples include mucosal scrapings, tracheal exudates, aspirates, pneumonic tissue, mastitic milk and fluids from joints or body cavities. Swabs from lesions or suspect material should be placed in mycoplasmal transport media for transfer to the laboratory.
- The presence of *Mycoplasma* species or mycoplasmal antigens in samples can be demonstrated immunologically or by nucleic acid procedures:
  – Fluorescent antibody techniques.
  – Peroxidase–antiperoxidase procedures on paraffin-embedded tissues.
  – Polymerase chain reaction techniques. These are particularly useful for poorly preserved samples or for samples from animals which have been treated with antibiotics, in which the organisms may not be viable.
- Inoculated mycoplasmal medium is incubated aerobically or in 5 to 10% CO_2, in a humid atmosphere at 37°C for up to 14 days.
- Fluid samples can be inoculated directly on to agar or into broth media. Tissue specimens such as lung should be freshly sampled and a cut surface moved across the surface of a solid medium. Alternatively, the tissue can be homogenized in broth and samples of the suspension used for inoculation of liquid or solid media.
- Identification criteria for isolates:
  – ‘Fried-egg’ microcolonies
  – Microcolony size
  – Cholesterol requirement for growth (digitonin sensitivity test)
  – Biochemical profile including urease production
  – Fluorescent antibody technique on microcolonies
  – Growth inhibition test with specific antisera.
- Antimicrobial susceptibility testing. Disc diffusion methods are not suitable for testing mycoplasmal organisms because of their slow-growing nature, and thus micro broth dilution methods are most commonly used. However, for mycoplasma organisms which grow on solid media, the E test method is an easily performed, satisfactory method.
- Serological tests:
  – Complement fixation tests for the major mycoplasmal diseases of ruminants are used for certification when animals are traded internationally.

– Tests based on ELISA are being developed for the diagnosis of economically important mycoplasmal diseases. A competitive ELISA for *Mycoplasma mycoides* subsp. *mycoides* has now been approved for the purposes of international trade (Anon., 2008a).
– Rapid plate agglutination tests are used for screening poultry flocks and for the field diagnosis of contagious bovine pleuropneumonia.
– Haemagglutination-inhibition tests can be used to determine the antibody levels in avian mycoplasmal diseases.

Clinical infections

Mycoplasmas are often implicated in disease processes involving mucosal surfaces and such disease frequently becomes chronic. Factors such as extremes of age, stress and intercurrent infection may predispose to tissue invasion. In addition, mycoplasmas may exacerbate disease initiated by other pathogens, particularly in the respiratory tract.

Mycoplasmal infections cause respiratory diseases of major economic importance in farm animals, especially in ruminants, pigs and poultry (Table 38.1). Infections associated with mastitis or conjunctivitis in cattle and with disease conditions in pets are usually of lesser importance (Table 38.2). Several mycoplasmas have been isolated from dogs and cats but their precise role in disease has not been clearly defined. They have been implicated in respiratory and urinary tract disease in dogs (Jang *et al*., 1984). *Mycoplasma cynos* is commonly associated with respiratory disease in dogs, and experimental infection resulted in pneumonia (Chalker, 2005). In cats, *M. felis* can cause conjunctivitis and *M. gateae* is associated with arthritis.

Contagious bovine pleuropneumonia

Contagious bovine pleuropneumonia (CBPP) is a severe contagious disease of cattle which has been recognized for more than 200 years and formerly had a worldwide distribution. It is caused by *M. mycoides* subsp. *mycoides* (small colony type), a member of the ‘mycoides cluster’.This cluster is composed of five closely related members including the *M. mycoides* and *M. capricolum* subspecies of sheep and goats (Table 38.1) and *M. leachii* (Table 38.2). Members of the cluster share biochemical, immunological and genetic characteristics which render differentiation of individual species and subspecies difficult (Egwu

et al., 1996).

Contagious bovine pleuropneumonia is endemic in central Africa, the Middle East and Asia. Sporadic outbreaks, usually of a less severe form of the disease, have recently occurred in Portugal, France, Italy and Spain. Recent genotyping of strains from Europe and Africa, using multilocus sequence analysis, has confirmed that the strains from both continents are different and that European strains did not originate in Africa (Yaya *et al*., 2008). The main method of transmission is by aerosols. Transmission of the disease requires close contact with clinically affected animals or asymptomatic carriers. Clinical signs become apparent 3 weeks after infection. The severity of the disease relates to strain virulence and the immune status of the host. Spread of infection can be relatively slow with peak morbidity (about 50%) at 7 to 8 months after introduction of infection into a herd. In severe outbreaks, the mortality rate may be high.

Clinical signs and pathology

Clinical signs in the acute form of CBPP include sudden onset of high fever, anorexia, depression, drop in milk yield, accelerated respiration and coughing. Animals adopt a characteristic stance with the head and neck extended and elbows abducted. Expiratory grunting and mucopurulent nasal discharge may be present. Death can occur 1 to 3 weeks after the onset of clinical signs. Arthritis, synovitis and endocarditis may be present in affected calves.

At post-mortem, the pneumonic lungs have a marbled appearance. Grey and red consolidated lobules alternate irregularly with pink emphysematous lobules and the interlobular septa are distended and oedematous. There may be abundant serofibrinous exudate in the pleural cavity. In chronic cases, fibrous encapsulation of necrotic foci is commonly found. These necrotic foci contain viable mycoplasmas, and breakdown of the capsules in chronically affected animals is a major factor in the persistence and spread of CBPP in endemic areas.

Diagnosis

- In endemic regions, clinical signs and characteristic post mortem findings allow a presumptive diagnosis.
- The fluorescent antibody test can be used on pleural fluid to confirm the presence of the pathogen.
- Isolation and definitive identification of the pathogen from broncho-

alveolar lavage, pleural fluid, lung tissue or the broncho-pulmonary lymph nodes is confirmatory.

- Polymerase chain reaction based tests may be useful confirmatory tests. The successful development of real-time PCR assays for the differentiation of members of the 'mycoides cluster' has been reported recently (Fitzmaurice *et al*., 2008).
- Serological tests:
  – Rapid field serum agglutination test
  – Passive haemagglutination screening test
  – Complement fixation test or competitive ELISA for determining disease status of animals crossing national boundaries
  – Dot-blot technique for confirmation (Nicholas *et al*., 1996).

Treatment and control

- Treatment with antimicrobial drugs is prohibited in countries where the disease is exotic but is often attempted in countries where the disease is endemic. It is generally unsatisfactory, especially for chronically affected animals, but has been reported as effective in reducing transmission of infection to healthy in-contact animals where all affected cattle are treated (Hubschle *et al*., 2004).
- In countries where CBPP is exotic, slaughter of affected and in-contact cattle is mandatory.
- In endemic regions, control strategies are based on prohibiting movement of suspect animals, mandatory quarantine and the elimination of carrier animals by serological testing and slaughter.
- Annual vaccination with attenuated vaccines is carried out to stimulate effective immunity in cattle in endemic areas. The virulence of attenuated vaccines varies with the strain of mycoplasma employed. Annual vaccination may be discontinued as eradication of the disease progresses.

Infections with *Mycoplasma bovis*

Strains of *M. bovis*, which is worldwide in distribution, can cause severe pneumonia in calves in the absence of other respiratory pathogens (Doherty *et al*., 1994) and can exacerbate respiratory disease caused by *Pasteurella* and *Mannheimia* species (Gourlay *et al*., 1989). *Mycoplasma bovis* is frequently

associated with chronic respiratory disease. There is good evidence that the organism plays a causal role in the lesions of caseonecrotic bronchopneumonia frequently observed in natural outbreaks of *M. bovis* infection and disease (Caswell and Archambault, 2007). It is suggested that the lesions observed in experimental infections are milder because animals are usually slaughtered within 2 weeks of infection. *Mycoplasma bovis* has also been associated with mastitis and polyarthritis. Arthritis usually occurs in association with respiratory disease or mastitis. Diagnostic techniques are similar to those used for other mycoplasmas. Treatment and control of respiratory disease are based on management practices and antimicrobial therapy although response to treatment is frequently poor in chronic cases. Although two inactivated vaccines are available in the USA as an aid in the prevention of respiratory disease, their efficacy is questionable. Mastitis caused by *M. bovis* may range from subclinical to severe and systemic involvement with arthritis may occur. A number of other *Mycoplasma* species cause sporadic mastitis in cattle (Table 38.2). There is often a dramatic loss of milk production and the serous or purulent mastitic exudate has a high leukocyte count. Mycoplasmal mastitis should be considered when other common bacterial causal agents have been excluded. Mastitis caused by *Mycoplasma bovis* is discussed in more detail in Chapter 93.

Contagious agalactia of sheep and goats

This severe febrile disease of sheep and goats, caused by *M. agalactiae*, is prevalent in parts of Europe, northern Africa and parts of Asia. It usually becomes evident immediately after parturition and is characterized by mastitis, arthritis and conjunctivitis. Pregnant animals may abort and the disease can be fatal in young animals due to pneumonic complications. The organism is shed in milk and may remain localized in the supramammary lymph nodes between lactations. Disease due to *M. agalactiae* must be distinguished from mastitis and arthritis associated with *M. capricolum* subsp. *capricolum* or *M. mycoides* subsp. *capri* (Gil *et al*., 1999). Inactivated and attenuated vaccines for *M. agalactiae* are commercially available.

Contagious caprine pleuropneumonia

Contagious caprine pleuropneumonia (CCPP), caused by *M. capricolum* subsp. *capripneumoniae* (formerly *Mycoplasma* strain F38), is present in northern and eastern Africa and in Turkey. The disease is characterized by pneumonia,

fibrinous pleurisy, profuse pleural exudate and a marbled appearance on the cut surface of affected lungs. Although similar in many respects to contagious bovine pleuropneumonia (CBPP), well developed necrotic areas in the lungs in chronic CCPP are rare. The disease is highly contagious and is transmitted by aerosols. Nomadic herds often carry infection to regions free of the disease. Pleuropneumonia in goats can occasionally be caused by *M. mycoides* subspecies *capri*. However, monoclonal antibody to *M. capricolum* subsp. *capripneumoniae* is specific for this organism in a growth inhibition disc test (Belton *et al*., 1994). More recent tests based on PCR are rapid and specific and are now widely used for identification of isolates and for direct detection of the organism in clinical specimens (Anon., 2008b). Inactivated vaccines give satisfactory protection.

Enzootic pneumonia of pigs

This economically important disease, caused by *M. hyopneumoniae*, occurs worldwide in intensively reared pigs. Poor ventilation, overcrowding and temperature fluctuations may precipitate an outbreak. Pigs of all ages are susceptible and the condition is characterized by coughing, poor growth rates and, in some cases, respiratory distress. At post-mortem, pulmonary consolidation is confined to the cranial and middle lobes with clear demarcation from normal lung tissue. Clinical, epidemiological and pathological findings are usually indicative of the presence of the condition. The gold standard for confirmation of disease is by isolation and identification of the pathogen. However, the organism is extremely difficult to isolate. Although *M. hyopneumoniae* can be demonstrated in lung tissue by immunofluorescence, PCR-based tests are now regarded as the most sensitive tests available. Infection on a herd basis may be demonstrated serologically, usually by ELISA. Appropriate antimicrobial drugs such as tylosin tartrate, lincomycin or tiamulin, when incorporated into the feed, are suitable for controlling herd infections. However, antimicrobial resistance to a number of agents has been reported, including resistance to tylosin and to fluoroquinolones (Vicca *et al*., 2004, 2007). Inactivated and subunit vaccines are available and are useful for reducing the development of clinical disease and production losses but they do not prevent infection. Prevention and control are primarily based on the development of specific-pathogen-free herds.

Other mycoplasmal diseases of pigs

Mycoplasma hyorhinis causes a chronic progressive polyserositis in pigs up to 10 weeks of age. It is characterized by fever, laboured breathing, lameness and swollen joints. At post-mortem, serofibrinous pleurisy, pericarditis and peritonitis are present. The disease can be confirmed by isolation and identification of the pathogen and by serology. Tylosin or lincomycin, administered early in the course of the disease, may be of therapeutic value.

A polyarthritis caused by *M. hyosynoviae* affects pigs from 10 to 30 weeks of age. This self-limiting arthritis and synovitis produce transient lameness. Confirmation relies on isolation and identification of the pathogen.

Mycoplasmal diseases of poultry

Mycoplasma gallisepticum causes chronic respiratory disease in chickens and infectious sinusitis in turkeys. The organism is transmitted through infection of the embryo in the egg or by aerosols. Clinical signs are consistent with upper respiratory tract involvement in chickens. In turkeys, there is swelling of the paranasal sinuses. Reduced egg production may be evident. Diagnosis is based on isolation and identification of the pathogen and on flock testing using the serum plate agglutination test. Several PCR-based methods for the detection of the organism in clinical samples and for identification of isolates have been published (Anon., 2008c). Haemagglutination inhibition and ELISA tests are also used in flocks to confirm infection. Although antimicrobial medication of feed is used during outbreaks, the establishment of specific-pathogen-free flocks is the preferred method for controlling the disease. Eggs used for hatching should be dipped in a tylosin solution to eliminate the pathogen. Modified live vaccines and bacterins are available.

Mycoplasma meleagridis may be egg-transmitted and may be present in turkey semen. Aerosol transmission is less important with this pathogen than with *M. gallisepticum*. The clinical features of the infection include reduced egg hatchability, airsacculitis in young poults and joint and bone deformities in growers. Confirmation requires isolation and identification of the pathogen. The serum plate agglutination test is used for flock testing. Tylosin, administered in the water for the first 10 days of life, is of therapeutic value. Eggs used for hatching should be dipped in tylosin solution. Semen should be obtained from *M. meleagridis*-free toms.

Mycoplasma synoviae, the cause of infectious synovitis in chickens and

turkeys, is transmitted mainly by aerosols. Egg transmission is much less important than in *M. gallisepticum* and *M. meleagridis* infections. Synovitis, arthritis and respiratory signs are the main clinical features. Confirmation requires isolation and identification of the pathogen or positive serological tests. In common with other mycoplasmas, PCR-based detection methods are available. Tetracycline medication of the feed is used for treatment and control. Eradication is possible through the development of specific-pathogen-free flocks.

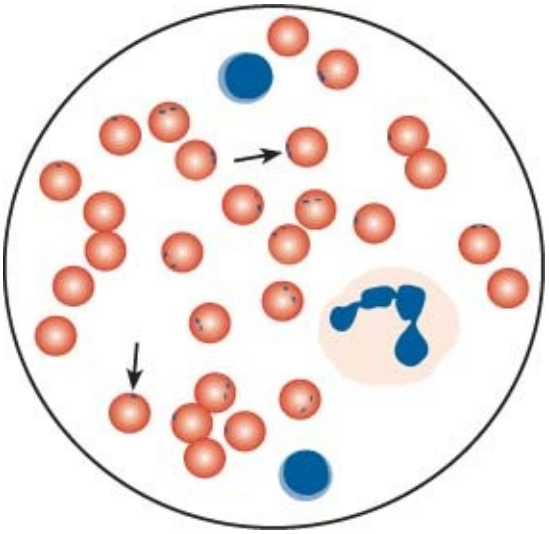
Haemotropic mycoplasmas

These organisms, which were formerly classified within the rickettsial group, are small bacteria that cannot be cultured *in vitro*. They were reclassified as mycoplasmas based on biological and phenotypic characteristics including their small size, lack of a cell wall and resistance to penicillin. Sequence analysis of the 16S ribosomal RNA gene confirmed the relationship of the species *Haemobartonella* and *Eperythrozoon* with mycoplasmas (Messick, 2004).

Feline infectious anaemia

Feline infectious anaemia, which is caused by *Mycoplasma haemofelis*, occurs worldwide. The pathogen is found on the surface of erythrocytes (Fig. 38.4). Two other haemotropic mycoplasmas (Candidatus *M. haemominutum* and Candidatus *M. turicensis*) have been detected in cats, but they are seldom associated with clinical disease (Willi *et al*., 2007a). The exact mode of transmission is uncertain. However, the disease is comparatively common in free-roaming tom cats between 1 and 3 years of age and transmission through bite-wounds or by biting arthropods has been suggested. Perinatal transmission to kittens has been recorded. Recovered cats may remain asymptomatic carriers. The prevalence of carrier cats in a population may approach 15% for *M. haemofelis* and may be as high as 38% for Candidatus *M. haemominutum* (Willi *et al*., 2007a).

Figure 38.4 A blood smear from a cat infected with *Mycoplasma haemofelis*. When stained with a Romanowsky stain, the organisms (arrows) appear as dark cocci or rods which are located on the surfaces of red blood cells.



Pathogenesis and clinical signs

The clinical presentation of the disease is variable and the mechanisms for production of anaemia and haemolysis are not completely understood. The attachment of the organism to the red blood cells may lead to direct damage. In addition, cold agglutinins have been observed in cats infected with *M. haemofelis* which suggests the involvement of immunological mechanisms in the destruction of red cells. In peracute disease, a profound anaemia associated with immunosuppression and an overwhelming parasitaemia rapidly results in death. The more commonly encountered acute form of the disease presents with fever, anaemia, depression, weakness and, occasionally, jaundice. A chronic form of the disease may follow, with affected animals exhibiting anaemia, lethargy and marked weight loss. In immunocompetent cats, successive waves of parasitaemia are gradually eliminated and a satisfactory regenerative bone marrow response develops. Immunosuppression resulting from infection with feline retroviruses is often a major factor in the development of severe feline infectious anaemia.

Diagnosis

- *Mycoplasma haemofelis* may be demonstrated on the surface of erythrocytes in Giemsa-stained blood smears (Fig. 38.4). Because of the cyclical nature of the parasitaemia, daily blood sampling may be necessary and the sensitivity of this technique is low, less than 20%.
- PCR analysis is the best method currently available for diagnosis of infection with haemotropic mycoplasmas. Several methods have been

published including a real-time PCR technique which can differentiate between the three haemotropic mycoplasmas known to infect cats (Tasker *et al*., 2003; Willi *et al*, 2007b).

- The pathogen can be demonstrated in blood smears by immunofluorescence.
- Haematological findings may include a reduced packed cell volume and evidence of regenerative anaemia.
- *Babesia felis* and *Cytauxzoon felis* should be considered in the differential diagnosis.

Treatment and control

- In acute disease, doxycycline therapy initiated early and continued for up to 21 days is effective for treatment of clinical signs but may not eliminate infection.
- Severely affected cats may require blood transfusions.
- Control measures should include flea control and the careful selection of feline donors for blood transfusion.

Infection with *Mycoplasma haemocanis*

Dogs infected with *Mycoplasma haemocanis* are usually asymptomatic. Immunosuppressive drug therapy, splenectomy, splenic dysfunction or severe immunosuppressive infections may activate latent infections resulting in the development of acute haemolytic anaemia.

Infection with *Mycoplasma suis*

Mycoplasma suis infection in pigs is one of the most common haemotropic mycoplasma infections of farm animals. Most infections are subclinical and the prevalence in some pig herds can approach 20%. Transmission involves biting arthropods such as lice. It may also result from the use of instruments contaminated with infected blood. Outbreaks of disease are sporadic and may be associated with stress factors. Signs include fever, haemolytic anaemia, weakness and jaundice. Disease can be particularly severe in young pigs (Henderson *et al*., 1997). Tetracycline therapy is effective.

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Chapter 39

Chlamydia and Chlamydophila species

Members of the order *Chlamydiales* are obligate intracellular bacteria with an unusual developmental cycle during which unique infectious forms are produced. They replicate within a non-acidified, cytoplasmic vacuole in eukaryotic host cells. The order *Chlamydiales* consists of four families, *Chlamydiaceae, Parachlamy-diaceae, Simkaniaceae and Waddliaceae. Waddlia chon-drophila and Parachlamydia* species have been implicated in bovine abortion (Ruhl *et al*., 2009), while*Neochlamydia hartmannellae*, an amoebic endosymbiont, has been associated with ocular disease in cats (von Bomhard *et al*., 2003). However, by far the most significant species from a veterinary and medical point of view are found in the family *Chlamydiaceae*. On account of their apparent difficulty with generating ATP and resultant dependence on host cell metabolism, chlamydiae have been termed ‘energy parasites’. Currently, two genera, *Chlamydia* and *Chlamydophila*. and nine species are described (Fig. 39.1). Formerly a single genus and four species, *Chlamydia trachomatis, C. psittaci, C. pneumoniae* and *C. pecorum*, were recognized. This classification was based on phenotypic characteristics such as host preference, inclusion morphology, iodine staining for the presence of glycogen, and sulphonamide susceptibility. However, nucleic acid sequencing studies of the 16S and 23S rRNA genes indicate two distinct lineages (Everett *et al*., 1999). Recently, it has been proposed to include all nine species in a single genus, *Chlamydia* species (Greub, 2010a, b).

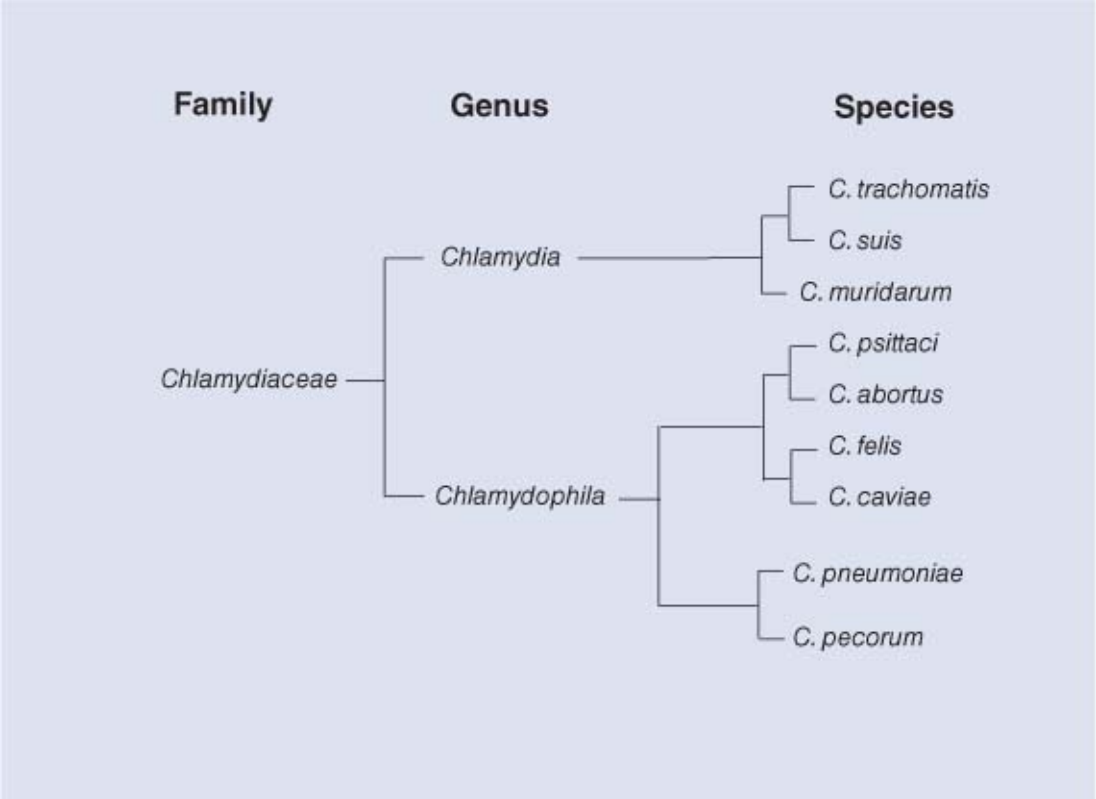
In the developmental cycle of chlamydiae, infectious and reproductive forms are morphologically distinct (Fig. 39.2). Infectious extracellular forms, called elementary bodies (EBs) are small (200 to 300 nm), metabolically inert and osmotically stable. Each EB is surrounded by a conventional bacterial cytoplasmic membrane, a periplasmic space and an outer envelope containing lipopolysaccharide. The periplasmic space does not contain a detectable peptidoglycan layer and the EB relies on disulphide cross-linked envelope proteins for osmotic stability (Hatch, 1996). Elementary bodies enter host cells

by receptor-mediated endocytosis Acidification of the endosome and fusion with lysosomes are prevented by mechanisms which are not fully understood. A process of structural reorganization within the pathogen, of several hours' duration, results in the conversion of an EB into a reticulate body (RB). The RB, about 1 μm in diameter, is metabolically active and osmotically fragile and replicates by binary fission within the vacuole. The vacuole and its contents, when stained, are called an inclusion. When a number of inclusions containing RBs of *C. trachomatis* are formed in an infected cell, fusion of these structures may occur. About 20 hours after infection, the developmental cycle becomes asynchronous, with some RBs continuing to divide and remaining in close association with the inclusion membrane while others detach from the inclusion membrane, condense and mature to form EBs. In general, replication continues for up to 72 hours after infection when the host cell lyses releasing several hundred bodies which include EBs, RBs and intermediate forms. Chlamydial replication may be delayed in the presence of gamma interferon or penicillin or when the availability of tryptophan or cysteine is limited, resulting in morphologically aberrant forms and persistent infection. Delayed replication of this type appears to be important in the development of the immunopathological changes in humans associated with trachoma and with pelvic inflammatory reactions.

Key points

- Spherical intracellular bacteria with unique developmental cycle
- Appropriate staining procedures include the modified Ziehl-Neelsen and Giemsa methods
- Unable to synthesize ATP and replicate only in living cells
- Cell walls lack peptidoglycan but contain family-specific lipopolysaccharide
- Species vary in virulence for particular hosts; some strains are associated with specific diseases in domestic animals
- Produce disease in the respiratory, enteric and reproductive tracts of animals and humans

Figure 39.1 Classification of chlamydiae on the basis of genetic relatedness (based on Everett *et al.*, 1999).



Usual habitat

The gastrointestinal tract appears to be the usual site of *Chlamydophila* species infection in animals. Intestinal infections are often subclinical and persistent. Faecal shedding of the organisms, which is typically prolonged, becomes intermittent with time. The EBs can survive in the environment for several days.

Pathogenesis and pathogenicity

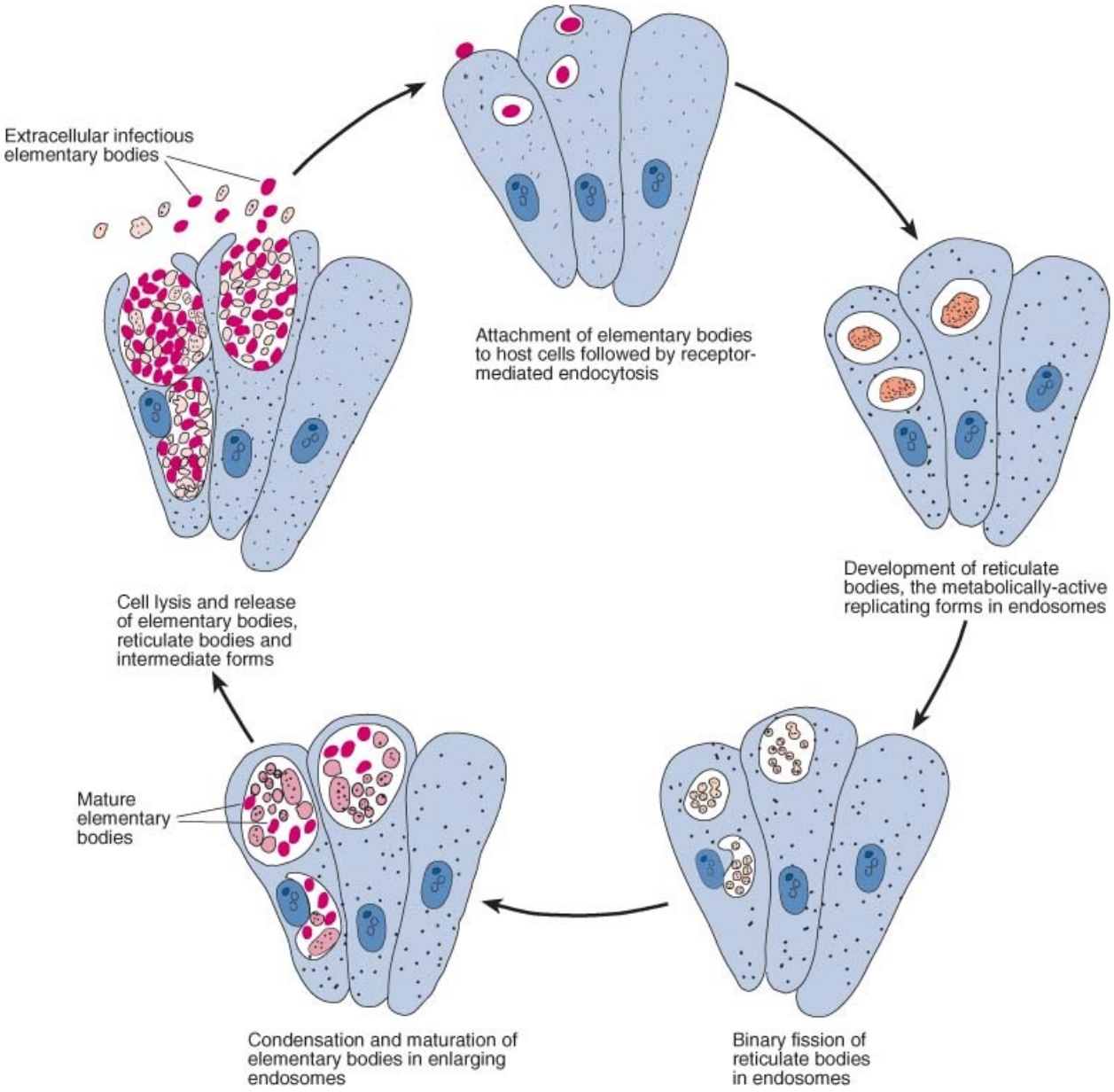
Chlamydiae infect over 450 species of birds and a large number of mammalian species including humans. In recent years, isolations have also been made from invertebrate species. Chlamydial species are usually associated with specific diseases in particular hosts. In sheep, *Chlamydophila abortus* is an important cause of abortion whereas infections with *C. pecorum* are frequently inapparent. Interspecies transmission is uncommon. When it occurs, the outcome of infection in the secondary host may be either similar, as in transmission from sheep to cattle, or severe, as in transmission from sheep to pregnant women.

Infection with *C. pecorum* is associated with conjunctivitis, arthritis and

inapparent intestinal infection. The type of clinical presentation relates to the route of infection and the degree of exposure. Environmental factors and management practices can influence the prevalence of some chlamydial infections such as enzootic abortion in ewes, which tend to be more prevalent in intensively managed lowland flocks.

Many chlamydial infections are asymptomatic, particularly when they are localized in superficial epithelia They may also persist for long periods without inducing protective immunity. However, chronic infections may repeatedly stimulate the host immune system. Chlamydiae possess a number of heat shock proteins which are partially homologous to heat shock proteins in other bacteria and to a number of human mitochondrial proteins. It is considered that repeated stimulation of the immune system with these proteins contributes significantly to the delayed-type hypersensitivity responses associated with trachoma and inflammatory pelvic disease in humans. The tissue damage in these diseases is more severe than would be expected from direct infection alone. Gamma interferon has been shown to contribute to the control of primary chlamydial infections. However, there is also evidence that gamma interferon can induce latent or persistent chlamydial infections which, in turn, may be responsible for increased heat shock protein expression (Ward, 1995).

Figure 39.2 Stages in the development of chlamydial forms in host cells.



Diagnostic procedures

Consideration of the history, clinical signs and pathological changes may suggest certain chlamydial infections such as feline chlamydiosis and enzootic abortion of ewes. Based on history and clinical findings, chlamydial involvement in a disease outbreak may be suspected but laboratory confirmation is required for a reliable diagnosis. A review of recent developments in the laboratory diagnosis of chlamydial infections has been published (Sachse *et al*., 2009).

- Specimens for isolation of the organism should be placed in suitable

transport medium such as sucrose–phosphate–glutamate (SPG) medium supplemented with foetal calf serum, aminoglycoside antibiotics and an antifungal agent (Spencer and Johnson, 1983). As chlamydiae are thermolabile, samples should be kept at 4°C. For long-term storage, samples should be frozen at −70°C. However, each cycle of freezing and thawing reduces the titre of the stored organisms.

- Direct microscopy is suitable for the detection of the organisms in smears or tissue sections containing moderate numbers of organisms. Smears or histo-logical sections of organs from aborted foetuses or from the liver and spleen in cases of avian chlamydiosis are suitable for direct examination. Placental smears from cases of chlamydial abortion typically contain large numbers of organisms. Suitable chemical staining procedures include the modified Ziehl-Neelsen, Giemsa, modified Machiavello and Castaneda methods. Methylene blue-stained smears can be examined by dark-field microscopy. Immunofluorescent staining improves the sensitivity of detection of chlamydial EBs in smears but usually does not identify the species involved because the monoclonal antibodies commonly used are directed against *Chlamydiaceae*- specific antigens such as chlamydial LPS.
- Several commercial kit sets employing ELISA methodology have been developed for the detection of *C. trachomatis*. Many of these kit sets detect chlamydial lipopolysaccharide (LPS) which is common to all *Chlamydia* and *Chlamydophila* species. Consequently, they can be used to detect the LPS of species in both genera.
- Chlamydiae can be isolated either in embryonated eggs, following inoculation into the yolk sac, or in a number of continuous cell lines such as McCoy, L929, baby hamster kidney and Vero. Cell lines vary in their susceptibility to infection by different chlamydial species. For ease of fixing and subsequent staining, tissue culture cells are usually grown in flat-bottomed vials containing coverslips. The attachment of chlamydiae to cells is greatly enhanced by centrifugation of the sample on to the monolayer. The sensitivity of the isolation procedure is also increased by the use of non-replicating cells. This is achieved by the addition of cytotoxic chemicals such as cycloheximide, 5-iodo-2-deoxyuridine, cytochalasin B and emetine to the cell culture medium. After 2 to 3 days' incubation at 37°C the monolayer is fixed, stained as described above and examined for the presence of chlamydial inclusions. Antibiotics to which chlamydiae are sensitive, such as oxytetracycline, erythromycin and penicillin, should not

be used in the cell culture medium.

- Polymerase chain reaction techniques have been developed for the detection of chlamydial DNA in samples. The primers are typically directed against the ribosomal RNA operon or the *ompA* gene. Using these methods it is possible to distinguish different chlamydial species by employing specific primers (Sheehy *et al*., 1996; Everett and Andersen, 1999; Sachse and Hotzel, 2003). Real-time protocols and a DNA microarray assay are also available for both detection and species identification (DeGraves *et al*., 2003 ; Ehricht *et al*., 2006).
- Several serological procedures available for the detection of antibodies to chlamydiae include complement fixation, ELISA, indirect immunofluorescence and micro-immunofluorescence. Although the complement fixation test is the most widely recognized serological test, it is time-consuming and only moderately sensitive. More sensitive assays based on ELISA methodology are now available. Because chlamydial infection is widespread, an exceptionally high or a rising antibody titre must be demonstrated in order to correlate infection with clinical signs. Interpretation of results is complicated by the fact that many of the available serological procedures detect antibodies against chlamydial LPS and therefore do not allow differentiation of the chlamydial species involved in the infection. In addition, there is cross-reactivity between the LPS of chlamydiae and that of some other Gramnegative bacteria.

Clinical infections

A wide range of animal species are susceptible to infections with chlamydiae (Vanrompay *et al*., 1995). Both the severity and the type of disease produced by chlamydiae are highly variable, ranging from clinically inapparent infections and local infections of epithelial surfaces to severe systemic infections (Table 39.1). Diseases associated with chlamydial infections include conjunctivitis, arthritis, abortion, urethritis, enteritis, pneumonia and encephalomyelitis. Clinical signs and their severity are influenced by factors related to both host and pathogen, and one type of clinical presentation usually predominates in outbreaks of disease.

Table 39.1 Chlamydial pathogens of veterinary and medical importance.

Pathogen	Hosts	Clinical conditions

Chlamydophila psittaci	Birds	Pneumonia and airsacculitis
		Intestinal infection and diarrhoea
		Conjunctivitis
		Pericarditis
		Encephalitis
	Humans (secondary hosts)	Psittacosis/ornithosis
C. abortus	Sheep	Enzootic abortion of ewes (EAE)
	Goats	Chlamydial abortion
	Cattle	Chlamydial abortion
	Pigs Humans	Chlamydial abortion Abortion
C. felis	Cats Humans	Conjunctivitis (feline pneumonitis) Conjunctivitis
C. caviae	Guinea-pigs	Guinea-pig inclusion conjunctivitis
C. pecorum	Sheep	Intestinal infection Conjunctivitis Polyarthritis
	Cattle	Sporadic bovine encephalomyelitis Polyarthritis Metritis
	Koalas	Conjunctivitis Urogenital infection
C. pneumoniae	Humans	Respiratory infection
	Horses	Respiratory infection
	Koalas	Conjunctivitis
Chlamydia trachomatis	Humans	Trachoma, inclusion conjunctivitis of infants
		Non-specific urethritis
		Respiratory disease of infants
		Proctitis
		Lymphogranuloma venereum
		Arthritis
C. suis	Pigs	Intestinal infection
C. muridarum	Mice	Respiratory infection

The species of *Chlamydophila* that infect humans differ in transmissibility. Although human infections can be acquired following contact with aborting ewes or cats with conjunctivitis, infected birds are considered to be more likely sources of infection. Human infections acquired from psittacine species are termed psittacosis while those from other avian species are termed ornithosis. Irrespective of the avian source of the infection, the condition typically presents as a respiratory illness.

Enzootic abortion of ewes

Enzootic abortion of ewes (EAE) caused by *C. abortus* is primarily a disease of intensively managed flocks. The disease is economically significant in most sheepproducing countries.Although abortion associated with *C. abortus* is best documented in sheep, it has also been reported in other domestic species including cattle, pigs and goats. Chlamydial infection in cattle and goats often

originates from sheep. The source of infection in pigs is less clearly defined (Schiller *et al*., 1997).

Epidemiology

Infection is usually introduced into clean flocks when infected replacement ewes abort. Large numbers of chlamydiae are shed in placentas and uterine discharges from affected ewes. Organisms can remain viable in the environment for several days at low temperatures. Infection occurs by ingestion. The role of infected rams in venereal spread is uncertain (Appleyard *et al*., 1985). Ewes infected late in pregnancy do not usually abort but may do so in the next pregnancy. Infection early in pregnancy can result in abortion during that pregnancy. Ewe lambs may acquire infection during the neonatal period and abort during their first pregnancy. As a result, the most dramatic outbreaks of EAE often occur in the year following the introduction of infection into a flock.

Pathogenesis

The site of persistence in non-pregnant ewes is unknown. The first signs of chlamydial infection of the placenta are detectable at about day 90 of gestation. The organism targets the trophoblast layer giving rise to inflammation, thrombotic vasculitis and tissue necrosis in the placenta. Dissemination to foetal tissues occurs but the pathological changes are mild. Abortion is considered to result from a combination of factors including reduced efficiency of foetal–maternal exchange, disruption of placental endocrine function and disruption of the immunological balance (‘immune expulsion’) between foetus and dam (Sammin *et al*., 2009).

Clinical signs

Enzootic abortion of ewes is characterized by abortion during late pregnancy or by the birth of premature weak lambs. Aborted lambs are well developed and fresh. Necrosis of cotyledons and oedema of adjacent intercotyledonary tissue in affected placentae is often present along with a dirty pink uterine exudate. Aborting ewes rarely show evidence of clinical disease and their subsequent fertility is usually unimpaired. Although up to 30% of animals in a fully susceptible flock may abort, a rate of 5 to 10% is more usual in flocks in which the disease is endemic.

Diagnosis

- Well-preserved aborted lambs and evidence of necrotic placentitis are suggestive of EAE.
- Large numbers of EBs can be demonstrated in placental smears using suitable staining procedures. If using MZN staining, care must be taken to avoid confusing chlamydial EBs with *Coxiella burnetii* which also causes abortion in ruminants and has similar staining properties.
- Commercial diagnostic kits are available for the detection of chlamydial antigen in samples.
- Isolation of chlamydiae in suitable cell lines or in the yolk sac of embryonated eggs is possible.
- Polymerase chain reaction techniques are available and can be carried out using species- specific primers to distinguish *C. abortus* and *C. pecorum* (Sachse and Hotzel, 2003). A real-time PCR protocol for *C. abortus* is available (Pantchev *et al*., 2009).
- A number of different serological tests can be used for the detection of chlamydial antibodies including the complement fixation test, ELISA and indirect immunofluorescence. *Chlamydophila abortus* shares some common antigens with *C. pecorum* and a number of Gram-negative bacteria. The use of recombinant antigens specific for *C. abortus* may improve the specificity of serological tests (Rodolakis *et al*., 1998; Wilson *et al*, 2009). The serological tests currently available do not distinguish between vaccinated and infected animals.

Treatment and control

Control measures for EAE have been comprehensively reviewed (Aitken *et al*., 1990).

- Chlamydiae are susceptible to a number of antibiotics which can be used during an outbreak. Administration of long-acting oxytetracycline to in-contact pregnant ewes has been shown to increase the number of live-born lambs. However, antibiotic treatment does not eliminate the infection and treated ewes may shed chlamydiae at parturition.
- Transmission of infection in an affected flock can be reduced by isolating all aborted ewes for 2 to 3 weeks, removing and destroying all placentas, thoroughly cleaning areas where abortions occurred and administering

long-acting oxytetracycline to ewes which have not yet lambed.

- A decision should be made either to vaccinate or attempt to eradicate the disease by culling. A live attenuated vaccine, containing a chemically induced temperature-sensitive mutant strain, is available and must be administered to ewes prior to breeding. An inactivated vaccine is also available which can be used in pregnant animals. The abortion rate and level of shedding of the organism is significantly reduced in vaccinated animals.
- *Chlamydophila abortus* infection is serious and potentially life-threatening for pregnant women who should avoid contact with ewes during the lambing season (Johnson *et al*., 1985; Buxton, 1986).

Feline chlamydiosis

Chlamydophila felis (formerly known as feline strains of *C. psittaci*) is associated with conjunctivitis and, less commonly, rhinitis. Feline pneumonitis, the original name for feline chlamydiosis, is now considered a misnomer because of the rarity of lower respiratory tract infection caused by *C. felis* in cats.

Epidemiology

Serological surveys have revealed that about 10% of unvaccinated cats have antibodies to *C. felis*. The primary target is the conjunctiva and *C. felis* can be isolated from up to 30% of cats with conjunctivitis, particularly cats with chronic conjunctivitis (Wills *et al*., 1988). Infection is transmitted by direct or indirect contact with conjunctival or nasal secretions. Organisms may also be shed from the reproductive tract (TerWee *et al*., 1998). Infection may be persistent with prolonged shedding of organisms and clinical relapses. The infection is most common in multi-cat households, particularly breeding catteries. The stress of parturition and lactation may trigger shedding of organisms by infected queens, facilitating transmission to their offspring. The majority of cases occur in cats less than 1 year of age.

Clinical signs

After an incubation period of about 5 days, unilateral or bilateral conjunctival congestion, clear ocular discharge, chemosis and blepharospasm become

evident. If secondary infection with organisms such as *Mycoplasma felis* and *Staphylococcus* species occurs, the ocular discharge may become mucopurulent. Conjunctivitis may be accompanied by sneezing and nasal discharge. The condition usually resolves without treatment in a few weeks. However, persistent infection with recurring clinical episodes also occurs.

Diagnosis

- The specimen of choice for detection of the organism is a conjunctival swab containing a large number of cells.
- Stained conjunctival smears may reveal intracyto-plasmic inclusions.
- The organism may be isolated in suitable cell lines or in embryonated eggs.
- Commercial diagnostic ELISA kits for detecting the family-specific lipopolysaccharide antigen are available.
- Conventional and real time polymerase chain reaction protocols have been developed for samples and are considered the methods of choice (von Bomhard *et al*., 2003; Dean *et al*, 2005).
- The complement fixation test, ELISA or indirect immunofluorescence test can be used to detect chlamydial antibody titres. Serology is useful for establishing if infection is endemic in a group of cats.

Treatment and control

- Chlamydiae are susceptible to several antibiotics, and systemic treatment is more effective than local treatment. Tetracyclines are generally considered the antibiotics of choice. To avoid relapses, treatment should continue for 2 weeks after the resolution of clinical signs. All in-contact cats should be treated at the same time.
- Both inactivated and modified live vaccines are available for parenteral inoculation. Vaccination reduces the clinical effects of natural infection but does not prevent infection or the shedding of organisms. Inadvertent intraocular administration of the live vaccine can result in conjunctivitis (Sturgess *et al*., 1995).
- A small number of cases of conjunctivitis in humans involving *C. felis* have been reported.

Sporadic bovine encephalomyelitis

This neurological disease, caused by *C. pecorum*, has been described in several regions of the world including the USA, Japan, Israel and Europe. Although intestinal infection in cattle with *C. pecorum* is considered to be common, sporadic bovine encephalomyelitis occurs haphazardly and the predisposing factors are unknown. Affected animals, which are usually under 3 years of age, develop a high fever and exhibit incoordination, depression, excessive salivation and diarrhoea. Terminally, animals may become recumbent and can develop opisthotonos. The course of the disease is about 2 weeks and the mortality rate may be up to 50%. Lesions associated with vascular damage are found in the brain and other organs. Diagnosis is based on clinical signs, the presence of a serofibrinous peritonitis, histopathological changes in the brain and isolation or detection of the organism in brain tissue. High doses of antibiotics such as tetracyclines and tylosin may be effective. Vaccines are not available and a control strategy has not been formulated.

Avian chlamydiosis

Infections with *C. psittaci* in psittacine birds were originally designated psittacosis, and ornithosis was reserved for chlamydial infection in other avian species. Avian chlamydiosis is currently the preferred designation for the condition. The disease has been recorded worldwide with the highest infection rates reported in psittacine birds (*Psittacidae*) and pigeons (*Columbiformes*).

Based on immunofluorescent reactions with a panel of monoclonal antibodies against major outer membrane protein (MOMP) epitopes, six avian serotypes of *C. psittaci* designated A to F are recognized (Andersen, 1991, 1997). The syndromes and host ranges of particular serotypes can be related to particular avian species: serotype A, psittacine birds; serotype B, pigeons; serotype C, ducks and geese; serotype D, turkeys; serotype E, pigeons and ratites; serotype F, parakeets. Based on PCR-RFLP data, serotypes have been assigned to equivalent genotypes (Vanrompay *et al*, 1997'. A further three genotypes are generally accepted, namely genotype E/B, a group of isolates from ducks, turkeys and pigeons (Geens *et al*., 2005); and two mammalian isolates, WC in cattle and M56 in muskrats. Sequence analysis of the *ompA* gene, which codes for MOMP, has revealed a further six genotypes of *C. psittaci* (Sachse *et al*., 2008).

Epidemiology

A wide range of both wild and domestic avian species are susceptible to

infection. The organism is present in respiratory discharges and faeces of infected birds. Infection is usually acquired by inhalation or by ingestion. Subclinical infection is common. Clinically affected and carrier birds may shed organisms intermittently for prolonged periods. Stress arising from captivity, transportation, egg-laying, overcrowding and intercurrent infection is important in precipitating both increased shedding and disease outbreaks.

Clinical signs

Avian chlamydiosis is a generalized infection, affecting particularly the digestive and respiratory tracts. The incubation period is up to 10 days. Clinical signs vary in nature and severity, depending on the strain of *C. psittaci* and the species and age of the affected birds. Signs include loss of condition, nasal and ocular discharges, diarrhoea and respiratory distress. The most frequent post-mortem findings are hepatosplenomegaly, airsacculitis and peritonitis.

Diagnosis

The diagnostic techniques for avian chlamydiosis have been reviewed by Andersen (2008).

- Organisms may be identified in stained impression smears of affected tissues.
- Chlamydial antigen may be detected using immunohistochemistry or ELISA kits.
- Chlamydial DNA may be demonstrated by the polymerase chain reaction (Van Loock *et al*., 2005 ; Laroucau *et al*., 2007). Real-time PCR protocols and a genotyping microarray for *C. psittaci* are available (Geens *et al*., 2005 ; Sachse *et al*., 2008).
- Isolation of *C. psittaci* is carried out in cell culture or embryonated eggs.
- Antibodies to *C. psittaci* may be detected using suitable serological tests including the complement fixation test and ELISA. However, interpretation of antibody titres can be difficult, particularly when single samples are tested. Results based on paired serum samples or samples from several birds in a flock are more reliable for diagnosis than a single serum sample.

Treatment and control

- Tetracyclines are the antibiotics of choice. An extended course of treatment

over several weeks is required.

- A commercial vaccine is not available.
- Imported birds, particularly psittacine species, should be held in quarantine and receive tetracycline-medicated feeds.
- Proper husbandry and suitable transportation minimize the occurrence of clinical disease.
- Avian chlamydial isolates are potentially zoonotic. Infection, which commonly follows aerosol inhalation, may be subclinical or result in systemic disease. Pulmonary involvement is common. Meningitis or meningoencephalitis may develop in severely affected individuals. Psittacosis in humans is a notifiable disease in the USA, Japan, Australia and many European countries.

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Chapter 40

Rickettsiales and Coxiella burnetii

Organisms in the order *Rickettsiales* form a diverse group of small (0.3 to 0.5 × 0.8 to 2.0 μm), non-motile, pleomorphic Gram-negative bacteria which replicate only in host cells. They can be cultured in the yolk sac of embryonated eggs or in selected tissue culture cell lines. Because they stain poorly with aniline dyes, these organisms should be stained by Romanowsky methods such as Giemsa or Leishman. In addition to host-cell dependence and poor affinity for basic dyes, a requirement for an invertebrate vector distinguishes them from conventional bacteria and the *Chlamydiales*.

The application of ribosomal RNA sequencing techniques and other precise analytical methods has led to extensive reclassification of the organisms in the *Rickettsiales*. The family *Bartonellaceae* has been removed from the order (Brenner *et al*., 1993) and *Coxiella burnetii*, which is genotypically and phenotypically distinct from other members of the group, is now placed in the gamma subgroup of the proteobacteria within the order *Legionellales*. *Coxiella burnetii* is dealt with in this chapter under a separate section. The genera *Haemobartonella* and *Eperythrozoon*, previously classified in the *Anaplasmataceae*, have been transferred to the genus *Mycoplasma* (Neimark *et al*., 2001, 2002).

At present, two families, *Rickettsiaceae* and *Anaplasmataceae*, comprise the *Rickettsiales* (Fig. 40.1). The species in the family *Rickettsiales* awaiting definitive assignment are in quotation marks in Fig. 40.1 and Table 40.1. The members of the family *Rickettsiaceae* have cell walls similar to those of other Gram-negative bacteria. Ultrastructural studies have shown that the *Anaplasmataceae* have outer membranes which are generally similar to those of Gram-negative bacteria but lack an obvious peptidoglycan layer. Organisms in the family *Rickettsiaceae*, referred to as rickettsiae, generally target endothelial cells. Although several new species of rickettsiae have recently been identified in domestic animals using molecular techniques, their pathogenicity is uncertain and currently the only species of veterinary importance in the family

Rickettsiaceae is *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever. Members of the *Anaplasmataceae* comprise four genera of veterinary importance, *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Aegyptianella*. Organisms in this family parasitize cells of haemopoietic origin, and species of veterinary importance are listed in Table 40.1. The organisms previously known as HGE (human granulocytic ehrlichiosis) agent, *Ehrlichia equi* and *Ehrlichia phagocytophila*, have been unified under the single species *Anaplasma phagocytophilum*.

Key points

- Minute, non-motile, Gram-negative bacteria
- Obligate intracellular pathogens, replicating only in cells
- Demonstrated in blood smears by Romanowsky stains
- Host specificity and tropism for particular cell types evident
- Extracellular survival brief for most members apart from *Coxiella burnetii*
- Cause systemic diseases, mainly arthropod-borne, in humans and animals
- *Rickettsiaceae*
  - – cell walls contain peptidoglycan
  - – cultured in specific cell lines or in fertile eggs
  - – tropism for vascular endothelium
- *Anaplasmataceae*
  - – lack cell walls, possess cell membranes
  - – have not been cultured *in vitro*
  - – tropism for cells of haemopoietic origin

Figure 40.1 Classification of members of the *Rickettsiales* of veterinary importance and the cell types which they target.

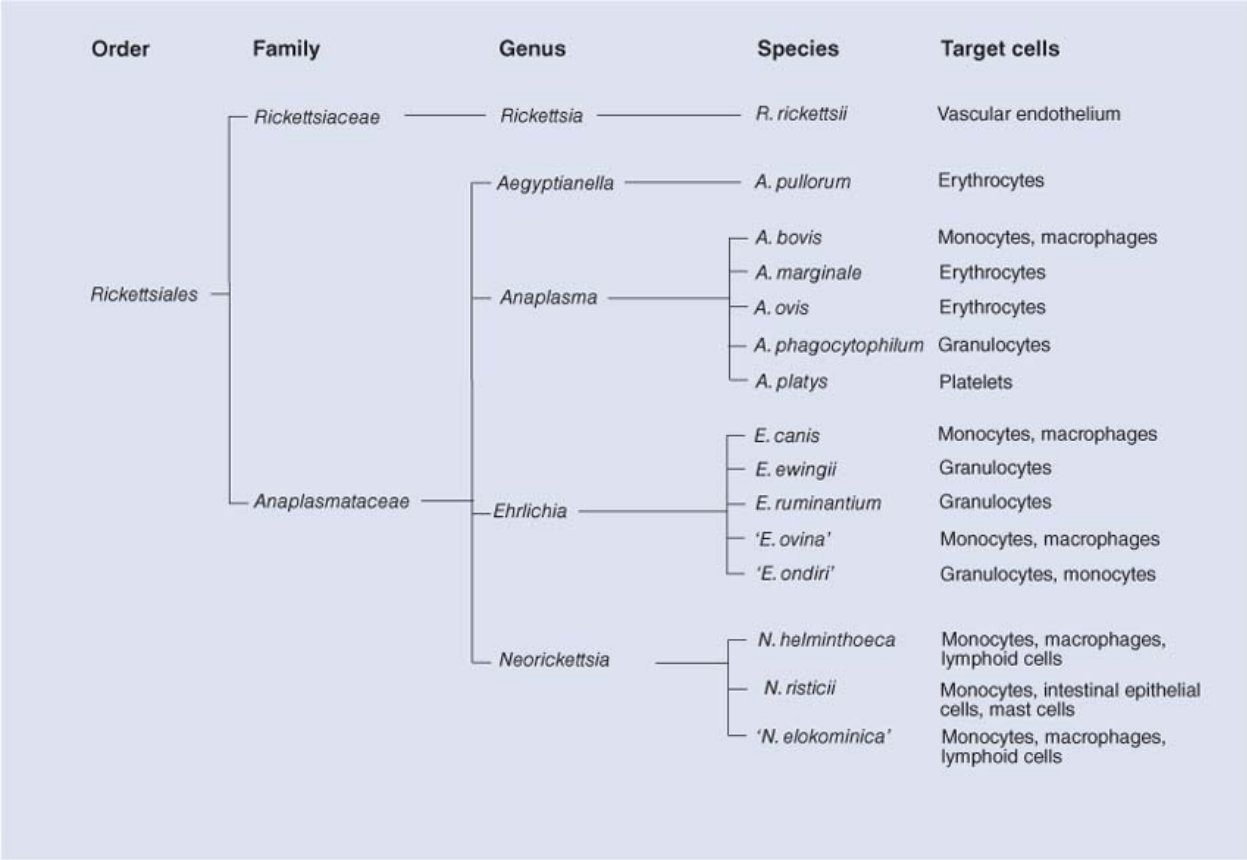


Table 40.1 Species of veterinary importance in the family *Anaplasmataceae*.

Pathogen	Hosts/Vectors	Disease	Geographical distribution
Aegyptianella pullorum	Poultry/Ticks	Aegyptianellosis	Africa, Asia, Mediterranean region
Anaplasma bovis	Cattle/Ticks	Bovine anaplasmosis	Africa, Middle East, Asia, South America
A. marginale	Ruminants/Ticks	Anaplasmosis	Tropical and subtropical regions
A. ovis	Sheep, goats/Ticks	Anaplasmosis	Asia, Africa, Europe, USA
A. phagocytophilum	Ruminants, horses, humans/Ticks	Tick-borne fever, equine and human granulocytic ehrlichiosis	Worldwide
A. platys	Dogs/Ticks suspected	Canine cyclic thrombocytopenia	Americas, Middle East, Mediterranean region
Ehrlichia canis	Dogs/Ticks	Canine monocytic ehrlichiosis	Tropical and subtropical regions
E. ewingii	Dogs/Ticks	Canine granulocytic ehrlichiosis	USA
E. ruminantium	Ruminants/Ticks	Heartwater	Sub-Saharan Africa, Caribbean islands
'E. ondiri'	Cattle/Ticks suspected	Bovine petechial fever	Highlands of East Africa
'E. ovina'	Sheep/Ticks	Ovine ehrlichiosis	Africa, Asia, Middle East
'Neorickettsia elokominica'	Dogs, bears, racoons/Flukes	Elokomin fluke fever	West coast of North America
N. helminthoeca	Dogs, bears/Flukes	Salmon poisoning disease	West coast of North America
N. risticii	Horses/Flukes	Potomac horse fever	North America, Europe

Epidemiology

Animal hosts and arthropod vectors are the reservoirs for most organisms in the *Rickettsiales*. Some organisms, including *Ehrlichia canis*, *Anaplasma marginale* and *A. phagocytophilum*, produce latent infections. In arthropods, rickettsiae replicate in the epithelial cells of the gut before spreading to other organs, including the salivary glands and ovaries, where further replication may occur. Organisms are transmitted when the arthropod feeds on the animal host. Some organisms such as *Rickettsia rickettsii* are maintained in a tick population by transovarial transmission. Transmission of *E. canis* and *A. phagocytophilum* occurs in ticks by trans-stadial but not transovarial routes. The majority of members of the *Rickettsiales* are transmitted by arthropods but the vectors of some *Ehrlichia* species have not yet been clearly defined (Table 40.1). Transmission by flukes has been confirmed for *Neorickettsia* species, with *N. risticii* having a complex life cycle involving an intermediate snail reservoir and a trematode vector. Members of the *Rickettsiales* are labile outside host cells, and for some species a silent cycle involving ticks and small wild mammals may constitute a possible source of infection for domestic animals.

Pathogenesis and pathogenicity

Many *Rickettsia* species including the causal agents of typhus (*R. prowazekii*), murine typhus (*R. typhi*) and scrub typhus (*R. tsutsugamushi*) are primarily human pathogens. Rocky Mountain spotted fever, caused by *Rickettsia rickettsii*, which is a common rickettsial disease of humans, also affects dogs. These highly pathogenic organisms have a predilection for the endothelial cells of small blood vessels, resulting in vasculitis and thrombosis in many organs. *Rickettsia* species produce phospholipase which damages the membranes of phagosomes allowing the organisms to escape into the cytoplasm. *Rickettsia rickettsii* replicates in both the cytoplasm and the nucleus of host cells, inducing cytotoxic effects.

Unlike *Rickettsia* species, all members of the *Anaplasmataceae* replicate within phagosomes in the host cell by inhibiting phagosome/lysosome fusion. Reticulate and dense-core forms are described and both divide by binary fission. Dense-core forms predominate late in infection and it is suggested that these may be the infectious forms, perhaps because they possess many adhesins. Organisms are released from cells by cell lysis or exocytosis. *Ehrlichia* species, with the exception of the human pathogens *E. chaffeensis* and *E. sennetsu*, are

pathogens of domestic and feral animals. These organisms have a predilection for leukocytes and, in the case of *E. ruminantium*, vascular endothelium. *Ehrlichia ruminantium*, the cause of heartwater in ruminants, probably parasitizes macrophages and other cell types in lymphoid tissues during the initial phase of infection. Organisms finally localize in membrane-bound vacuoles in endothelial cells throughout the body.

Two species in the genus *Neorickettsia* cause acute febrile disease in dogs. These organisms, which localize predominantly in lymph nodes, produce a generalized lymphadenopathy.

Many *Anaplasma* species and *Aegyptianella pullorum* parasitize red blood cells and are found within vacuoles in this cell type. Other members of the genus, including *A. phagocytophilum* and *A. bovis*, target granulocytes and *A. platys* infects platelets. The ability of some members of this family to survive and replicate within granulocytes is unique among bacterial organisms.

Recognition and differentiation of members of the *Rickettsiales*

Definitive classification of the members of the *Rickettsiales* is based on 16S ribosomal RNA sequencing, lipopolysaccharide content and metabolic requirements (Woldehiwet and Ristic, 1993). In diagnostic laboratories, identification of these organisms is based on the species affected, cell predilection, microscopic appearance and molecular techniques. Some members of the *Rickettsiales* can be cultured in embryonated eggs or tissue culture cells. These difficult procedures are usually performed only in laboratories engaged in research or vaccine production.

- Blood or tissue smears stained by the Giemsa technique can be used to demonstrate the morphology of members of the *Anaplasmataceae*. They occur as purplish blue, small, individual organisms, sometimes in clusters, or as morulae up to 4.0 μm in diameter. Those *Ehrlichia* and *Anaplasma* species that are found in granulocytes or platelets can be demonstrated in blood smears from animals in the early stages of disease. Species that target mono- cytes are present less frequently in blood smears.
- Fluorescent antibody techniques can be used to identify *R. rickettsii* and specific members of the *Anaplasmataceae* in smears.
- Some organisms can be isolated in the yolk sac of embryonated eggs or in

defined tissue culture cell lines. Many of those *Ehrlichia* and *Anaplasma* species that parasitize monocytes grow comparatively readily in the yolk sac. *Ehrlichia* and *Anaplasma* species which affect granulocytes and *Anaplasma* species parasitizing red blood cells have not been grown *in vitro*.

- Molecular methods, such as nucleic acid probes and polymerase chain reaction techniques, including real-time PCR techniques, have been developed to detect members of the *Rickettsiales* in host tissues.
- In outbreaks of major diseases such as bovine anaplasmosis, susceptible domestic animals can be inoculated with infected blood or tissue in order to identify an organism or confirm a diagnosis.

Clinical infections

Rickettsial organisms are relatively host-specific. Because definitive arthropod or fluke vectors are involved in the transmission of members of the *Rickettsiales*, diseases associated with these organisms tend to occur in defined geographical regions (Table 40.1). In many instances, the clinical signs reflect the targeting of a particular cell type by the causal agent. Human granulocytic anaplasmosis caused by variants of *Anaplasma phagocytophilum* and Rocky Mountain spotted fever are important zoonotic diseases, as is Q fever.

Rocky Mountain spotted fever in dogs

Rocky Mountain spotted fever, caused by *Rickettsia rickettsii*, affects mainly humans and dogs. In North America, the main tick vectors are *Dermacentor variabilis* and *D. andersoni*. *Rhipicephalus sanguineus* and *Amblyomma cajennense* are the main vectors in Central and South America. Ticks acquire the pathogen while feeding on infected small wild mammals. *Rickettsia rickettsii* is maintained in the tick population by transovarial and trans-stadial transmission and thus the tick acts as both a reservoir and a vector of the organism. An infected tick must remain attached for up to 20 hours before salivary transmission to the host occurs. The organisms, which replicate in endothelial cells of infected dogs, produce vasculitis, increased vascular permeability and haemorrhage.

Clinical signs

The incubation period of the disease is 2 to 10 days and the course is usually less than 2 weeks. Clinical signs include fever, depression, conjunctivitis, retinal haemorrhages, muscle and joint pain, coughing, dyspnoea and oedema of the extremities. Neurological disturbance, which occurs in about 80% of affected dogs, presents as stupor, ataxia, neck rigidity, seizures and coma. Dogs with mild disease and those treated early following infection usually recover. In severe disease, death may result from cardiovascular, neurological or renal damage. At post-mortem, there is widespread haemorrhage, splenomegaly and generalized lymphadenopathy.

Diagnosis

- Rocky Mountain spotted fever should be considered in dogs with systemic disease which have been exposed to ticks in endemic areas.
- Indirect fluorescent antibody test or ELISA demonstrating a rising antibody titre to *R. rickettsii* is diagnostic. Antibodies are not demonstrable until at least 10 days after infection.
- A marked thrombocytopenia and leukopenia may be present during the acute phase of the disease.
- The disease must be differentiated from acute canine monocytic ehrlichiosis.
- PCR detection in canine and tick tissues has been described by a number of workers.

Treatment and control

- Tetracycline therapy, which usually produces clinical improvement within 24 hours, must be continued for 2 weeks.
- Supportive therapy is necessary for severely debilitated dogs.
- Frequent removal of ticks is recommended. Because the disease is zoonotic, gloves should be worn during this procedure or a forceps should be used.

Canine monocytic ehrlichiosis

Canine monocytic ehrlichiosis, a generalized disease of *Canidae* caused by *Ehrlichia canis*, is confined to tropical and subtropical regions. *Rhipicephalus sanguineus*, the brown tick, is one of the main vectors and trans-stadial transmission occurs. After detachment from an infected host, ticks can transmit

the agent to susceptible dogs for up to 5 months. Dogs often remain carriers for more than 2 years after recovery from acute disease. Human ehrlichiosis is caused by *E. chaffeensis* which is closely related to *E. canis*.

Clinical signs

Following an incubation period lasting up to 3 weeks, the disease can progress through acute, subclinical and chronic phases (Fig. 40.2). The acute phase, in which signs range from mild to severe, is characterized by fever, thrombocytopenia, leukopenia and anaemia. Most affected dogs recover but some progress to a subclinical phase lasting months or years during which low blood cell values persist but clinical signs are minimal. A minority of these dogs later develop a severe form of the disease known as tropical canine pancytopenia. Persistent bone marrow depression, along with haemorrhages, neurological disturbance, peripheral oedema and emaciation, are characteristic of this phase of the disease. Hypotensive shock may ultimately develop, leading to death (Rikihisa, 1991). Progression to this chronic phase of the disease may be influenced by factors such as breed susceptibility, immunosuppression and the virulence of the infecting strain of *E. canis*.

Diagnosis

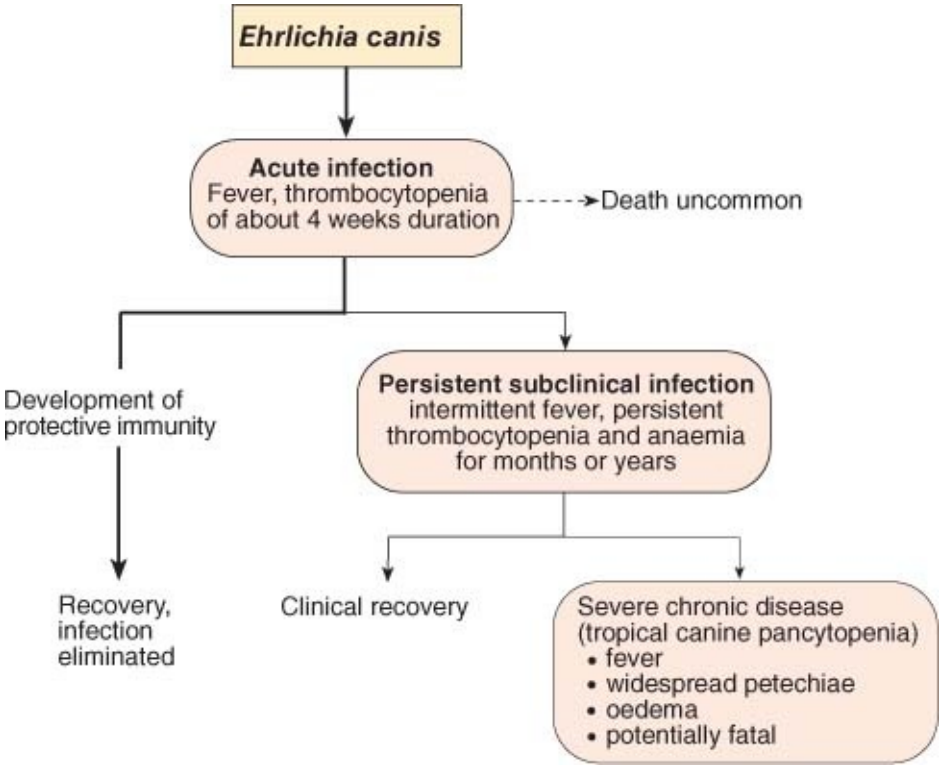
- Typical clinical and haematological features in dogs exposed to ticks in an endemic area may suggest canine monocytic ehrlichiosis.
- Morulae of *E. canis* may be detected in mononuclear cells in Giemsa-stained smears of the buffy- coat layer prepared from peripheral blood.
- Seroconversion can be demonstrated 3 weeks after infection using indirect immunofluorescence. Antibody titres of 1:10 or greater are considered to be indicative of infection.
- Culture of *E. canis* in a canine macrophage cell line is possible although time-consuming.
- Detection using PCR-based methods is now the more common method used for demonstration of the infection.

Treatment and control

- Doxycycline therapy for 10 days is recommended. Tetracyclines and chloramphenicol are also effective.

- Fluid replacement therapy or blood transfusions may be necessary.
- Tetracyclines can be administered to susceptible dogs entering an endemic area as a short-term prophylactic measure.

Figure 40.2 Possible consequences of infection with *Ehrlichia canis*.



Canine granulocytic ehrlichiosis

This disease, described in both dogs and humans in the USA, is caused by *Ehrlichia ewingii* (Anderson *et al*., 1992). Neutrophils are the primary target cells for the pathogen. Infected dogs, which exhibit mild clinical signs, recover uneventfully.

Canine cyclic thrombocytopenia

Anaplasma platys, the cause of this condition, parasitizes platelets. In infected dogs thrombocytopenia recurs at intervals of about 10 days, usually without evidence of clinical disease. Seroconversion, detected by indirect immunofluorescence, can be demonstrated about 2 weeks after infection.

Potomac horse fever

Potomac horse fever, also known as equine monocytic ehrlichiosis and equine ehrlichial colitis, is caused by *Neorickettsia risticii*. Originally described in 1970 in horses near the Potomac river in Virginia and Maryland, the disease has now been reported throughout North America and in some European countries. Potomac horse fever occurs during summer months and the life cycle involves a fluke vector in which transovarial transmission occurs, and an intermediate snail reservoir. Transmission to horses probably occurs following ingestion of aquatic insects infested with infected metacercariae. *Neorickettsia risticii* infects epithelial cells of the crypts in the colon and also targets monocytes, tissue macrophages and mast cells.

Clinical signs

Fever, anorexia, depression, colic, leukopenia and laminitis may be evident. The case fatality rate can reach 30%. Transplacental transmission of *E. risticii* may occur and the agent may induce abortion (Holland and Ristic, 1993). Patchy hyperaemia of the large intestine may be found at post- mortem (Rikihisa, 1991).

Diagnosis

- Clinical signs, although non-specific, may suggest the disease in endemic areas.
- A rising antibody titre detected by indirect immunofluorescence or ELISA tests is consistent with active infection.
- PCR can be used to amplify DNA from the organism in blood or faeces.

Treatment and control

- Oxytetracycline intravenously for 7 days is therapeutically effective.
- Inactivated vaccines, commercially available in North America, are not fully effective.

Bovine petechial fever

Bovine petechial fever, also called Ondiri disease, which occurs in both wild and domestic ruminants, is caused by *‘Ehrlichia ondiri’*. Clinical disease is most common in cattle imported into endemic areas. The disease is limited to highland areas of Kenya and other East African countries, and the vector is considered to be a species of tick with restricted distribution. *‘Ehrlichia ondiri’*

is thought to replicate initially in the spleen and to spread subsequently to other organs. Clinical signs include high fluctuating fever, depressed milk yield and widespread petechiation of visible mucous membranes. Oedema and petechiation of the conjunctiva produces ‘poached-egg’ eye, a feature typical of severe cases. Death often results from pulmonary oedema. Recovered animals, which become carriers, are resistant to reinfection for at least 2 years. The organisms are often found in granulocytes in smears of peripheral blood stained by the Giemsa method. Tetracyclines are effective only when administered during the incubation period of the disease.

Tick-borne fever

Tick-borne fever (TBF) is a rickettsial disease of domestic and wild ruminants caused by variants of *Anaplasma phagocytophilum*. The disease tends to be endemic on certain tick-infected upland farms in some European countries. The main vector is the tick, *Ixodes ricinus*, in which trans-stadial transmission occurs. Transmission to ruminant hosts occurs through the bites of infected ticks and, less commonly, through contaminated instruments. Recovered animals are immune to challenge with the homologous strain of *A. phagocytophilum* but remain infected for up to 2 years and act as reservoirs of infection for ticks. Since the maintenance of immunity appears to be related to repeated exposure to *A. phagocytophilum*, removal of animals from tick- infected pastures results in a decline in protective immunity. Recent findings indicate that several species of small rodents such as shrews and voles are competent hosts of *A. phagocytophilum* and thus may be important reservoirs of infection (Woldehiwet, 2006).

Pathogenesis

Anaplasma phagocytophilum is unusual in its ability to survive and multiply within neutrophils, eosinophils and monocytes. The ability to survive in these cells is related to its ability to inhibit lysosomal fusion with the cytoplasmic vacuoles within which it multiplies. In addition, it delays apoptosis of infected cells which allows the organism to complete its replication in spite of the short lifespan of these cells. Up-regulation of the chemokine IL-8 by *A. phagocytophilum* helps multiplication of the organism as it recruits uninfected neutrophils to the site of infection. These neutrophils then become infected and further disseminate the organism.

Carrier animals which have recovered from infection show recurrent bouts of bacteraemia. Persistence of infection may be related to antigenic variation of outer membrane proteins and immune evasion. Immunosuppression is characteristic of infection with *A. phagocytophilum* and is a consequence of severe leukopenia and impaired function of neutrophils and lymphocytes.

Clinical signs

Clinical signs, which develop after an incubation period of up to 13 days, include fever, inappetence and a reduced growth rate in young animals. A drop in milk production and abortions or stillbirths may occur in naive, pregnant animals after transfer to farms where the disease is endemic (Jones and Davies, 1995). Most affected animals recover within 2 weeks. However, *A. phagocytophilum* depresses both antibody-mediated and cell-mediated immune responses, increasing the susceptibility of young lambs to tick pyaemia and louping ill, diseases which are also tick-transmitted. Haematological changes in tick-borne fever include leukopenia and transient thrombocytopenia.

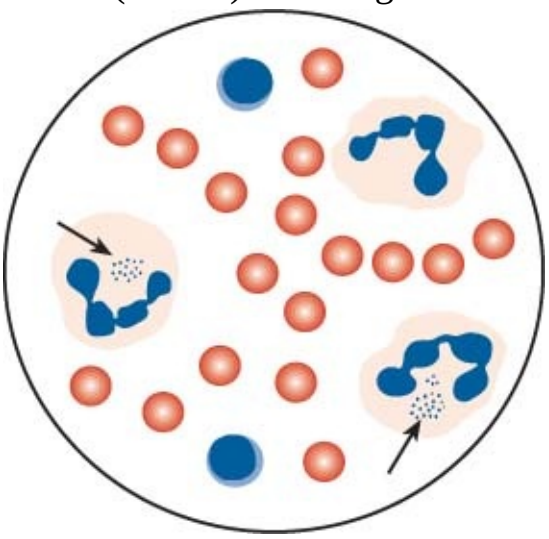
Diagnosis

- The disease should be considered in sick ruminants on tick-infected pastures in endemic regions.
- In Giemsa-stained blood smears, more than 70% of neutrophils contain intracytoplasmic blue morulae during the febrile period of the disease (Fig. 40.3).
- Indirect immunofluorescence is used to detect rising antibody titres.
- PCR-based techniques, including real-time RT- PCR, have been described (Sirigireddy *et al*., 2006).
- Differentiation of variants of *A. phagocytophilum* causing TBF from variants that infect humans, dogs and horses can be achieved by sequencing amplified fragments of the 16S rRNA gene (Woldehiwet, 2006).

Treatment and control

- Affected lactating cows should be treated with oxytetracycline.
- Tick control is an essential part of disease prevention.
- Long-acting tetracyclines, administered to lambs in the first 2 to 3 weeks of life, may protect against infection with *A. phagocytophilum*.

Figure 40.3 A blood smear from a sheep infected with *Anaplasma phagocytophilum*. When stained with a Romanowsky stain, groups of basophilic bodies (arrows) are recognizable in the cytoplasm of many neutrophils.



Equine granulocytic ehrlichiosis

This disease, often known as equine ehrlichiosis, is caused by variants of *Anaplasma phagocytophilum* which infect horses. The organism was previously thought to be a different species from the agent causing tick-borne fever and was known as *Ehrlichia equi*. It has been reported in the USA, some European countries and Israel. Clinical signs include fever, depression, ataxia, limb oedema, icterus and petechial haemorrhages on mucous membranes. The disease is relatively mild, the mortality rate is low and cases tend to occur in late autumn and in winter. Transmission is by *Ixodes* species of ticks. Diagnosis is based on the demonstration of morulae in neutrophils during the acute phase of the disease. Elevated antibody titres, demonstrated by indirect immunofluorescence, and marked leukopenia are additional indicators of infection. Tetracycline therapy is effective.

Heartwater

Heartwater, caused by *Ehrlichia ruminantium*, is a severe disease of ruminants limited to regions of sub- Saharan Africa and some Caribbean islands. Ticks belonging to *Amblyomma* species are the main vectors. Wild ruminants such as wildebeest become subclinically infected and the disease can be comparatively mild in indigenous breeds of domestic cattle in which a carrier state can be

maintained for up to 8 months. Clinical disease develops in calves and lambs and in newly introduced breeds of *Bos taurus*.

Ehrlichia ruminantium replicates in reticuloendothelial cells, particularly macrophages, and in endothelial cells of capillaries, especially those of the central nervous system. Damage to vascular endothelium results in increased permeability and widespread petechial haemorrhage.

Clinical signs

Sudden onset of fever occurs following an incubation period of 1 to 4 weeks. Neurological signs are common and include chewing movements, twitching of eyelids, high-stepping gait, circling and recumbency. Death often occurs during convulsions in acute cases. In subacute disease, lesions include hydropericardium, hydrothorax, pulmonary oedema and congestion. Splenomegaly and extensive mucosal and serosal haemorrhages may be evident.

Diagnosis

- In endemic regions, nervous signs and post mortem findings provide a presumptive diagnosis.
- Squash preparations of brain tissue, stained by the Giemsa method, may disclose the organisms located close to nuclei of endothelial cells.
- Nucleic acid probes, including polymerase chain reaction techniques, can be used on tissues from clinically affected cattle.
- Indirect immunofluorescence, ELISA and western blot procedures are used to demonstrate antibodies to *E. ruminantium*.
- PCR assays, including quantitative real-time PCR, have been described (Steyn *et al*., 2008).

Treatment and control

- Tetracycline therapy administered early in the disease may be effective.
- Immunization by inoculating blood from infected sheep, along with tetracycline therapy, may be used.
- Tick control is expensive and often impractical. However, as a consequence of tick control measures, immunity in indigenous stock may decline due to the lack of challenge with the infectious agent through repeated exposure to ticks.

Salmon poisoning disease

Salmon poisoning disease, caused by *Neorickettsia helminthoeca*, is an acute and frequently fatal infection of *Canidae*. The pathogen passes through the developmental stages in a snail–fish–dog cycle of the fluke, *Nanophyetus salmincola*. Dogs become infected by ingesting raw salmon containing fluke metacercariae. *Neorickettsia helminthoeca* enters the blood-stream following attachment of the fluke to the intestinal mucosa of the canine host. Replication of the bacterium in lymphoid tissues results in generalized lymphadenopathy. The disease is limited to the northwest Pacific coast of North America and occurs close to rivers into which the salmon migrate.

Clinical signs

Signs of illness develop abruptly about 7 days after ingestion of raw fish. Fever, anorexia, weakness and depression are followed by persistent vomiting and bloody diarrhoea. Death ensues in 7 to 10 days in up to 90% of untreated dogs. Animals that survive are usually resistant to reinfection.

Diagnosis

- A history of access to raw fish in endemic areas and the presence of fluke eggs in the faeces of severely ill dogs are suggestive of the infection.
- Organisms can be demonstrated in macrophages in lymph node aspirates stained by the Giemsa method.
- Infection with canine parvovirus 2 and canine distemper virus may be considered in the differential diagnosis.

Treatment and control

- Tetracyclines, sulphonamides or chloramphenicol may be effective if administered early in the course of disease.
- Supportive therapy may be necessary in dehydrated or anaemic animals.
- Raw fish should not be fed to dogs in endemic areas.
- A vaccine is not available.

Elokomin fluke fever

‘Neorickettsia elokominica’, the cause of Elokomin fluke fever, is

morphologically indistinguishable from *N. helminthoeca* and has the same fluke vector. The disease is milder than salmon poisoning disease and has a wider host range which includes *Canidae*, bears, racoons and ferrets. '*Neorickettsia elokominica'* infection may be concurrent with *N. helminthoeca* infection and there is no cross-protection between the two organisms.

Bovine anaplasmosis

Bovine anaplasmosis, or gall sickness, caused by *Anaplasma marginale*, affects cattle in tropical and subtropical regions. The disease, which is characterized by fever, anaemia and icterus, is often inapparent in animals in endemic areas. In young calves, infections are mild and result in the development of a carrier state. Carrier animals may develop mild clinical signs when stressed. Although severe clinical disease may develop in susceptible yearlings introduced into an endemic area, most recover. In contrast, the mortality rate in naive adult cattle may approach 50%. Morulae of *Anaplasma marginale* are located inside erythrocytes close to the cell membrane. The main vectors are ticks of *Boophilus* species but transmission may also occur through biting diptera. Instruments contaminated with infected blood may also be a source of infection. *Anaplasma marginale* has six major outer surface proteins, three of which vary antigenically. However, although several genotypes of *A. marginale* have been identified, infection exclusion occurs, with only one genotype establishing in an individual animal. The development of vaccines capable of inducing cross- protection against multiple genotypes is important as new genotypes of the organisms may be introduced into an area when animals harbouring distinct genotypes of the pathogen are introduced into a region for the first time (Kocan *et al*., 2004).

Clinical signs

The incubation period ranges from 2 to 12 weeks. Clinical signs include inappetence, depression and reduced milk yield. Marked anaemia and jaundice develop in the absence of haemoglobinuria, and weight loss is pronounced. Affected cattle may die suddenly from hypoxia if they are handled roughly. Resistance of recovered animals to re-infection is dependent on persistence of *A. marginale* in tissues.

Diagnosis

- Clinical signs and haematological findings in stressed indigenous cattle or

in naive cattle introduced into an endemic area may suggest the condition.

- Giemsa-stained blood smears may contain densely staining bodies (0.3 to 1.0 μm in diameter) located near the periphery of erythrocytes. The organisms are most numerous about 10 days after the onset of fever, when up to 50% of erythrocytes can be affected.
- The organisms can be identified in blood smears by immunofluorescence.
- A radioactive RNA probe and a polymerase chain reaction-based method are sensitive techniques used for detecting the pathogen.
- Serological tests are of particular value in detecting latent infections. These tests include the complement fixation test, the card agglutination test, ELISA and dot enzyme-linked immunosorbent assay.

Treatment and control

- Long-acting oxytetracycline or imidocarb dipropionate, administered early in the disease, are effective.
- Supportive therapy is essential in severe cases.
- In endemic areas, control measures are aimed at minimizing stress in indigenously reared cattle.
- Prior to introduction into an endemic region, animals must be vaccinated. A live *A. centrale* vaccine, which provides partial protection against *A. marginale*, is used only in calves. Attenuated and inactivated *A. marginale* vaccines are also available.

Aegyptianellosis in poultry

This disease, caused by *Aegyptianella pullorum*, affects poultry and wild birds. The vector is a tick of the genus *Argus*. Infected birds have ruffled feathers, anorexia, diarrhoea, anaemia and hyperthermia. Lesions include hepatosplenomegaly and punctiform haemorrhages on serosal surfaces. Control of ticks is important and tetracyclines are effective for therapy.

Q fever

The acute form of Q (query) fever, caused by *Coxiella burnetii*, is an influenza- l ike occupational disease of farmers, abattoir workers, veterinarians and others in contact with farm animals and their products. Subclinical disease is more common than clinical infection, and endocarditis is the principal form of the

chronic disease. Outbreaks of disease have been reported in humans without apparent contact with farm animals, perhaps resulting from windborne transmission of the organism (Arricau-Bouvery and Rodolakis, 2005). Although an obligate intracellular pathogen, *C. burnetii* forms small resistant forms during replication and these endospore-like forms remain viable in the environment for up to 150 days. *Coxiella burnetii* grows preferentially in the acid environment of phagolysosomes of macrophages and monocytes and many of its metabolic activities are detectable only at pH 5 or lower (Redd and Thompson, 1995). Most infections are acquired by inhalation of aerosols originating from parturient sheep, goats or cattle. *Coxiella burnetii* localizes and replicates in the female genital tract and mammary glands of ruminants with intermittent or continuous shedding of organisms in uterine discharges, foetal fluids, urine and milk. Rare outbreaks of Q fever have been associated with exposure to parturient cats (Langley *et al*., 1988). Laboratory infections are common. Although several genera of ticks serve as carriers of *C. burnetii*, infection following tick bites is relatively rare. Ingestion of milk or milk products contaminated with *C. burnetii* usually results in asymptomatic infections in humans. Vertical and sexual transmission of infection is possible in animals. Most infections in domestic animals are subclinical. However, rare sporadic abortions have been described in sheep, goats, cattle and cats. In ruminants, infection may also result in infertility or the birth of weak offspring. Placentitis or endometritis may be evident. Foetal lesions include hepatitis, myocarditis and interstitial pneumonia (Campbell, 1994).

Diagnosis

To prevent human infections, specimens must be collected and handled with care, and diagnostic procedures must be carried out in a biohazard safety cabinet.

- Smears from placental tissue and uterine discharges stained by the MZN method reveal small clumps of red coccobacillary bodies. Demonstration of the organism by the MZN staining method combined with serological results from the affected group of animals provide an adequate basis for diagnosis of infection (Anon., 2008).
- Immunofluorescence can be used to demonstrate the organisms in placental smears.
- Polymerase chain reaction procedures are used to detect small numbers of organisms in different sample types including aborted materials, milk and

faeces (Anon., 2008).

- *Coxiella burnetii* can be cultured in the yolk sac of 5 to 7-day-old embryonated eggs.
- Serological tests for *C. burnetii* include the complement fixation test, indirect immunofluorescence, ELISA and a competitive immunoassay (Soliman *et al*., 1992).

Control

- Segregation of parturient ruminants and careful disposal of placentas and aborted foetuses are essential following confirmation of disease.
- Inactivated egg-yolk vaccines are available for annual vaccination of non-pregnant ruminants.
- A vaccine suitable for laboratory and abattoir workers who are at high risk of infection is available.

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Chapter 41

Bacterial species of limited pathogenic significance

This heterogeneous group of Gram-negative bacteria contains organisms which occasionally produce disease in domestic animals (Table 41.1) and also a number of bacterial species of uncertain pathogenicity which are commonly isolated from clinical specimens (Table 41.2). Apart from *Neisseria* species which are Gram-negative cocci, the organisms in the group are Gram-negative rods.

Acinetobacter species

Organisms belonging to the *Acinetobacter* species are Gram-negative coccobacilli, which are easily isolated on standard culture media. They are environmental organisms which cause opportunistic infections in humans and animals, especially in hospital settings. *Acinetobacter baumannii, A. calcoaceticus* and *A. Iwoffii* are the most commonly isolated species. Many strains are resistant to multiple antimicrobial agents and strains have been isolated from human patients which are resistant to all commercially available agents (Munoz-Price and Weinstein, 2008). All *A. baumannii* isolates contain chromosomally-encoded cephalosporinases. The *AmpC* gene encoding these enzymes is normally expressed at low levels but clinical resistance occurs when a promoter insertion sequence close to the *AmpC* gene causes its overexpression. Efflux pumps are important mechanisms of resistance in *Acinetobacter* species and can expel β-lactam antibiotics, quinolones, tetracyclines and chloramphenicol. Acquired resistance to the carbapenem group of anti-microbials through the action of metallo-β-lactamases is an emerging problem in human medicine, and strains which have acquired these enzymes have been reported in many parts of the world. In animals, *Acinetobacter* species have been isolated from different clinical conditions including post-operative wound infections and septicaemia (Abbott *et al*., 2005; Brachelente *et al*., 2007; Weese,

2008). Antimicrobial treatment of infection can be challenging when an isolate is multi-drug resistant. Control of *Acinetobacter* infections is most successful when a point source can be identified, but general control measures include thorough cleaning of physical facilities, improved infection control measures, surveillance and isolation (Munoz-Price and Weinstein, 2008).

Bartonella species

The genus *Bartonella*, which was formerly classified in the order *Rickettsiales*, contains 19 recognized species at present (Table 41.3). The organisms are thin, Gram-negative, slightly curved rods, which grow only on blood-enriched media. Growth is slow and colonies may require up to 4 weeks to develop.

Epidemiology

Bartonella species are carried by a number of wild and domestic animals (Table 41.3) and transmission usually occurs by means of an arthropod vector. *Bartonella henselae* is carried by healthy cats and is transmitted from cat to cat by the cat flea, *Ctenocephalides felis*, without causing clinical signs. Infection is more common in warmer climates than in temperate countries and affects younger animals. Serological surveys in humans and animals indicate increasing prevalence of infection from temperate to tropical regions. Kittens may be bacteraemic for weeks to months. Transmission to humans usually occurs by means of a scratch and less commonly by means of cat bites. It is suggested that the organism may enter the scratch from contaminated flea faeces which adheres to the claw of the cat (Chomel *et al*., 2006).

Table 41.1 Bacteria of limited veterinary importance.

Bacterial species	Host species	Pathogenicity
Achromobacter species	Humans	Nosocomial pathogen
	Dogs, laboratory rabbits	Occasional reports of opportunistic infections
Acinetobacter species	Principally hospitalized domestic animals and humans	Opportunistic nosocomial pathogen of animals and humans. Frequently resistant to multiple antibiotics
Aeromonas salmonicida	Salmonid fish, goldfish	Furunculosis, ‘ulcer disease’
A. hydrophila	Amphibians	‘Red-leg’ syndrome
	Snakes (captive)	Ulcerative stomatitis, pneumonia, septicaemia
	Freshwater fish	Haemorrhagic septicaemia
	Cattle	Abortion
	Young dogs	Septicaemia
	Humans	Food poisoning

Bartonella henselae	Cats, humans	No clinical signs evident in cats. Causes cat scratch disease in humans
Chromobacterium violaceum	Pigs, dogs, sheep	Saprophyte in soil and water in tropical regions; may cause opportunistic infections
Listonella anguillarum	Marine fish, eels	Skin lesions, septicaemia
Ornithobacterium rhinotracheale	Chickens, turkeys	Respiratory disease
Plesiomonas shigelloides	Fish, reptiles	Septicaemia
	Harbour seals	Diarrhoea
	Humans	Diarrhoea, neonatal meningitis
Riemerella anatipestifer	Ducklings	Septicaemia
Streptobacillus moniliformis	Turkeys	Septicaemia following rat bites.
		Normal inhabitant of the upper respiratory tract of rodents
Vibrio cholerae	Humans	Cholera
V. parahaemolyticus	Humans	Food poisoning associated with seafood
V. metschnikovii	Chickens	Severe enteric disease

Table 41.2 Bacteria commonly isolated from clinical specimens but of uncertain pathogenicity.

Bacterial species	**Comments**
Achromobacter species	Commonly found in the environment. *A. xylosoxidans* is an emerging nosocomial pathogen in humans.
	Occasional reports of opportunistic infections in animals
Alcaligenes species	Saprophytes, occasionally isolated from the intestinal tract of vertebrate animals
Flavobacterium species	Present in soil and water. Nosocomial infections in humans. Some species are pathogens of fish
Neisseria species	Present in nasopharynx and on conjunctiva of many animal species. Occasional reports of localized opportunistic infections; occasionally associated with infection of dog-bite wounds in humans

Table 41.3 Selected *Bartonella* species, their reservoir host and significance.

Bartonella species	**Reservoir**	**Significance**
B. alsatica	Rabbits	Human disease reported
B. bacilliformis	Humans	Bartonellosis in humans
B. bovis	Cattle, cats	Endocarditis in cattle
B. clarridgeiae	Cats	Cat scratch disease in humans.
		Endocarditis, hepatitis recorded in dogs and cats
B. elizabethae	Rats	Endocarditis in humans
B. grahamii	Mice, voles	Human disease reported
B. henselae	Cats	Cat scratch disease in humans.
		Endocarditis, hepatitis and other disease syndromes recorded in dogs; disease seldom recorded in cats
B. koehlerae	Cats	Cat scratch disease in humans
B. quintana	Humans	Trench fever in humans
B. vinsonii subsp. arupensis	Mice	Bacteraemia and vasculitis in humans reported
B. vinsonii subsp. berkoffii	Dogs	Endocarditis and other disease syndromes in dogs

Pathogenesis

These bacteria are present in red blood cells and vascular endothelial cells. Mändle *et al*. (2005) reported that the organism invades the red-cell progenitor cells in the bone marrow, resulting in the presence of the organism in circulating red blood cells. In humans, infection with *B. henselae*, *B. clarridgeiae* or *B. koehlerae* causes cat scratch disease, with signs developing 1 to 3 weeks after the scratch or bite of a cat. In some cases, a small skin lesion which progresses to an ulcer and then heals is visible at the site of inoculation. Other signs include lymphadenitis and systemic signs such as fever, malaise and headaches. Usually the condition resolves without treatment but complications may occur. *Bartonella henselae* causes bacillary angiomatosis in immunocompromised individuals. Disease caused by *Bartonella* species in animals has been recorded mainly in dogs and includes reports of endocarditis, polyarthritis, uveitis and inflammatory lesions in organs such as lymph nodes and liver (Table 41.3).

Diagnostic procedures

- *Bartonella* species are readily identified in tissues with silver stains such as Warthin-Starry stain.
- These organisms are difficult to isolate; they grow on blood-enriched media following several weeks of incubation at 37°C. Isolation from bacteraemic cats may be enhanced by lysis of the feline red blood cells by freezing the blood sample before it is cultured.
- Isolates cannot be identified using conventional biochemical tests but cell wall fatty acid profiles can be used for identification. Increasingly, identification is by molecular methods such as PCR or partial sequencing techniques (Boulouis *et al*., 2005).
- Isolation of the organism from clinical specimens is even more difficult than from the blood of bacteraemic cats. Bacterial detection in such specimens is usually by extraction of DNA from tissues followed by PCR-based techniques.
- Serology is used for the diagnosis of clinical disease but is not useful for the detection of bacteraemia in cats as such animals may be seronegative when sampled. The most frequently used serological tests are IFA and ELISA.

Treatment and control

Antimicrobial therapy of cat scratch disease in immunocompetent people is not required. Prolonged treatment with antibiotics is needed for bacillary angiomatosis affecting immunocompromised individuals. *In vitro* susceptibility testing, which can be carried out using agar dilution or E test methods (Dörbecker *et al*., 2006), does not always reflect *in vivo* results, probably due to the intracellular nature of the organism. Effective regimes for the elimination of bacteraemia in cats have not been established although treatment with doxycycline or enrofloxacin for 2 to 4 weeks has been used for this purpose (Breitschwerdt, 2008). Control of infection in cats is based largely on elimination of the flea vector.

Aeromonas species, *Plesiomonas shigelloides* and *Vibrio* species

Aeromonas species, *Plesiomonas shigelloides* and *Vibrio* species are Gram-negative bacteria with a number of common attributes. They are found in aquatic environments, possess some similar biochemical characteristics and morphological features, and are opportunistic pathogens of fish, reptiles and rarely mammals. *Vibrio cholerae*, an important human pathogen, produces cholera which is a severe, life-threatening, enteric infection.

Morphologically, *Aeromonas* and *Plesiomonas* species are straight, medium-sized rods, unlike *Vibrio* species which are curved. Most members of these genera are catalase-positive, oxidase-positive, facultative anaerobes which are motile by polar flagella. The positive oxidase reaction distinguishes this group of organisms from members of the *Enterobacteriaceae*. Although *Aeromonas* species and *Plesiomonas shigelloides* grow on non-enriched media, many *Vibrio* species are halophilic. The optimal temperature for growth of some species in the group is lower than 37°C. Microaerophilic organisms, formerly classified as *Vibrio* species, are now assigned to the genus *Campylobacter*.

Aeromonas species and *P. shigelloides* are found in fresh water and are present in the oral cavity and on the skin of fish and reptiles. Most *Vibrio* species are found in brackish and salt water.

Clinical infections

Members of these genera are primarily pathogens of fish and reptiles, although

some species can infect mammals and birds. Infections are usually opportunistic, requiring stress factors for the initiation of disease. Species which have been associated with disease processes are included in Table 41.1. *Aeromonas hydrophila*, *P. shigelloides* and *V. metschnikovii* are the opportunistic pathogens which have been most often encountered in domestic animals and humans.

The pathogenetic mechanisms involved in the production of disease are poorly understood. *Aeromonas hydrophila* produces a number of virulence factors including adhesins, exoenzymes, haemolysin and enterotoxins. This organism has been associated occasionally with disease conditions in domestic animals. Abortion attributed to *A. hydrophila* was recorded in cattle (Wohlgemuth *et al*., 1972). The organism has also been isolated from a young dog with septicaemia (Pierce *et al*., 1973). Experimentally, A. *hydrophila* produces haemorrhagic colitis in rabbits (Hibbs *et al*., 1971).

Pathogenic strains of *Plesiomonas shigelloides*, which cause diarrhoea in humans, produce enterotoxins. The geographical distribution of this organism is limited to tropical and subtropical regions.

Apart from the important human pathogen, *V. cholerae*, there are at least five other species which cause enteric infections in humans. Food poisoning caused by *Vibrio parahaemolyticus* is associated with the consumption of raw or undercooked seafoods. *Vibrio metschnikovii* causes enteric disease in chickens. *Listonella anguillarum* (*Vibrio anguillarum*) and some *Vibrio* species are pathogens of fish.

Diagnostic procedures

A definitive diagnosis requires the isolation and identification of the pathogen from lesions. Because of the widespread distribution of the majority of these bacteria in the environment, laboratory results should be interpreted with caution.

Treatment

Antibiotic therapy should be based on susceptibility testing of *Aeromonas* species and *P. shigelloides*. Cephalosporins may be of therapeutic value. Gentamicin and nalidixic acid are usually effective for the treatment of infections caused by *Vibrio* species.

Chromobacterium violaceum

Chromobacterium violaceum is a motile, Gram-negative rod which grows on MacConkey agar and on nutrient agar with the production of a non-diffusible violet pigment. This bacterium is a catalase-positive, oxidase- positive facultative anaerobe which is found in soil and water of subtropical and tropical regions. Septicaemic infections with *C. violaceum* have been recorded in humans, pigs and dogs (Gogolewski, 1983). The organism has been associated with acute pleuropneumonia in Barbary sheep (Carrasco *et al*., 1996) and pigs (Liu *et al*., 1989).

Ornithobacterium rhinotracheale

This organism has been associated with respiratory disease in chickens and turkeys (Hinz *et al*., 1994) but has been isolated from many wild and domesticated species of birds. It is a pleomorphic Gram-negative rod which grows on blood agar producing small, grey, non-haemolytic colonies. Although *Ornithobacterium rhinotracheale* grows in an aerobic environment, growth is enhanced in 5 to 10% CO_2. The organism is oxidase- positive and catalase-negative (Charlton *et al*., 1993). Transmission of infection can take place by horizontal and vertical routes. Clinical infections are most severe in older chickens and turkeys and severity is also dependent on the presence of concurrent infections and management factors. Antimicrobial susceptibility varies greatly between strains but amoxicillin administered in drinking water for 3 to 7 days at 200 ppm is usually effective.

Riemerella anatipestifer

This organism, previously designated *Pasteurella anatipestifer*, is a non-motile, asaccharolytic Gram-negative rod which grows optimally on enriched media in an atmosphere of 5 to 10% CO_2. It is non- haemolytic on blood agar and does not grow on MacConkey agar. Infection with this organism can cause septicaemia primarily affecting ducklings up to 6 weeks of age although older waterfowl, turkey poults, chickens and pheasants can also be affected. The disease in ducklings is usually precipitated by stress. Clinical signs include ocular and nasal discharges, head and neck tremors and incoordination.

Mortality may reach 70%. Fibrinous pericarditis and peritonitis are common post-mortem findings. Meningitis and fibrinous airsacculitis may also be present. Twenty-one serotypes of this organism are currently recognized and multiple serotypes may occur on a single farm. Because of this, serotyping may not always provide useful epidemiological data and newer typing methods such as PCR-based techniques or *Sma*I macrorestriction analysis may provide more useful information (Kiss *et al*., 2007). Early treatment with ampicillin, amoxicillin or tetracyclines may be effective. A bacterin and a live avirulent vaccine are available.

Streptobacillus moniliformis

Streptobacillus moniliformis, a highly pleomorphic Gram-negative rod, is a normal inhabitant of the upper respiratory tract of rodents. The organism occasionally causes outbreaks of bronchopneumonia in laboratory rats and mice and cervical lymphadenitis in guinea-pig colonies. Rare cases of synovitis and deaths are reported in turkey flocks associated with rat bites. The bacterium is responsible for Haverhill fever and rat-bite fever in humans. Using PCR, the organism has been identified in the mouths of dogs with known contact with rats but the role of dogs as a source of infection in humans has not been established (Wouters *et al*., 2008).

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Section IV
Mycology

Chapter 42

General features of fungi associated with disease in animals

It is estimated that there are probably 1.5 million species in the kingdom Fungi, of which more than 80,000 species have been described. Approximately 400 fungal species are pathogenic for humans and animals. Fungi are eukaryotic, non-photosynthetic heterotrophs which produce exoenzymes and obtain nutrients by absorption. The taxonomy of the kingdom Fungi is in a state of flux, due to recent research based on DNA comparisons of species. Traditionally, classification has been based on morphological differences, physiology, the presence of structural macromolecules and sexual mating. However, in several instances molecular studies have revealed morphologically indistinguishable fungi in the same habitat to be different species. In 2007, a comprehensive phylogenetic classification of the kingdom Fungi was proposed (Hibbett *et al*., 2007) and this new classification is reflected in the *Dictionary of the Fungi* (Kirk *et al*., 2008) and the *Index Fungorum* (www.indexfungorum.org). Five phyla in the kingdom are recognized: *Microspora* (*Microsporidia*), *Ascomycota*, *Basidiomycota*, *Blastocladiomycota* and *Glomeromycota*. The phyla *Ascomycota* and *Basidiomycota* have been placed in the newly created sub-kingdom Dikarya. Two new phyla have been proposed: *Chytridiomycota* and *Neocallimastigomycota*. The phylum *Zygomycota* is not recognized in this classification scheme pending clarification of the relationships among the clades that are traditionally included in this phylum. Most zygomycetes of veterinary importance have been placed in the sub- phylum *Mucormycotina* which is currently listed as *incertae sedis* (Latin, of uncertain placement). It is possible that the phylum *Zycomycota* will be resurrected and validated to include *Mucormycotina* in the future as the term is still in use.

Key points

- Eukaryotic, non-photosynthetic microorganisms in the kingdom Fungi

- Widely distributed in the environment
- Cell walls contain chitin and other polysaccharides
- Heterotrophs; produce exoenzymes and obtain nutrients by absorption
- Branching hyphae and unicellular yeasts are the two major forms
- Reproduce both sexually and asexually with the production of spores
- Grow aerobically at 25°C; some moulds are strict aerobes
- Tolerate high osmotic pressures and low pH values; grow on Sabouraud dextrose agar, pH 5.5
- Resistant to antimicrobial drugs which are effective against bacteria
- Majority are saprophytes; some cause opportunistic infections
- Dermatophytes are pathogens that cause ringworm in animals and humans

Traditional classification of the fungi relies heavily on morphology and sexual reproduction. The form of a fungal species during its sexually reproductive life cycle is termed its teleomorph, while its asexual form is referred to as its anamorph. The preferred term for fungi that lack a meiotic stage is mitosporic fungi. About a fifth of all fungi, including many *Aspergillus, Malassezia, Penicillium* and *Coccidioides* species, have no known sexual stage. Formerly these fungi were placed in a heterogeneous group called the Class Deuteromycota or Fungi Imperfecti. Molecular methods are increasingly being used to produce phylogenetic trees which demonstrate evolutionary relationships and to assign fungal species to their appropriate grouping, even where there is no known sexual form. Such methods usually involve comparisons of the nucleotide sequences of highly conserved ribosomal RNA, especially the small (18S) subunit (SSU) and the large (26S) subunit (LSU) of ribosomal DNA. Many fungi formerly assigned to the Fungi Imperfecti have been transferred to the *Ascomycota*. A dual naming system has been used for many years with separate teleomorphic and anamorphic names. This system arose because many fungi were identified before their sexual reproductive mode was recognized. In many instances the anamorphic name is better known because it is the asexual form which is associated with disease production. This is illustrated by the dermatophyte *Microsporum canis* whose teleomorph name is *Arthroderma otae*. It is expected that, with advances in diagnostic molecular methods, the dual naming system will eventually become unnecessary. Fungi of veterinary importance are found in the three phyla *Ascomycota, Basidiomycota* and *Zygomycota*. Members of the *Chytridiomycota* cause skin infections in frogs which interfere with their ability to respire through their skin (Rosenblum *et al*., 2010). Chytridiomycosis, first reported in Australia, has subsequently been reported in many continents and has resulted in high mortality rates in

amphibians in California, Central America and regions of South America. The glomeromycetes, which are the smallest group of fungi, are of major ecological importance as plant symbionts which form arbuscular mycorrhizal associations with plant roots. The phylum *Blastocladiomycota* contains zoosporic fungi.

The two main morphological fungal forms are moulds and yeasts (Fig. 42.1). Moulds grow as branching filaments called hyphae (2 to 10 µm in diameter) whereas the unicellular yeasts have an oval or spherical appearance (3 to 5 µm in diameter). Dimorphic fungi occur in both mould and yeast forms. Environmental factors usually determine the form in which a dimorphic fungus occurs. Fungi such as *Candida albicans*, which produce forms additional to the two major forms, are described as polymorphic.

Fungi grow aerobically and many are strict aerobes. Temperatures appropriate for the optimal growth of different groups of pathogenic fungi and the incubation time required for the development of distinctive colonial features are indicated in Table 42.1. Reproduction by spore formation may be either sexual or asexual. In some species both types of spore formation occur. Fungi tolerate high osmotic pressures and acidic environments as low as pH 5.0.

Figure 42.1 Microscopic appearance of the two main fungal forms: A, Septate branching hypha of a mould. A mass of interlacing hyphae forms a mycelium. B, Budding cells of a yeast.

Table 42.1 Incubation conditions appropriate for the aerobic culture of fungi.

Fungal group	**Incubation conditions**	
	Temperature (°C)	**Time**
Dermatophytes	25	2 to 4 weeks
Aspergillus species	37	1 to 4 days
Yeasts (pathogenic)	37	1 to 4 days
Dimorphic fungi		
mould phase	25	1 to 4 weeks
yeast phase	37	1 to 4 weeks
Zygomycetes	37	1 to 4 days

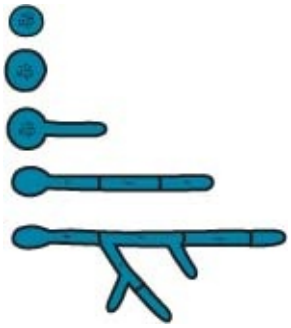
Fungal species may be saprophytic, parasitic or mutualistic. Mutualistic fungi have obligatory associations with other microorganisms and are non- pathogenic. Saprophytic fungi, which are widespread in the environment and are involved in

the decomposition of organic matter, cause sporadic opportunistic infections in animals. The parasitic dermatophytes are pathogens, causing ringworm in animals. Overgrowth of yeasts, which are often commensals on skin and mucous membranes, may cause localized lesions.

Structure

Hyphal cell walls, which impart rigidity and osmotic stability, are mainly composed of carbohydrate components including chitin macromolecules with cellulose cross-linkages. In yeasts, cell walls contain protein complexed with polysaccharides and, in some species, a range of lipid compounds. In the bilayered cell membrane, which lines the cell wall in the fungi, the predominant sterol is ergosterol in contrast to cholesterol, which predominates in the cell membranes of animals. Both moulds and yeasts have nuclei with well defined nuclear membranes, mitochondria and networks of microtubules. Septa (cross-walls) are often present in hyphae.

Figure 42.2 Stages in the germination of a fungal spore leading to the development of a branched hypha.



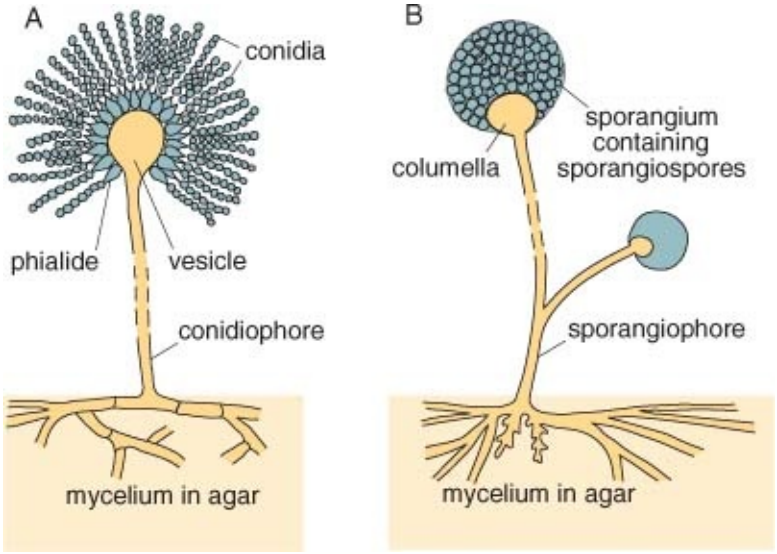
Growth, reproduction and colonial formation

Airborne fungal spores germinate in locations where environmental conditions are favourable. Spores swell and their metabolic activity increases prior to the production of tubular projections which develop into branched hyphae (Fig. 42.2). The hyphal wall is thin and plastic at its tip and, as apical growth occurs, cross-linkage of wall constituents results in maturation of the structure. Lateral branches develop from hyphae at localized areas of plasticity which allow outgrowth from the rigid mature cell wall. Septa, formed by inward growth of the cell wall, have central pores through which nutrients and organelles may

pass. Extension of hyphae and their lateral branches results in the formation of a mycelium, an interlacing network of hyphae.

Moulds tend to form large colonies with growth and extension of hyphae at their peripheries. In some species, mature elements at the centre of colonies produce specialized aerial hyphae which support spore-bearing structures and facilitate dispersal of mature spores. In this asexual reproduction, two main types of spores, conidia and sporangiospores, are recognized. Conidia are formed on conidiophores, and sporangiospores are formed within a sporangium, a sac-like structure borne on an aerial hypha termed a sporangiophore (Fig. 42.3). Sporangiospores are formed only by fungi in the phylum *Zygomycota*. In dermatophytes, multicellular structures called macroconidia and single-celled microconidia are produced in cultures from lateral hyphal branches, whereas arthroconidia are formed from the disintegration of hyphae within keratinized structures. Asexual spores produced by fungi are illustrated in Fig. 42.4.

Figure 42.3 Fungal growth on agar illustrating vegetative mycelia and aerial hyphae with sporing heads. A, *Aspergillus* species. B, *Rhizopus* species.



In most yeasts, asexual division is by budding. Daughter cells separate from parent cells after the formation of a cross-wall at the point of budding. The colonies of yeast-like fungi are soft, smooth and round.

Demonstration of the sexual stage of fungi, which is usually conducted in specialized laboratories, is essential for the taxonomic classification of phyla. A summary of the features of the sexual spores of the *Ascomycota, Basidiomycota* and *Zygomycota* is presented in Table 42.2.

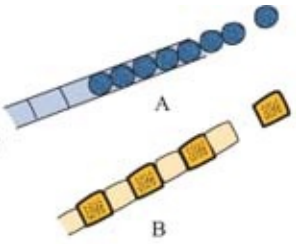
General features of fungal disease

The pathogenetic mechanisms by which fungi produce disease are listed in Box 42.1. The fungal diseases which result from tissue invasion (mycoses) can be conveniently categorized according to the sites of lesions (Table 42.3). Superficial mycoses are classified either as dermatomycoses or as dermatophytoses. In the dermatomycoses, opportunistic infections of the skin or muco-cutaneous junctions result from overgrowth of fungi such as *Candida* species or *Malassezia pachydermatis*. The dermatophytoses, which are clinically more important because of their communicability and zoonotic potential, are associated with invasion and destruction of keratinized structures by dermatophytes such as *Microsporum* species and *Trichophyton* species. Subcutaneous mycoses result from localized fungal invasion of the dermis and subcutis, often following penetration by a foreign body. When infection is caused by pigmented (dematiaceous) fungi, the condition is termed phaeohyphomycosis. Tumour-like granulomatous lesions are called mycetomas when caused by saprophytic fungi, and pseudomycetomas when associated with dermatophyte invasion. Systemic mycoses, which often originate in the respiratory or digestive tracts, usually follow opportunistic infection by saprophytic fungi. Factors that predispose to infection include alteration in the normal microbial flora as a result of prolonged antimicrobial therapy, immunosuppression following corticosteroid therapy or viral infection, and exposure to high infective doses of spores in confined spaces (Box 42.2).

Figure 42.4 Asexual spores produced by fungi of veterinary importance.

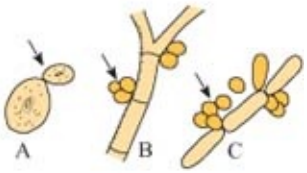
Arthroconidia (arthrospores)

Spores which are formed and subsequently released during the process of hyphal fragmentation. Spores may be formed successively as in dermatophytes (A), or with intervening empty cells as in *Coccidioides immitis* (B)



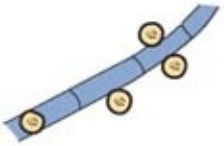
Blastoconidia (blastospores)

Conidia (arrows) which are produced by budding, as in *Candida albicans*, from a mother cell (A), from hyphae (B) or from pseudohyphae (C)



Chlamydoconidia (chlamydospores)

Thick-walled, resistant spores which contain storage products. These structures are formed by some fungi in unfavourable environmental conditions



Macroconidia

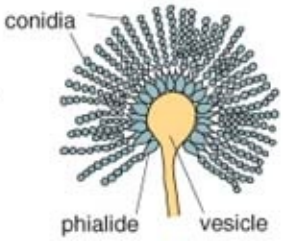
Large multi-celled conidia which are produced by dermatophytes in culture

Microconidia

Small conidia which are produced by certain dermatophytes

Phialoconidia

Conidia produced from phialides. The phialides of *Aspergillus* species arise from a vesicle



Sporangiospores

Spores (arrow), formed by zygomycetes such as *Rhizopus* species, are released when a mature sporangium ruptures

Table 42.2 Sexual spores of fungi in the phyla *Ascomycota*, *Basidiomycota* and

Zygomycota.

Spores	Comments
Ascospores	Produced by members of *Ascomycota*; develop in a sac-like structure called an ascus. Asci may be enclosed in well defined structures termed ascocarps
Basidiospores	Produced by members of *Basidiomycota* on club-shaped structures called basidia
Zygospores	Produced by members of *Zygomycota*; develop in a thick-walled zygosporangium, formed from the fusion of side projections of two compatible hyphae

Box 42.1 Mechanisms involved in fungal diseases.

- Tissue invasion (mycosis)
- Toxin production (mycotoxicosis)
- Induction of hypersensitivity

Table 42.3 Fungal diseases categorized according to sites of lesions.

Category	Sites of lesions
Superficial mycoses	Epidermis, other keratinized structures and mucous membranes
Subcutaneous mycoses	Subcutaneous tissues
Systemic mycoses	Respiratory and digestive tracts and other organ systems

Mycotoxicoses constitute an important group of diseases resulting from the ingestion of fungal toxins which have been pre-formed in stored food or standing crops. Although hypersensitivity reactions to fungal infections are rare in domestic animals, they can be associated with chronic pulmonary disease in cattle and horses.

Box 42.2 Factors which may predispose to fungal invasion of tissues.

- Immunosuppression
- Prolonged antibiotic therapy
- Immunological defects
- Immaturity, ageing and malnutrition
- Exposure to heavy challenge of fungal spores
- Traumatized tissues
- Persistent moisture on skin surface
- Some neoplastic conditions

Diagnosis of fungal diseases

Mycological cultural procedures should be performed in a biohazard cabinet because of the risk of human infection from spore aerosols. Culture of *Coccidioides immitis* should be attempted only in reference laboratories because highly infective arthrospores are produced in cultures at both 25°C and 37°C.

- Clinical signs and history may point to a presumptive diagnosis particularly in the dermatophytoses.
- Specimens for diagnosis include hair and skin scrapings from superficial mycoses and biopsy or post-mortem specimens from subcutaneous and systemic mycoses. Before treatment commences, specimens should be collected from the periphery of active lesions in an aseptic manner.
- Direct microscopic examination of wet preparations may be confirmatory:

  – Ringworm arthrospores surrounding infected hairs or hyphae in infected tissues may be demonstrable after clearing specimens in a few drops of 10% KOH under a coverslip for some hours.

  – *Cryptococcus neoformans* can be demonstrated, in cerebrospinal fluid mixed with India ink or nigrosin, as budding cells with wide capsules.

  – Sporing heads can be examined under a coverslip after mounting a sample from a colony in a drop of lactophenol cotton blue. Other methods for direct examination include slide culture and transparent adhesive tape techniques. Yeast cells can be stained with methylene blue or by the Gram method.
- Fungi are usually isolated on Sabouraud dextrose agar (pH 5.5) which inhibits growth of most bacteria. The addition of chloramphenicol and cycloheximide increases selectivity by inhibiting some of the fast-growing contaminating fungi such as the zygomycetes. To stimulate growth of the yeast phase of dimorphic fungi, enriched media, such as brain–heart infusion agar with 5% blood, and incubation at 37°C are required. Incubation times and temperatures for culture of the various fungal groups are listed in Table 42.1.
- Histopathological demonstration of fungal hyphae or yeast forms is usually necessary for confirmation of the significance of isolates from deep mycotic infections. The periodic acid-Schiff (PAS) reaction or methenamine silver impregnation can be used to demonstrate fungal elements in tissue sections.

Table 42.4 Differentiating features of fungi implicated in mycotic diseases.

Feature	Phylum		
	Ascomycota	*Basidiomycota*	*Zygomycota*
Sexual spores	ascospores	basidiospores	zygospores
Asexual spores	conidia	conidia	sporangiospores
Septate hyphae	+	+	–

Differentiation of fungal species

The main morphological features used for differentiating fungi implicated in mycotic diseases are presented in Table 42.4. In addition, molecular and immunological characterization of fungal pathogens is being developed for species differentiation.

- The form of the sexual stage (teleomorph) is used for assigning a fungus to a phylum (Table 42.2).
- Examination of sporing heads for conidial arrangement and the type and morphology of spores may allow initial differentiation. The presence of a mature sporangium identifies the fungus as a zygomycete (Fig. 42.3).
- Features of vegetative hyphae which can be used for differentiation include:
  - Presence or absence of septa
  - Either hyaline (colourless) or dematiaceous (pigmented)
  - Specific hyphal structures such as racquet-shaped and spiral hyphae.
- Colonial characteristics:
  - Size and appearance after specified incubation time
  - Colour of both obverse and reverse sides
  - Surface elevations or depressions.
- Yeasts can be differentiated by colonial appearance and the size and shape of individual cells. Biochemical reactions are also used for differentiation.
- Dimorphic fungi grow as moulds when cultured on Sabouraud dextrose agar at 25°C and as yeasts when cultured on enriched media at 37°C.
- Soluble antigens produced by dimorphic fungi can be used for identification in immunological tests.
- Specific nucleic acid probes have been developed for rapid and reliable identification of dimorphic fungi.
- Molecular diagnostic methods, including PCR and specific probes, are

increasingly being applied for the identification of specific fungal species. However, conventional methods will remain important due to the large number of fungi in the environment capable of causing disease.

Antifungal chemotherapy

The eukaryotic cells of fungi and animals have cell structures and metabolic pathways that are often similar. Since the plasma membranes of most fungi differ from those of animal cells in having ergosterol as a main sterol component, they are the primary target of many antifungal therapeutic agents. The classification of antifungal drugs and their modes of action are reviewed in Chapter 53.

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Chapter 43

Dermatophytes

The dermatophytes, a group of septate fungi which occur worldwide, invade superficial keratinized structures such as skin, hair and claws. More than 30 species of dermatophytes are recognized in three anamorphic genera: *Microsporum*, *Trichophyton* and *Epidermophyton*. Originally, dermatophytes were classified in the Fungi Imperfecti. However, despite their ecological and phenotypic variation, molecular studies have shown that all dermatophytes are genetically closely related to each other and are members of the family *Arthrodermataceae* in the phylum *Ascomycota*. Several species have been shown to be capable of reproducing sexually and have been placed in the teleomorphic genus *Arthroderma* with dual names referring to the anamorphic and teleomorphic forms, respectively. The name of the anamorphic form which is still the most widely recognized will be used in this chapter. The species *Epidermophyton floccosum* is primarily a human pathogen.

Arthrospores (arthroconidia) are the infectious forms most often associated with tissue invasion by this group of fungi. They are released by fragmentation of hyphae in keratinized structures. These resistant forms can remain viable for more than 12 months in suitable environments in buildings. Dermatophytes are strict aerobes, most of which grow slowly on standard Sabouraud dextrose agar. A few require special growth factors which are supplied by the addition of yeast extract to the Sabouraud dextrose agar. Macroconidia and microconidia are produced in culture. The colonies of many dermatophytes are pigmented. Colonial morphology and the type of macroconidia produced are used for identification.

Key points

- Members of the phylum *Ascomycota*
- Affinity for keratinized structures; colonize and invade skin, hair and nails
- Grow slowly on specially formulated laboratory media such as Sabouraud dextrose agar; some require additional growth factors
- Aerobic, tolerate cyclohexamide in media

- Colonies often pigmented
- Macroconidia formed in cultures
- Arthrospores, shed from infected animals, remain infective for many months
- Zoophilic and anthropophilic dermatophytes are obligate pathogens; geophilic dermatophytes are saprophytes in soil
- Cause characteristic circular skin lesions termed ringworm

Dermatophytosis (ringworm) affects many animal species (Table 43.1). The disease is a zoonosis and most human infections are caused by *Microsporum canis* contracted from infected cats (Pepin and Oxenham, 1986).

Usual habitat

Dermatophytes can be grouped on the basis of their habitats and host preferences as geophilic, zoophilic or anthropophilic (Table 43.2). Geophilic dermatophytes inhabit and replicate in the soil in association with decomposing keratinous materials such as hairs or feathers (Weitzman and Summerbell, 1995). Animals can acquire infection with geophilic dermatophytes from soil or from contact with infected animals. Zoophilic and anthropophilic dermatophytes are obligate pathogens which are unable to replicate in soil. Their existence as pathogens of keratinized structures usually corresponds with an inability to reproduce sexually. Dermatophytes growing on keratinized structures rarely produce macroconidia and consequently rely on the production of arthrospores for transmission. Each zoophilic species tends to parasitize a particular animal species.

Table 43.1 Dermatophytes of animals, their main hosts and reported geographical distribution.

Dermatophyte	**Hosts**	**Geographical distribution**
Microsporum canis (var. *canis*)	Cats, dogs	Worldwide
M. canis var. *distortum*	Dogs	New Zealand, Australia, North America
M. canis (syn. *M. equinum*)	Horses	Africa, Australasia, Europe, North and South America
M. gallinae	Chickens, turkeys	Worldwide
M. gypseum	Horses, dogs, rodents	Worldwide
M. nanum	Pigs	North and South America, Europe, Australasia
M. persicolor	Field voles	Europe, North America
Trichophyton equinum	Horses	Worldwide
T. equinum var. *autotrophicum*	Horses	Australia and New Zealand
T. mentagrophytes var. *erinacei*	European hedgehogs, dogs	Europe, New Zealand
T. mentagrophytes var.	Rodents, dogs, horses and many other animal	

mentagrophytes	species	Worldwide
T. mentagrophytes var. *quickeanum*	Mice	Australia, Canada, Eastern Europe, Italy
T. simii	Monkeys, poultry, dogs	India, Brazil, Guinea
T. verrucosum	Cattle	Worldwide

Table 43.2 Dermatophytes grouped according to host preference or habitat.

Zoophilic group	**Geophilic group**	**Anthropophilic group**[a]
Microsporum canis	*Microsporum cookei*	*Epidermophyton floccosum*
M. gallinae	*M. gypseum*	*M. audouinii*
Trichophyton equinum	*M. nanum*	*M. ferrugineum*
T. mentagrophytes	*M. persicolor*	*T. rubrum*
T. verrucosum	*T. simii*	*T. schoenleinii*

a, Anthropophilic dermatophytes rarely infect animals

Laboratory recognition and differentiation

Individual species are identified mainly by colonial morphology and the microscopic appearance of macroconidia, chlamydospores or other structures (Table 43.3, Figs 43.1, 43.2).

- The colonial morphology of dermatophytes commonly isolated from animals is described in Table 43.3. The obverse and reverse of each colony should be examined.
- Macroconidial morphology is assessed under low or high dry magnification in preparations or transparent adhesive tape mounts of colony samples stained with lactophenol cotton blue (Figs 43.1, 43.2). Other structures such as spiral hyphae, microconidia or chlamydospores can be used for differentiation.
- Special growth requirements can be determined using commercially available trichophyton agar. Control medium, designated trichophyton agar 1 (T1), is a casein basal agar. Other media, produced by adding growth factors to the basal agar, are T3 containing thiamine and inositol, T4 containing only thiamine and T5 containing nicotinic acid.
  - *Trichophyton verrucosum*, which has a requirement for thiamine and sometimes for inositol, usually grows on T3 or T4 media.
  - *Trichophyton equinum* requires nicotinic acid for growth whereas *T. equinum* var. *autotrophicum* does not. Culture on T1 and T5 media can be used to differentiate these variants.
  - *Trichophyton mentagrophytes* hydrolyses urea when grown on

Christensen urea agar.

- Temperature tolerance tests are useful for differentiating *T. verrucosum* and *T. mentagrophytes*, which grow well at 37°C, from other dermatophytes which do not tolerate this temperature.
- *In vitro* hair perforation tests are sometimes used to distinguish atypical isolates of *T. mentagrophytes* from *T. rubrum* and atypical *M. canis* from *T. equinum*. Sterilized blonde hairs from a child, placed on a culture of the dermatophyte under test, are incubated at 25°C. The hairs, stained with lactophenol cotton blue, are examined microscopically from the seventh day onwards. *Microsporum canis* and *T. mentagrophytes* penetrate the hair shafts forming wedge-shaped dark blue structures (Fig. 43.3).
- Dermatophyte test medium (DTM) has been formulated to differentiate dermatophytes from contaminating fungi. Phenol red is used as a pH indicator in this medium. Growth of dermatophytes results in alkaline metabolic products and the colour of the medium changes to red. Other fungal media should be used in conjunction with DTM because some contaminating fungi can also induce a colour change. In addition, the colour change in DTM can obscure the characteristic pigmentation required for differentiation of dermatophyte species.

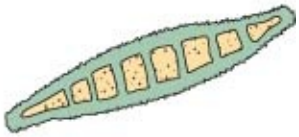
Table 43.3 Colonial appearance and growth characteristics of dermatophytes isolated from animals.

Dermatophyte	Colonial appearance on Sabouraud dextrose agar	Comments
Microsporum canis	Obverse, white to buff with bright orange periphery; reverse, yellowish orange or yellowish brown	Heavy sporulation occurs on rice grain media. Colony size up to 50 mm after incubation for 10 days
M. gypseum	Obverse, buff to cinnamon with white border and powdery; reverse, buff to reddish brown	Colony size up to 50 mm after incubation for 10 days. Mouse-like odour
M. nanum	Obverse, cream to tan and powdery; reverse, reddish brown	Colony size up to 35 mm after incubation for 10 days
Trichophyton equinum	Obverse, initially white and fluffy, later buff and folded; reverse, yellow to dark reddish brown	Nicotinic acid required for growth. Colony size up to 35 mm after incubation for 10 days
T. mentagrophytes	Obverse, cream–tan to buff and powdery; reverse, buff–tan to dark brown	Colony size up to 30 mm after incubation for 10 days. Urease-positive; grows well at 37°C
T. verrucosum	Obverse, white, heaped and velvety; reverse, white or pale buff	Growth slow, colony size up to 10 mm after incubation for 20 days. Requires thiamine and sometimes inositol for growth. Grows at 37°C

Figure 43.1 Morphological features of the macroconidia of some *Microsporum* species.

Microsporum canis

Spindle-shaped macroconidium (40-120 x 8-20 µm), rough, thick-walled, up to 15 septa



Microsporum gypseum

Boat-shaped macroconidium (25-60 x 7-15 µm), rough, thin-walled, up to 6 septa

Microsporum nanum

Pear-shaped or ovoid macroconidium (10-30 x 6-13 µm), rough, thin-walled, usually 1 septum

Figure 43.2 Morphological features of the macroconidia of *Trichophyton mentagrophytes* and the chlamydospores of *T. verrucosum*.

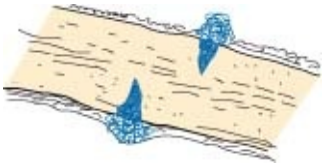
Trichophyton mentagrophytes

Cigar-shaped macroconidium (20-50 x 4-8 µm), smooth, thin-walled, up to 7 septa

Trichophyton verrucosum

Chlamydospores in chains; macroconidia rare

Figure 43.3 The *in vitro* hair perforation test. Wedge-shaped areas along a hair shaft stain darkly with lactophenol cotton blue. Some dermatophytes such as *M. canis* and *T. mentagrophytes* produce this hair perforation pattern.



Pathogenesis and pathogenicity

Dermatophytes invade keratinized structures such as the stratum corneum of the epidermis, hair follicles, hair shafts and feathers. Lesion development is influenced by the virulence of the dermatophyte and the immunological competence of the host. Young, aged, debilitated and immunosuppressed animals are particularly susceptible to infection, which occurs either directly by contact with an infected host or indirectly through infected epithelial debris in the environment. Infective arthrospores adhere to keratinized structures and germinate within 6 hours. Minor trauma such as gentle rubbing of the skin or

bites from arthropods may facilitate infection. Damp skin surfaces and warmth favour germination of spores. Metabolic products of hyphal growth may provoke a local inflammatory response. Hyphae grow centrifugally from the initial lesion towards normal skin, producing typical ringworm lesions. Alopecia, tissue repair and nonviable hyphae are found at the centres of lesions as they develop. Growth of hyphae can result in epidermal hyperplasia and hyperkeratosis. Secondary bacterial infection sometimes follows mycotic folliculitis.

The development of a strong cell-mediated response correlates with the onset of a delayed-type hypersensitivity which usually results in elimination of the dermatophyte, resolution of the lesion and local resistance to reinfection. Immunity to dermatophytosis is transient and reinfection may occur if the challenge dose is large (Moriello and De Boer, 1995). Other mechanisms which may be associated with the elimination of infection include an increased rate of desquamation from the stratum corneum and an increase in the permeability of the epidermis allowing penetration of inflammatory fluids (Wagner and Sohnle, 1995).

Animals with ringworm develop antibodies against dermatophyte glycoprotein antigens. Antibody- mediated responses do not appear to be protective. Strong humoral immune-mediated responses and weak cell-mediated responses have been demonstrated in persistently infected cats (Moriello and DeBoer, 1995).

Diagnostic procedures

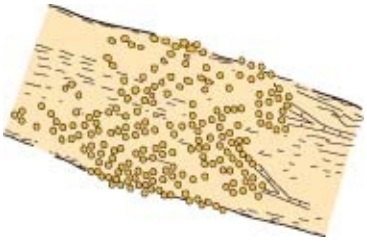
Laboratory investigation of dermatophytosis is often necessary because diagnosis on clinical grounds can be difficult.

- As dermatophyte species tend to parasitize particular hosts, the animal species affected may indicate the dermatophyte most likely to be involved (Table 43.1).
- Specimens suitable for laboratory examination include plucked hair, deep skin scrapings from the edge of lesions, scrapings from affected claws, and biopsy material from pseudomycetomas. Suitable material from cats can also be collected on a large sheet of paper by brushing the coat with a clean toothbrush.
- Hairs and skin scrapings treated with KOH should be examined microscopically for the presence of arthrospores. The arrangement of arthrospores on hair shafts is typically ectothrix (Fig. 43.4). Mites, such as

Demodex species, may also be detected in these specimens.

- Histological sections of skin or pseudomycetomas can be stained by the PAS or methenamine silver techniques to demonstrate fungal structures.
- Specimens are cultured on Emmon's Sabouraud dextrose agar (pH 6.9) with the addition of 2 to 4% yeast extract, 0.05g/litre chloramphenicol and 0.4 g/litre cycloheximide. Inoculated plates are incubated aerobically at 25°C to 27°C and examined twice weekly for up to 5 weeks.
- Identification criteria for isolates:
  – Colonial morphology
  – Microscopic appearance of macroconidia
  – Supplementary tests including growth on DTM medium.
- Several DNA-based techniques have been developed for the identification of isolated fungi and for the detection of fungal DNA in dermatological specimens (Nardoni *et al*., 2007; Kanbe, 2008).
- In cats and dogs with suspicious lesions, examination with Wood's lamp should always be carried out because *M. canis* infections are comparatively common in these species. A characteristic apple-green fluorescence from infected hairs is evident in more than 50% of affected dogs and cats (Sparkes *et al*., 1993). Detection of fluorescence depends on factors such as stage of infection and the characteristics of the infecting strain. In cats with inapparent infections, hairs should be cultured.
- Laboratory investigations to exclude other pathogens which cause skin conditions should be considered.

Figure 43.4 Surface (ectothrix) arthrospores on a hair shaft following clearance with 10% potassium hydroxide.



Clinical infections

Dermatophytosis is a comparatively common clinical condition in both companion and farm animals. Because of the zoonotic nature of the

dermatophytoses, affected animals should be handled with care.

Dermatophytosis in cats and dogs

Most infections in cats are caused by *M. canis*. Clinical features of the disease include classical ringworm lesions, miliary dermatitis, pseudomycetomas (Medleau and Rakich, 1994), onychomycosis and, rarely, generalized lesions in immunosuppressed animals. Inapparent infections are known to occur and cats may also carry arthrospores physically in their coats (Moriello *et al*., 1994). Persian cats appear to have a heritable predisposition and may present with mycetoma-like lesions. The dermatophytes which commonly affect dogs are listed in Box 43.1. The disease usually presents as areas of alopecia, scaling and broken hairs surrounded by inflammatory zones. Less commonly encountered lesions include folliculitis and onychomycosis. Lesion distribution on the muzzle may relate to certain behavioural activities such as compulsive digging in soil, rat-catching and attacking hedgehogs. These activities often determine the species of dermatophyte involved in the infection, for example *T. mentagrophytes* var. *erinacei* is usually acquired from hedgehogs and *M. gypseum* from the soil. Generalized infection is uncommon in dogs and is often associated with conditions such as hyperadrenocorticism and immunosuppression.

Box 43.1 Dermatophytes of dogs.

- *Microsporum canis*
- *M. gypseum*
- *Trichophyton mentagrophytes*
- *T. mentagrophytes* var. *erinacei*

Treatment and control

Because the dermatophytoses are zoonoses, treatment and control are particularly important in domestic carnivores.

- If lesions are limited in extent, treatment with preparations such as lime sulphur or miconazole shampoo may be effective (Moriello and De Boer, 1995). A solution of 0.2% enilconazole is approved in most countries for use in dogs, cats, horses and cattle.
- Clipping of the haircoat is advisable, particularly if lesions are extensive.

The clippings, which contain numerous infective arthrospores, must be disposed of carefully.

- Itraconazole, fluconazole or terbinafine, administered orally, are the drugs of choice for systemic therapy. Because they are potentially teratogenic, azole drugs should not be given to pregnant animals. Although griseofulvin has been used for many years for the treatment of dermatophytosis, it is used less frequently because of the risks of teratogenicity. In addition, griseofulvin can induce neutropenia and should not be given to cats with feline immunodeficiency virus infection.
- Animals with suspicious lesions should be isolated.
- Early laboratory confirmation is essential.
- In-contact animals should be examined under a Wood's lamp and closely monitored for skin lesions.
- Contaminated areas should be vacuum-cleaned to remove infected skin debris and hairs.
- Contaminated bedding should be burnt and grooming equipment should be disinfected with 0.5% sodium hypochlorite.
- A number of vaccines for use in dogs and cats have been produced over the years but a vaccine of proven efficacy still appears to be lacking (Lund and DeBoer, 2008).

Dermatophytosis in cattle

Trichophyton verrucosum is the usual cause of ringworm in cattle. Calves are affected most commonly and often develop characteristic lesions on the face and around the eyes. In heifers and cows, lesions may be present on the neck and limbs. Oval areas of affected skin are alopecic with greyish white crusts. Infection is most common in winter months, with a number of animals usually affected. Bovine dermatophytosis is usually self-limiting. However, individual valuable animals may require treatment. Topical preparations such as 5% lime sulphur, captan (1:300) or natamycin may be effective. Individual lesions can be treated with fluconazole, itraconazole or terbinafine. Commercial vaccines containing an attenuated strain of *T. verrucosum* (LTF-130 or CCM 8165) have been used successfully for the control of bovine dermato- phytosis in Europe and Russia (Gordon and Bond, 1996 ; Lund and DeBoer, 2008).

Dermatophytosis in horses

Trichophyton equinum is the main cause of ringworm in horses. *Trichophyton equinum* var. *autotrophicum* is a variety that lacks the requirement for nicotinic acid and although relatively specific for the horse is uncommon and is limited in geographical distribution. *Microsporum equinum*, now considered to be identical to *M. canis* (Graser *et al*., 2000), occurs from time to time in young horses. Transmission occurs by direct contact or from contaminated harness and grooming gear. The distribution of the skin lesions may indicate the likely source of the infection. Lesions may be limited to the girth strap or saddle regions or may be widely distributed if grooming gear is contaminated. Infection caused by *M. gypseum* can be acquired from rolling in soil, with lesions usually confined to the dorsum. *Trichophyton mentagrophytes* is occasionally isolated from horses, and *T. verrucosum* infections may be acquired from contact with infected cattle. Horses under 4 years of age are particularly susceptible to dermatophytosis. Treatment with topical preparations such as 5% lime sulphur, natamycin or azole compounds is usually effective. Affected animals must be isolated and contaminated harness and grooming gear should be disinfected with 0.5% sodium hypochlorite. In some European countries, vaccination is used to prevent transmission of infection in groups of animals (Lund and DeBoer, 2008).

Dermatophytosis in pigs

Dermatophytosis in pigs is uncommon and is usually caused by M. nanum. The condition, which can be endemic in a herd, may not be recognized, particularly in pigs with pigmented skin (Ginther, 1965). All ages are susceptible and lesions can occur anywhere on the body surface as thick brownish crusts. Ringworm in pigs is not of economic importance.

Favus in poultry

Gallinaceous birds are occasionally infected with *M. gallinae*, the cause of avian ringworm or favus. White patchy crusts develop on the comb and wattles. If the disease is severe, feather follicles may be invaded and affected birds may show signs of systemic illness.

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Chapter 44

Aspergillus species

Among the saprophytic moulds, *Aspergillus* species are widely distributed. Although the genus contains more than 190 species, only a limited number of these have been implicated in opportunistic infections in animals and humans. *Aspergillus fumigatus* is the species most often involved in tissue invasion. Aspergillosis may also be caused by other potentially invasive species including *A. niger, A. flavus, A. terreus, A. deflectus, A. nidulans* and *A. flavipes*. *Aspergillus* species are members of the phylum *Ascomycota*. The teleomorph or sexual stage is not known for many *Aspergillus* species. Recently, the sexual reproductive cycle of *Aspergillus fumigatus* was demonstrated and the teleomorph *Neosartorya fumigata* was described (O'Gorman *et al*., 2008). The hyphae are septate, hyaline and up to 8.0 µm in diameter. Unbranched conidiophores develop at right angles from specialized hyphal foot cells. The tip of the conidiophore enlarges to form a vesicle which becomes partially or completely covered with flask-shaped phialides. The phialides produce chains of round pigmented conidia (phialoconidia) which may be smooth or rough and are up to 5.0 µm in diameter (Fig. 44.1). Aspergilli are aerobic and grow rapidly, forming distinct colonies after incubation for 2 to 3 days. The colour of the obverse side of colonies, which may be bluish green, black, brown, yellow or reddish, varies with individual species and with cultural conditions. *Aspergillus fumigatus*, a thermotolerant species, grows at temperatures ranging from 20°C to 50°C.

Key points

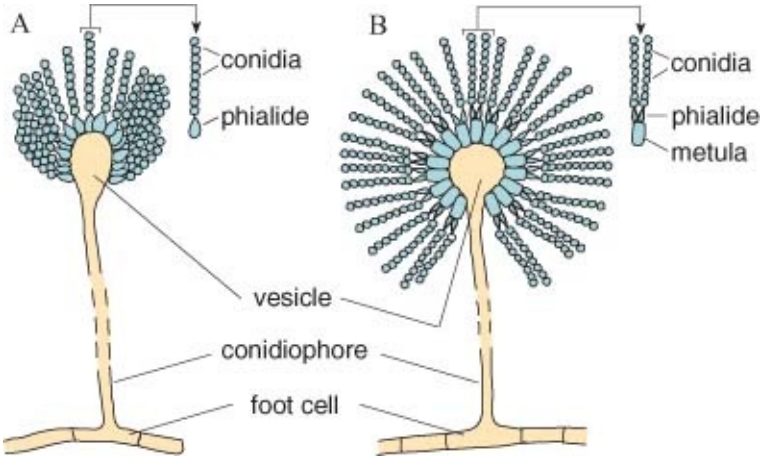
- embers of the phylum *Ascomycota*
- biquitous, saprophytic moulds with septate hyaline hyphae
- apidly growing pigmented colonies
- igmented conidia formed from phialides borne on vesicles
- espiratory pathogens, acquired by inhalation of spores
- *Aspergillus fumigatus* responsible for the majority of infections in animals
- oxins elaborated by *Aspergillus flavus* in stored food cause aflatoxicosis

Respiratory infections may occur following inhalation of spores. Less commonly, infection can result from ingestion of spores or following tissue trauma. Systemic infection is invariably associated with immunosuppression. Species such as *A. flavus*, which elaborate potent toxins when growing in cereals and other foods, cause mycotoxicosis (see Chapter 51).

Usual habitat

Aspergilli are common soil inhabitants and are also found in large numbers in decomposing organic matter. *Aspergillus fumigatus* often occurs in overheated, poor quality hay and in compost heaps. Spores of *Aspergillus* species are present in dust and air.

Figure 44.1 Sporing heads of two *Aspergillus* species. Differences in the shape of the vesicles and conidial arrangement is evident. The phialides of *A. fumigatus* (A) are borne directly on the vesicle (uniseriate) whereas those of *A. niger* (B) are borne on metulae (biseriate).

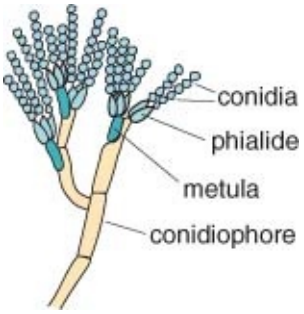


Recognition of *Aspergillus* species

Aspergillus species grow on standard laboratory media such as Sabouraud dextrose agar. Because the genus contains a large number of species, differentiation is difficult. A small number of the species are responsible for the majority of infections in animals and a presumptive identification may be made on the basis of colonial appearance and the conidial arrangement on sporing heads.

- Colonies can be up to 5 cm in diameter after incubation for 5 days. The colour of the reverse side is pale yellow to light tan. The colour of the obverse side is determined by the pigmentation of the conidia:
  – *A.fumigatus* colonies rapidly become velvety or granular and bluish green with narrow white peripheries. Older colonies are slate grey.
  – *A. niger* colonies are black and granular, features imparted by their large pigmented sporing heads.
  – *A. flavus* colonies are yellowish green with a fluffy texture.
  – *A. terreus* colonies are cinnamon brown with a granular texture.
- Sporing heads, stained with lactophenol cotton blue and examined with low and high dry magnification, have characteristic features. These include size and shape of vesicles, position of phialides, and the size, shape and colour of conidia. Differentiating features of *A. fumigatus* and *A. niger* sporing heads are illustrated in Fig. 44.1.
- Because their colonies can be similar in appearance, microscopic differentiation of *A. fumigatus* from some *Penicillium* species may be necessary. The conidiophores of *Penicillium* species often possess secondary branches (metulae), bearing several phialides (Fig. 44.2).
- For definitive identification it may be necessary to induce and examine the teleomorphic form of an *Aspergillus* isolate, a procedure carried out in reference laboratories.

Figure 44.2 Sporing head of *Penicillium* species. Colonies can resemble those produced by *Aspergillus fumigatus*.



Pathogenesis and pathogenicity

Infection with *Aspergillus* species, mainly *A. fumigatus*, has been recorded in many species of animals. Aspergillosis, which is primarily a respiratory infection, follows spore inhalation. Because the spores of *A. fumigatus* are small,

they can pass through the upper respiratory tract and may be carried to the terminal parts of the bronchial tree (Amitani *et al*., 1995). Germination of inhaled spores and hyphal invasion of tissues depend on a number of factors. A true virulence factor has not been demonstrated but rather a number of factors appear to combine to facilitate disease production (Tomee and Kauffman, 2000). *Aspergillus* spores adhere to collagen, fibrinogen, fibronectin and laminin. Pathogenic *Aspergillus* species grow well at 37°C and produce a wide variety of extracellular enzymes. Proteases with elastase, fibrinolytic and anticoagulative properties are considered to be important. In addition, a metabolite of *A. fumigatus*, gliotoxin, inhibits both the activity of cilia and phagocytosis by macrophages.

Immune competence of the host largely determines the outcome of infection. Factors that may modify immune competence include corticosteroid therapy and long-term treatment with antimicrobial drugs. Interference with both neutrophil and monocyte function may predispose to tissue invasion. Hyphal invasion of blood vessels leads to vasculitis and thrombus formation. Mycotic granulomas may develop in the lungs and occasionally in other internal organs.

Diagnostic procedures

- Certain specific clinical conditions such as guttural pouch mycosis may suggest the involvement of *Aspergillus* species.
- Endoscopic examination can be used to detect lesions in the nasal cavity and guttural pouch.
- For confirmation of aetiological involvement, tissue invasion by fungi must be demonstrated in biopsy specimens or tissues taken at post-mortem, and *Aspergillus* species must be isolated from specimens.
- Tissue sections stained by methenamine silver or by the PAS method may reveal hyphal invasion.
- For isolation, small tissue specimens are applied to the scarified surface of Sabouraud dextrose agar and incubated aerobically at 37°C for 2 to 5 days. Hyphae grow from specimens forming colonies.
- Identification criteria:
  - Colonial morphology
  - Appearance of sporing heads including conidia (Fig. 44.1)
  - Growth at 45°C to 50°C (thermotolerant species).

- Molecular procedures, such as the polymerase chain reaction technique, are being developed to detect *A. fumigatus* in clinical specimens (Spreadbury *et al*., 1993; O'Sullivan *et al*., 2003; Peeters *et al*., 2008).
- Serological tests are based on growth phase or hyphal-specific antigens of *A. fumigatus*. As a consequence of constant exposure, most animals develop antibodies to conidial antigens and serological results must be interpreted with caution. In dogs, the most sensitive serological test is considered to be the ELISA. A commercial human ELISA that detects a cell wall component, β-1,3-D-glucan, in blood and bronchoalveolar lavage samples has been applied with some success to avian serum samples (Cray *et al*., 2009).

Clinical infections

Clinical cases of aspergillosis are comparatively uncommon and usually sporadic. Birds appear to be more susceptible to aspergillosis than mammals and this susceptibility has been attributed to differences in innate and acquired immunity to the fungus in avian and mammalian species. Infections often involve the respiratory tract although localized infections with *A. fumigatus* have been recorded in other organs. Mycotic mastitis occasionally results from the accidental introduction of *A. fumigatus* spores into the mammary gland on an intramammary tube. *Aspergillus fumigatus* is sometimes involved in mixed infections associated with otitis externa. The clinical conditions caused by *Aspergillus* species in domestic animals are summarized in Table 44.1. Rarely, other fungi such as *Penicillium* species, *Paecilomyces* species and *Scedosporium apiospermum* may cause opportunistic infections similar to those caused by *Aspergillus* species (Watt *et al*., 1995). The spores of *Aspergillus fumigatus* are among the allergens reported to be capable of inducing the allergic condition chronic obstructive pulmonary disease (COPD) in horses, also known as recurrent airway obstruction and 'heaves' (McGorum *et al*., 1993).

Table 44.1 Clinical conditions caused by *Aspergillus* species in domestic animals.

Hosts	Condition	Comments
Birds	Brooder pneumonia	Occurs in newly hatched chickens in incubators
	Pneumonia and airsacculitis	Chickens and poults up to 6 weeks of age are most susceptible; older birds sometimes affected
	Generalized aspergillosis	Dissemination of infection usually from the respiratory tract

Horses	Guttural pouch mycosis	Confined to guttural pouch, often unilateral
	Nasal granuloma	Produces a nasal discharge and interferes with breathing. Fungi other than *Aspergillus* spp. may initiate this condition
	Keratitis	Localized infection following ocular trauma
Cattle	Mycotic abortion	Occurs sporadically; produces thickened placenta and plaques on skin of aborted foetus
	Mycotic pneumonia	Uncommon condition of housed calves
	Mycotic mastitis	May result from the use of contaminated intramammary antibiotic tubes
Dogs	Nasal aspergillosis	Invasion of nasal mucosa and turbinate bones; occurs periodically
	Otitis externa	*Aspergillus* species may constitute part of a mixed infection
	Disseminated aspergillosis	Uncommon; may result in osteomyelitis or discospondylitis
Cats	Systemic aspergillosis	Rarely encountered; immunosuppressed animals are at risk

Brooder pneumonia in young chickens

This disease affects newly hatched chickens which are exposed to high numbers of *A. fumigatus* spores. Affected chickens develop somnolence and inappetence, and many die. Yellowish nodules are present in the lungs, air sacs and, occasionally, in other organs. Histopathological evidence of tissue invasion by fungi and culture of *A. fumigatus* from lesions are required for confirmation. Strict hygiene and routine fumigation of incubators are effective control measures.

Aspergillosis in mature birds

Infection in mature birds frequently follows inhalation of spore-laden dust derived from contaminated litter or feed. Poultry and captive penguins, raptors and psittacine birds may be affected. Penguins are susceptible to infection if kept at unsuitably high ambient temperatures, whereas infection in raptors has been attributed to *A. fumigatus* spores from shredded wood bark on aviary floors. Clinical signs, which are variable, include dyspnoea and emaciation. Yellowish nodules resembling lesions of avian tuberculosis can be observed in the lungs and air sacs. Dissemination may occur to other internal organs. Diagnosis is confirmed by histopathology and culture.

Guttural pouch mycosis

This condition, which is frequently associated with *Aspergillus* species infection, particularly *A. fumigatus*, is usually unilateral (Ludwig *et al*., 2005). Lesions, often plaque-like, develop in the mucosa of the pouch wall. When fungal hyphae penetrate to deeper tissues, they cause tissue necrosis, thrombosis, erosion of

blood vessel walls and neural damage. The clinical signs include epistaxis, dysphagia and laryngeal hemiplegia. Postauricular swelling and unilateral nasal discharge may follow accumulation of inflammatory exudates in the pouch. Diagnosis is based on clinical signs, radiographic evidence of fluid accumulation in the pouch and demonstration of characteristic lesions by endoscopy. Confirmation is based on demonstration of fungal hyphae in biopsy specimens and isolation of *A. fumigatus* from lesions. Therapeutic options include infusion of antifungal agents such as itraconazole into the pouch and surgical intervention to deal with serious haemorrhage. Oral or systemic antifungal therapy is used infrequently because of potential toxicity and excessive cost.

Nasal aspergillosis in dogs

Canine nasal aspergillosis is encountered in young to middle-aged dolichocephalic breeds. Clinical signs, which are often unilateral, include persistent, profuse sanguinopurulent nasal discharge with sneezing and bouts of epistaxis. Radiography may reveal an increased radiolucency of turbinate bones. Serology may suggest aspergillosis (Billen *et al*., 2009) but culture and histopathological examination of biopsy material are essential for confirmation.

Administration of itraconazole through tubes inserted surgically in the frontal sinuses and nasal chambers may be used together with systemic treatment with fluconazole or voriconazole which should continue for 6 to 8 weeks.

Mycotic abortion in cows

This form of abortion occurs sporadically and its prevalence may be influenced by poor quality contaminated fodder harvested in wet seasons. *Aspergillus fumigatus* can proliferate in damp hay, in poor quality silage and in brewers' grains. Infection, which reaches the uterus haematogenously, causes placentitis leading to abortion late in gestation. Affected cows usually show no signs of systemic illness. Intercotyledonary areas of the placenta are thickened and leathery and the cotyledons are necrotic. Aborted foetuses may have raised cutaneous plaques resembling ringworm lesions. Diagnosis is based on culture of *A. fumigatus* from foetal abomasal contents and histopathological evidence of mycotic placentitis.

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Chapter 45

Yeasts and disease production

Yeasts are eukaryotic, round or oval, single-celled organisms. During asexual reproduction, blastoconidia, also referred to as buds or daughter cells, develop. Blastoconidia, produced linearly without separation, may elongate to form a pseudohypha. Yeasts, such as *Candida* species, can produce true septate hyphae in animal tissues or when growing deeply in agar media. Yeasts grow aerobically on Sabouraud dextrose agar, and the species capable of tissue invasion grow well at 37°C. Colonies, which are usually moist and creamy in texture, resemble large bacterial colonies. Formerly, yeasts were classified as Fungi Imperfecti. Based on demonstration of a teleomorph or by utilizing sequencing data, the genera of veterinary importance have been placed in either phylum *Ascomycota* (*Candida, Macrorhabdus* and *Geotrichum*) or phylum *Basidiomycota* (*Cryptococcus, Malassezia* and *Trichosporon*).

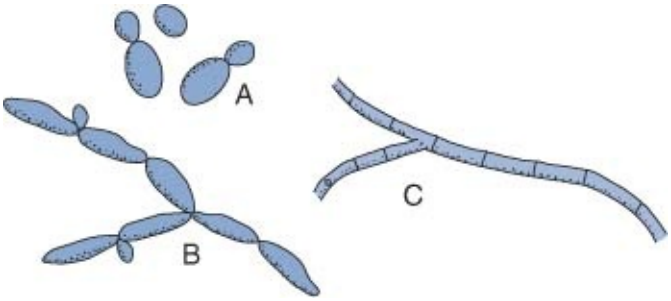
Yeasts are found in the environment, often on plants or plant materials. They may also occur as commensals on the skin or mucous membranes of animals. They cause opportunistic infections which are categorized as exogenous, when derived from the environment, or endogenous, when resulting from overgrowth of commensals. Immunosuppression or factors such as prolonged antimicrobial therapy which disturb the resident flora on mucosal surfaces may facilitate yeast overgrowth leading to tissue invasion. Yeasts of importance in animal disease are *Candida* species (particularly *C. albicans*), *Cryptococcus neoformans* and *Malassezia pachydermatis*. *Macrorhabdus ornithogaster* (formerly referred to as ‘megabacteria’) is a yeast found in the proventriculus of several avian species. It is associated with ‘going light’ in budgerigars, a fatal disease characterized by progressive weight loss. Other yeasts, such as *Trichosporon beigelii,* and the yeast-like mould *Geotrichum candidum* rarely cause infection.

Key points

- Eukaryotic, unicellular budding cells
- Asexual reproduction by blastoconidia

- Pseudohyphae or true hyphae may be formed
- Teleomorphs belong to either phylum *Ascomycota* or phylum *Basidiomycota*
- *Candida albicans:*
  - – Grows at 37°C on a wide range of media
  - – Chlamydospores produced on cornmeal agar
  - – Germ tubes formed in serum within 2 hours at 37°C
  - – Resistant to cycloheximide
  - – Commensal on mucocutaneous surfaces; uncommon in the environment
  - – Opportunistic infections, related to immunosuppression, in animals and humans
- *Cryptococcus neoformans:*
  - – Large mucopolysaccharide capsule produced
  - – Grows at 37°C on a variety of media, producing mucoid colonies
  - – Teleomorph is a basidiomycete
  - – Utilizes creatinine in bird droppings
  - – Opportunistic infection derived from environmental sources
  - – Localized granulomas or sometimes disseminated disease in cats, dogs, horses and cattle
- *Malassezia pachydermatis:*
  - – Bottle-shaped cells
  - – Monopolar budding
  - – Commensal on the skin of mammals and birds
  - – Associated with canine seborrhoeic dermatitis and otitis externa

Figure 45.1 Three forms of the polymorphic yeast *Candida albicans:* budding yeast cell (A); pseudohypha (B); true septate hypha (C).



Candida species

In the genus *Candida* there are more than 200 species. *Candida albicans* is the species most often implicated in animal disease. It grows aerobically at 37°C on a wide range of media including Sabouraud dextrose agar. Colonies are

composed of budding oval cells approximately 5.0 × 8.0 µm. In animal tissues, *C. albicans* may exhibit polymorphism in the form of pseudohyphae or hyphae (Fig. 45.1). On certain media, it characteristically produces thick-walled resting cells known as chlamydospores (chlamydoconidia).

Usual habitat

Candida species occur worldwide on plant materials and, as commensals, in the digestive and urogenital tracts of animals and humans. *Candida albicans* is isolated from environmental sources less frequently than other *Candida* species, suggesting adaptation towards a parasitic rather than a saprophytic existence.

Differentiation of *Candida* species

- Most *Candida* species have a similar colonial appearance. Colonies, which are whitish, shiny and convex, are 4 to 5 mm in diameter after incubation for 3 days.
- Subculturing on to an indicator medium allows presumptive identification of *C. albicans*, *C. krusei* and *C. tropicalis* on the basis of colonial appearance (Odds and Bernaerts, 1994).
- Carbohydrate assimilation and fermentation tests, which are usually performed in reference laboratories, allow definitive species identification.
- Commercially available biochemical test kits, giving results within 24 to 48 hours, are usually used for species differentiation in diagnostic laboratories.
- Features of *C. albicans* used for presumptive identification include:
  – Growth at 37°C
  – Production of chlamydospores in submerged cultures on cornmeal agar (Fig. 45.2)
  – Production of germ tubes within 2 hours when incubated in serum at 37°C (Fig. 45.3)
  – Growth on Sabouraud dextrose agar containing cycloheximide.

Figure 45.2 Thick-walled resting cells of *Candida albicans*, called chlamydospores (chlamydoconidia). These resting cells are formed from pseudohyphae when submerged colonies grow in cornmeal agar. The smaller cells are blastoconidia (arrow).

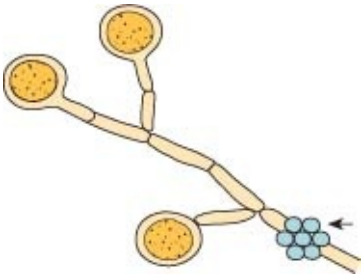
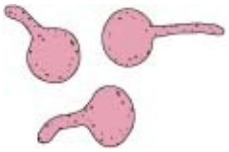


Figure 45.3 Germ tubes form within 2 hours when cells of *Candida albicans* are incubated in serum at 37°C.



Pathogenesis and pathogenicity

Candida albicans, the principal yeast involved in animal disease, possesses a number of putative virulence factors (Cutler, 1991). The organism has surface integrin-like molecules which allow adhesion to matrix proteins. In addition, surface structures can bind fibrinogen and complement components. Production of proteases and phospholipases may aid tissue invasion. Phenotypic switching and biofilm formation, which have been demonstrated in *C. albicans*, may facilitate evasion of host defence mechanisms.

During the early stages of infection, phagocytic clearance mechanisms eliminate most of the yeast cells. Cells that are not cleared rapidly convert to hyphal forms. This transition from budding to hyphal forms probably facilitates tissue penetration and increases resistance to phagocytosis due to the larger size of the hyphae. Phospholipases, concentrated in hyphal tips, appear to enhance invasiveness. The localized mucocutaneous form of candidiasis is associated with overgrowth of resident *C. albicans* in the oral cavity or gastrointestinal and urogenital tracts. Predisposing factors include defects in cell-mediated immunity, concurrent disease, disturbance of the normal flora by prolonged use of antimicrobial drugs and damage to the mucosa from indwelling catheters. Affected mucosa is thickened and often hyperaemic.

Haematogenous spread may occur following vascular invasion by hyphae or pseudohyphae, producing systemic lesions.

Diagnostic procedures

- Suitable specimens for culture and histopathology include biopsy or post-mortem tissue samples and milk samples.
- Tissue sections, stained by PAS or methenamine silver methods, may reveal budding yeast cells or hyphae.
- Culture is carried out aerobically at 37°C for 2 to 5 days on Sabouraud dextrose agar, with or without cycloheximide.
- Identification criteria for isolates:
  – Characteristic colonies yielding budding yeast cells.
  – Growth on media containing cycloheximide (specific for *C. albicans*).
  – Colonial appearance on CHROMagar Candida.
  – Biochemical profile.
  – Chlamydospore and germ tube production (specific for *C. albicans*).
- Molecular techniques have been used for the identification of *Candida* species in clinical veterinary samples but are not routinely applied (Kano *et al*., 2002).

Clinical infections

Opportunistic infections with *Candida* species, which occur sporadically, are usually associated with immunosuppression or the prolonged use of antimicrobial drugs. The clinical conditions attributed to *Candida* species are presented in Table 45.1. Overgrowth of commensal *Candida* species may result in localized mucosal damage in parts of the digestive or urogenital tracts.

Thrush of the oesophagus or crop in young chickens may be associated with prolonged antibiotic administration, debilitating conditions such as intestinal coccidiosis, or unsanitary, overcrowded housing conditions. Mycotic stomatitis has been reported in pups, kittens and foals (McClure *et al*., 1985). *Candida albicans* has been implicated in gastro-oesophageal ulceration in pigs and foals (Kadel *et al*., 1969; Gross and Mayhew, 1985). Rarely, disseminated candidiasis may occur in pigs, calves, dogs and cats.

Table 45.1 Clinical conditions associated with *Candida albicans*.

Hosts	**Clinical conditions**
Pups, kittens, foals	Mycotic stomatitis
Pigs, foals, calves	Gastro-oesophageal ulcers
Calves	Rumenitis
Dogs	Enteritis, cutaneous lesions
Chickens	Thrush of the oesophagus or crop

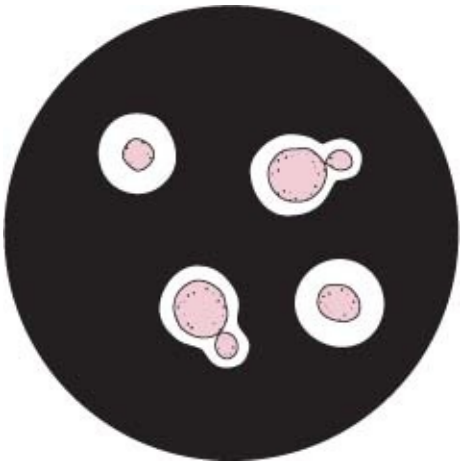
Geese, turkeys	Cloacal and vent infections
Cows	Reduced fertility, abortion, mastitis
Mares	Pyometra
Cats	Cystitis, pyothorax
Cats, horses	Ocular lesions
Dogs, cats, pigs, calves	Disseminated disease

Bovine abortion caused by *Candida* species has been recorded (Foley and Schlafer, 1987). In addition, a number of *Candida* species have been isolated from cases of bovine mastitis (Richard *et al*., 1980). Mycotic mastitis occurs sporadically either as a consequence of contaminated intramammary preparations or from heavy environmental contamination (Elad *et al*., 1995). Usually one quarter is involved and spontaneous elimination of the infection frequently occurs. Rarely, yeast cells may be shed for up to 12 months.

Cryptococcus species

Although the genus *Cryptococcus* contains more than 30 recognized species, only *C. neoformans* produces opportunistic infections. On the basis of polysaccharide capsular antigens, five serotypes of *C. neoformans* are recognized (A, B, C, D and AD hybrid). Originally two variants of *C. neoformans* were described, C. *neoformans* var. *neoformans* (serotypes A, D and AD) and *C. neoformans* var. *gattii* (serotypes B and C). However, two morphologically distinct teleomorphs, *Filobasidiella neoformans* and *F. bacillispora*, produced by these two variants have been demonstrated and *C. gattii* is now considered a separate species. In addition, significant genetic variations have been shown between serotypes A and D resulting in the recognition of serotype A as a separate variant, *C. neoformans* var. *grubii*. Currently, *C. neoformans* is considered to be a species complex consisting of two variants, *C. neoformans* var. *grubii* (A), *C. neoformans* var. *neoformans* (D) and subspecies *C. gatii* (B, C). Based on genetic analysis, nine molecular types have been described within the species complex and further taxonomic refinements are expected. The majority of infections in humans, which occur predominantly in immunocompromised individuals, are associated with *C. neoformans* var. *grubii*.

Figure 45.4 Cells of *Cryptococcus neoformans* as they appear in an India ink preparation. A narrow neck joins the mother cell and its bud. Prominent mucopolysaccharide capsules are a characteristic feature of this yeast.



The yeast cells are round to oval and 3.5 to 8.0 μm in diameter. A daughter cell is formed as a bud, on a narrow neck, from the mother cell. When recovered directly from affected animals, the yeasts have thick mucopolysaccharide capsules which can be demonstrated in India ink preparations (Fig. 45.4). The capsules can also be observed in tissue sections stained with mucicarmine. *Cryptococcus* species are aerobic, non-fermentative organisms which form mucoid colonies on a variety of media including Sabouraud dextrose agar. The ability to grow at 37°C distinguishes *C. neoformans* from other *Cryptococcus* species. Filamentous forms (hyphae and pseudohyphae) have been described but are rare. On account of their small size it has been suggested that basidiospores may be important infectious forms.

The clinical conditions caused by *C. neoformans* in domestic animals are presented in Table 45.2.

Usual habitat

Cryptococcus neoformans var. *grubii* and *C. neoformans* var. *neoformans* can be isolated from the droppings of pigeons and other birds and from soil enriched by these droppings. Creatinine, present in the droppings, is utilized by this yeast. Pigeons with *C. neoformans* in their intestinal tracts can excrete the organism for several months without developing disease.

Table 45.2 Clinical conditions caused by *Cryptococcus neoformans* in domestic animals.

Hosts	Clinical condition
Cats	Respiratory, cutaneous, neural and ocular infections
Dogs	Disseminated disease with neural and ocular signs
Cattle	Mastitis, nasal granulomas

Horses	Nasal granulomas, sinusitis, cutaneous lesions, pneumonia, meningoencephalitis, abortion

Cryptococcus gattii can be isolated from a number of tree species, particularly *Eucalyptus* species in Australia, and has been spread in timber products.

Laboratory recognition of *C. neoformans*

- Colonies of *Cryptococcus* species, which are mucoid when first isolated due to the presence of capsular material, become dry with age. They may have a cream, tan or yellowish appearance.
- Budding yeasts with wide capsules can be demonstrated in India ink preparations (Fig. 45.4).
- Most *Cryptococcus* species produce urease, rapidly hydrolysing urea to ammonia.
- Differentiation of species is possible using carbohydrate assimilation tests or commercially available biochemical kits.
- Identification criteria for *C. neoformans*:
  – Ability to grow at 37°C
  – Brown colonies on birdseed agar as a result of phenol oxidase production
  – Melanin demonstrable in cell walls using the Masson–Fontana stain on tissue sections.
- *Cryptococcus gattii* can utilize glycine as the sole source of nitrogen and is resistant to canavanine. In contrast, *Cryptococcus neoformans* var. *grubii* and *C. neoformans* var. *neoformans* cannot utilize glycine as a sole source of nitrogen and are susceptible to canavanine.

Pathogenesis and pathogenicity

Infection occurs through inhalation of *C. neoformans* cells in contaminated dust. Some yeast cells may be trapped in the nasal cavities or sinuses, while others are deposited in the lungs. Virulence factors of *C. neoformans* include the capsule, which is antiphagocytic, the ability to grow at mammalian body temperature and the production of phenol oxidase. Mutants that have lost one of these attributes are avirulent. The virulence arising from phenol oxidase activity may relate to the degradation of catecholamine which results in the accumulation of melanin in the yeast cell walls protecting against the toxic effects of free radicals (Jacobson and Emery, 1991). Production of phospholipases, involved in

membrane disruption during tissue invasion, is also thought to be an important virulence factor. Phenotypic switching has been described for *C. neoformans* (Jain and Fries, 2008).

Immunocompetent animals can mount an effective cell-mediated response to *C. neoformans*. Dissemination from the respiratory tract to brain, meninges, skin and bones is usually associated with defective cellmediated immunity. Lesions associated with *C. neoformans* infection range from discrete granulomas to tumour-like myxomatous masses composed of yeast cells in a connective tissue matrix. Small granulomas may be present in the lungs of clinically normal animals.

Diagnostic procedures

Care must be exercised when handling material from suspect *C. neoformans* cases because of the risk of acquiring infection.

- Suitable specimens for laboratory examination include exudates, cerebrospinal fluid and biopsy or post-mortem tissues.
- Budding yeasts with characteristic, thick capsules can be demonstrated in fluid samples using India ink preparations (Fig. 45.4).
- In tissue sections, yeast capsules are demonstrated by Mayer's mucicarmine method. Melanin can be detected in cell walls of *C. neoformans* by the Masson–Fontana technique.
- Specimens, cultured on Sabouraud dextrose agar with chloramphenicol but without cyclohexamide, are incubated aerobically at 37°C for up to 2 weeks.
- Identification criteria for isolates:
  - Mucoid colonies
  - Presence of capsules
  - Urease activity
  - Brown colonies on birdseed agar and growth at 37°C (specific for *C. neoformans*).
- A latex agglutination test or ELISA, which detect soluble capsular material of *C. neoformans* within 3 weeks of infection, can be used on samples of cerebrospinal fluid, serum and urine.
- A PCR assay for the detection of *C. neoformans* in cats has been described (Kano *et al*., 2001).

Clinical infections

Apart from sporadic cases in cats and dogs, cryptococcosis in domestic animals is relatively rare (Table 45.2). In companion animals, clinical signs of cryptococcosis usually relate to the nasal cavity or skin involvement. The disease in dogs, which is less common than in cats, is often disseminated with neurological and ocular signs (Jergens *et al*., 1986). Cryptococcosis has been recorded infrequently in horses. Clinical signs include nasal granulomas and sinusitis (Scott *et al*., 1974), pneumonia (Hilbert *et al*., 1980), meningoencephalitis and abortion (Blanchard and Filkins, 1992). *Cryptococcus neoformans* is a rare cause of mastitis in dairy cattle. Avian cryptococcosis has occasionally been described (Malik *et al*., 2003).

Feline cryptococcosis

Nasal, cutaneous, neural and ocular forms of cryptococcosis are recognized in cats. The nasal form, which accounts for approximately 70% of cases, is characterized by flesh-coloured, polyp-like granulomas in the nasal cavity. Cutaneous lesions, often affecting the face, head and neck, are reported in about 30% of cases. Peripheral lymphadenopathy is common. Neurological signs are evident in about 25% of cases and, in some instances, chorioretinitis may be evident.

Surgical removal combined with parenteral antifungal drugs is the usual method for treating cutaneous cryptococcosis. There may be a favourable response to amphotericin B with flucytosine or to ketoconazole, itraconazole or fluconazole (Medleau *et al*., 1990; Malik *et al*., 1992). Therapy should continue for at least 2 months. The latex agglutination test can be used to monitor the effects of antifungal therapy and declining capsular antigen levels indicate a favourable response to treatment (Medleau *et al*., 1990).

Malassezia pachydermatis

Malassezia species, commensals on the skin of animals and humans, are aerobic, non-fermentative, urease- positive yeasts which grow at 35°C to 37°C. There are 11 species recognized currently in the genus (*M. equi*, found on the skin of normal horses, has not yet been formally recognized). All species, with the exception of *M. pachydermatis*, are lipid-dependent on account of an inability to synthesize C14 or C16 fatty acids *de novo* (Ashbee, 2007). One species in

particular, *Malassezia pachydermatis* (formerly *Pityrosporum canis*) is of veterinary importance. The cells of *M. pachydermatis*, which are bottle-shaped, thick walled and up to 5 µm in length, reproduce by monopolar budding on a broad base. Multiple budding may occur from the same site on a mother cell. After repeated budding, a distinct collarette forms at this site (Fig. 45.5). Pseudohyphae may be produced infrequently in tissues (Guillot *et al*., 1998).

Figure 45.5 Bottle-shaped cells of the yeast, *Malassezia pachydermatis*. Monopolar budding on a broad base, with the formation of a prominent collarette, is a characteristic of this yeast.

Usual habitat

Malassezia pachydermatis can be found on the skin of mammals and birds, particularly in areas rich in sebaceous glands. The anal region, external ear canal, lips and interdigital skin of dogs are frequently colonized by this yeast (Bond *et al*., 1995b). Three lipid-dependent species, *M. furfur, M. globosa* and *M. sympodialis*, have been isolated from healthy cats. In many instances, mixed cultures containing two or even three *Malassezia* species, *M. pachydermatis, M. furfur* and *M. sympodialis*, are recovered from canine and feline specimens (Raabe *et al*., 1998).

Identification of *Malassezia pachydermatis*

- The unique budding pattern is demonstrable in microscopic preparations stained with methylene blue.
- *Malassezia pachydermatis* is the only member of the genus which grows on Sabouraud dextrose agar without lipid supplementation. Colonies, which are dull, opaque and cream- coloured, have a smooth surface.

Pathogenesis and pathogenicity

Malassezia pachydermatis is an opportunist and, in dogs, has been associated with two clinical conditions, otitis externa and dermatitis. Colonization and growth of the organism in these locations may be associated with

immunosuppression and other predisposing factors which alter the microclimate of the skin or ear canal. The yeast cells produce sebum-altering lipases and when present in high numbers they apparently induce excessive sebaceous secretion, a feature of seborrhoeic dermatitis (Akerstedt and Vollset, 1996). Zymogens in the yeast cell wall can activate the complement cascade leading to damage to keratinocytes, inflammation and pruritus. In otitis externa, the production of proteolytic enzymes by *M. pachydermatis* results in damage to the mucosa of the ear canal. Excessive production and retention of wax, a consequence of ceruminous gland hypersecretion, combined with the activity of *M. pachydermatis* and other microorganisms, contribute to inflammatory changes. Inflammatory exudate and necrotic debris accumulate in the canal.

Genetic characterization of *M. pachydermatis* isolates indicates that up to four different genetic types occur. While a single genetic type predominates, the other three types have been isolated only from the ear canals of dogs (Aizawa *et al*., 2001; Castellá *et al*., 2005).

Diagnostic procedures

- Involvement of *M. pachydermatis* should be considered in otitis externa and in canine seborrhoeic dermatitis. Cytological examination is the most useful technique for assessing increases in populations of *M. pachydermatis*. Small numbers of yeast cells may not be significant and as a general guide the finding of one or more organisms per oil-immersion field in association with clinical signs can be considered significant (Chen and Hill, 2005).
- Exudate from affected ear canals should be submitted for laboratory examination.
- Clear adhesive strips pressed on to skin lesions several times, impression smears, vigorous swabbing of the skin surface or superficial skin scraping may be used to collect suitable material for staining and examination for yeast cells. In severe dermatitis, biopsy of skin may be considered.
- Characteristic yeast cells are demonstrable in exudates stained with methylene blue (Fig. 45.5).
- *Malassezia pachydermatis* can be cultured aerobically at 37°C for 3 to 4 days on Sabouraud dextrose agar containing chloramphenicol. Dixon’s agar, a lipid- supplemented agar, supports the growth of both *M. pachydermatis* and the lipid-dependent *Malassezia* species.
- Identification criteria for isolates:

– Colonial appearance

– Growth without lipid supplementation (consistent with *M. pachydermatis*)

– Characteristic microscopic appearance

– Molecular methods are available for the differentiation of *Malassezia* species (Mirhendi *et al*., 2005).

- In otitis externa, blood agar and MacConkey agar plates should be inoculated with exudate to isolate bacterial pathogens aetiologically associated with *M. pachydermatis*.

Clinical infections

Malassezia pachydermatis has been implicated in canine seborrhoeic dermatitis and also in skin infections secondary to epidermal dysplasia, a genetic disorder of West Highland terriers (Akerstedt and Vollset, 1996). This yeast is one of many organisms which may contribute to otitis externa in dogs. The condition occurs infrequently in cats.

M. sympodialis has also been associated with otitis in cats, while *M. pachydermatis* has been linked to feline chin acne. *Malassezia* species isolated from cattle with otitis include *M. globosa, M. sympodialis, M. furfur* and *M. slooffiae*.

Canine seborrhoeic dermatitis

Factors that predispose to canine seborrhoeic dermatitis include hypersensitivity disorders, keratinization defects, immunosuppression and persistently moist skin folds. Lesions tend to occur more frequently and with greater severity in skin folds. Pruritus and erythema are accompanied by a foul-smelling, greasy exudate with matting of hair. Concurrent bilateral otitis externa may be present (Bond *et al*., 1995b). Treatment with miconazole–chlorhexidine shampoo (Bond *et al*., 1995a) or a combination of topical and oral ketoconazole may be effective.

Canine otitis externa

Otitis externa is characterized by a dark pungent discharge from the ear canal and intense pruritis with head shaking, scratching and rubbing of the ears. Damage to the pinna may manifest as a haematoma. The mucosa of the ear canal is painful and swollen. The aetiology of this condition is complex. Poor ear

conformation, wax retention and immunosuppression are among the factors that may predispose dogs to the disease. *Malassezia pachydermatis*, which is present in low numbers in the ear canal of clinically normal dogs, may proliferate in otitis externa. Predisposing causes should be investigated and eliminated or treated (Little, 1996). The fungal and bacterial pathogens causing the inflammatory response should be identified by microscopic examination and culture of aural exudate. Antibiotic susceptibility testing should be carried out on the bacterial isolates prior to initiating therapy. Proprietary ear drops, containing drugs effective against the bacteria and fungi usually involved and also against *Otodectes cynotis*, may be beneficial. In chronic cases, surgical intervention may be required.

‘Megabacteria’ (*Macrorhabdus ornithogaster*)

‘Megabacteria’ are large (20 to 50 × 3.0μm) Gram- positive rods which are found in the superficial mucosal glands of the lower portion of the proventriculus in budgerigars with megabacteriosis, a chronic wasting disease. They are also found in the proventriculus of clinically normal budgerigars (Baker, 1997). Initially believed to be a bacterium, phylogenetic analysis of sequences of the 18S rDNA and 26S rDNA has shown the organism to be an anamorphic ascomycetous yeast. The organism has been named *Macrorhabdus ornithogaster* (Tomaszewski *et al*., 2003) and is also referred to as avian gastric yeast. Infections have been documented in a wide range of avian species including budgerigars, canaries, finches, parrots, quail, cockatiels and ostriches. Large numbers of organisms are present in clinically affected birds whereas relatively few are found in asymptomatic birds. Factors that predispose to disease include poor hygiene, overcrowding and a genetic predisposition (Phalen, 2005). Clinical signs may include weight loss, diarrhoea and vomiting. The pH in the proventriculus changes from pH 2 to pH 7 or 8 (Simpson, 1992). Clinical signs and demonstration of large numbers of the organism in faeces or proventricular scrapings stained with Gram’s stain or Romanowsky stains are suggestive of proliferation of this yeast. Definitive diagnosis is based on postmortem examination and histopathology. The organism grows on blood agar in an atmosphere of 10% CO_2 and small haemolytic colonies are detectable after incubation for 2 days. These yeasts are catalase- negative, oxidase-negative, facultative anaerobes. Amphotericin B administered by gavage has proved effective for treatment (Christensen *et al*., 1997).

Trichosporon beigelii

Trichosporon beigelii (*T. cutaneum*), a soil saprophyte, produces yeast cells (blastoconidia), pseudohyphae, true hyphae and arthrospores (Fig. 45.6). After inoculation of Sabouraud dextrose agar, colonies appear in about 1 week. This yeast, which is non-fermentative and urease-positive, causes white piedra, a fungal infection of the hair shaft in humans. Rare infections in animals include skin lesions in horses and monkeys, and mastitis in cattle. Nasal granuloma, mycotic cystitis and disseminated trichosporonosis have been described in cats infected with feline leukaemia virus (Doster *et al*., 1987).

Figure 45.6 Fungal forms and structures of *Trichosporon beigelii*: yeast cells (A); pseudohyphae (B); true hyphae (C); arthrospores (D).

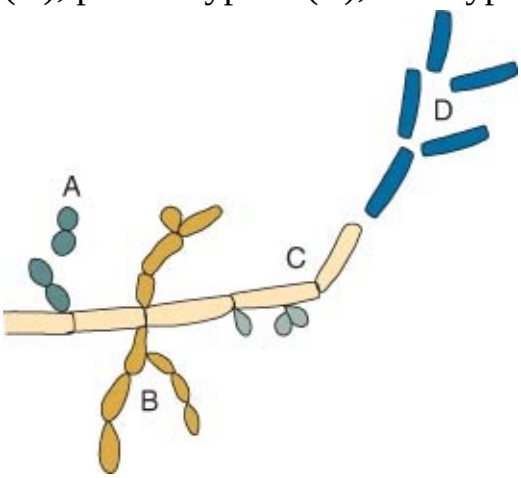
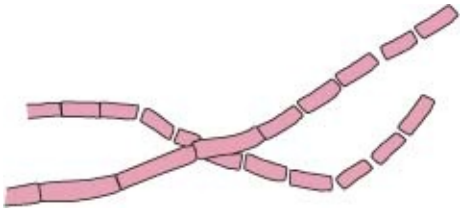


Figure 45.7 Rectangular arthrospores produced by the yeast- Iike mould, *Geotrichum candidum*.



Geotrichum candidum

The mould *Geotrichum candidum* has a yeast-like colonial morphology. The hyphae fragment into chains of rectangular arthrospores (Fig. 45.7). *Geotrichum candidum* is a saprophyte in soil and decaying organic matter. It can be isolated

from faeces of clinically normal animals. The fungus has occasionally been implicated in diarrhoea in dogs and apes, lymphadenitis in pigs and disseminated geotrichosis in dogs (Rhyan *et al*., 1990).

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Chapter 46

Dimorphic fungi

Some fungi, referred to as dimorphic fungi, occur in two distinct forms, a mould form and a yeast form. They exist as moulds in the environment and when cultured on Sabouraud dextrose agar at 25°C to 30°C. In animal tissues and when cultured at 37°C on brain– heart infusion agar with the addition of 5% blood, most grow as yeasts after conversion from the more stable mould form. The dimorphic fungi most often associated with disease in domestic animals are *Blastomyces dermatitidis, Histoplasma capsulatum* and *Coccidioides immitis* (Table 46.1). The spores of these dimorphic fungi usually enter hosts by the respiratory route and infection may be disseminated throughout the body. A variant of *H. capsulatum, H. capsulatum* var. *farciminosum*, hereafter referred to as *H. farciminosum*, generally enters through skin abrasions and produces lympho-cutaneous lesions, epizootic lymphangitis, in horses and mules. *Sporothrix schenckii*, which can also infect dermal tissues following trauma, produces occasional opportunistic infections.

Rare asymptomatic infections, caused by *Paracoccidioides brasiliensis* (Costa *et al*., 1995) and *Emmonsia* species (adiaspiromycosis), have been recorded in domestic animals. A clinical case of para- coccidioidomycosis has been reported in a dog (Ricci *et al*., 2004), while adiaspiromycosis may be of importance in wildlife (Borman *et al*., 2009).

Blastomyces dermatitidis

Blastomyces dermatitidis is a dimorphic fungus which causes blastomycosis, mainly in dogs and humans.

Key points

- Occur as moulds in the environment and as yeast forms in animal tissues
- Saprophytes in soil and in decaying vegetation
- Produce opportunistic infections in animals and humans

- *Blastomyces dermatitidis*:
  – Saprophyte in soil enriched with organic matter
  – Cells budding on a broad base in tissues
  – Causes blastomycosis in dogs and humans
- *Coccidioides immitis*:
  – Saprophyte in arid soils
  – Large spherules containing endospores demonstrable in infected tissues
  – Causes coccidioidomycosis in dogs, horses, cats and humans; sporadic infections in a wide range of other species
- *Histoplasma capsulatum*:
  – Saprophyte in soil enriched with bird faeces
  – Small yeast cells demonstrable in macrophages
  – Causes histoplasmosis in dogs, cats and humans; uncommon in other species
- *Histoplasma farciminosum*:
  – Saprophyte in soil
  – Small yeast cells in macrophages
  – Causes epizootic lymphangitis in *Equidae*
- *Sporothrix schenckii*:
  – Saprophyte on vegetation
  – Cigar-shaped yeast cells demonstrable in infected tissues and exudates
  – Causes sporotrichosis in horses, cats, dogs, humans and other species

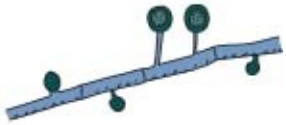
Table 46.1 Dimorphic fungi which are associated with disease in animals and humans.

Feature	*Blastomyces dermatitidis*	*Histoplasma capsulatum*	*Histoplasma farciminosum*	*Coccidioides immitis*	*Sporothrix schenckii*
Disease	Blastomycosis	Histoplasmosis	Epizootic lymphangitis	Coccidioidomycosis	Sporotrichosis
Geographical distribution	Eastern regions of North America, sporadic cases in India and the Middle East	Endemic in the Mississippi and Ohio river valleys, sporadic cases in some countries	Africa, Middle East, Asia	Semi-arid regions of southwestern USA, Mexico, Central and South America	Worldwide, most common in subtropical and tropical regions
Usual habitat	Acid soil rich in organic matter	Soil enriched with bat or bird faeces	Soil	Desert soils at low elevation	Dead vegetation, rose thorns, wooden posts, sphagnum moss
Main hosts	Dogs, humans	Dogs, cats, humans	Horses, other *Equidae*	Dogs, horses, cats, humans	Horses, cats, dogs, humans
Site of lesions	Lungs, metastases to skin and other tissues	Lungs, metastases to other organs	Skin, lymphatic vessels, lymph nodes	Lungs, metastases to bones, skin and other tissues	Skin, lymphatic vessels

Figure 46.1 The mould and yeast forms of *Blastomyces dermatitidis*.

Mould form

Oval or pear-shaped conidia (2-10 µm in diameter) form on conidiophores or directly on septate hyphae when cultured at 25°C



Yeast form

Thick-walled yeast cells (8-10 µm in diameter) form when cultured at 37°C. Daughter cells bud on a broad base

The mould and yeast forms of this fungus are shown in Fig. 46.1. The teleomorph of *B. dermatitidis* is a member of the phylum *Ascomycota* designated *Ajellomyces dermatitidis*.

Usual habitat

Although the precise natural habitat of *B. dermatitidis* is unknown, it has been isolated from moist, acid soils rich in organic matter (Archer *et al*., 1987).

Recognition and laboratory diagnosis

- When incubated at 25°C to 30°C on Sabouraud dextrose agar, mould colonies are white and cottony, usually becoming brown with age. Oval or pear-shaped conidia (2 to 10 µm in diameter) are borne either on conidiophores or directly on the hyphae.
- When incubated at 37°C on brain–heart infusion agar with added cysteine and 5% blood, yeast colonies are cream to tan, wrinkled and waxy. The yeast cells (8 to 10 |m in diameter) are thick-walled and typically bud on a broad base.
- A soluble exoantigen of *B. dermatitidis* can be identified by agar gel immunodiffusion using specific antiserum (Di Salvo, 1998).
- Commercially available nucleic acid probes for use on cultures of dimorphic fungi are sensitive and specific (Stockman *et al*., 1993).
- Yeast cells may be demonstrated in cytological and histopathological preparations from affected tissues by staining with the PAS stain or with methenamine silver. Exudates or aspirates for cytological examination should be stained with methylene blue or by the Giemsa method. Alternatively, immunofluorescent staining can be used to specifically identify the yeast form in tissues.
- Polymerase chain reaction protocols are available for the detection and

identification of the fungus in clinical specimens (Bialek *et al*., 2003).

- Serological procedures, suitable for demonstrating rising antibody titres in affected dogs, are ELISA and counter- immunoelectrophoresis. Detection of fungal antigen in serum and urine by enzyme immunoassay has also been described (Spector *et al*., 2008).

Clinical infections

Blastomycosis most commonly affects dogs and humans (Legendre *et al*., 1981). Infection in other species is uncommon but it has been recorded in the cat (Breider *et al*., 1988). The disease is encountered in North America, Africa, the Middle East and India.

Canine blastomycosis

Young male dogs of sporting breeds are particularly prone to infection because of frequent exposure to the fungus in the environment. Infection usually occurs by inhalation of aerosolized spores or mycelial fragments. Conversion to the yeast form occurs in the tissues. The yeast form is much more resistant to neutrophils and mononuclear phagocytes than the mould form. The best studied virulence factor is BAD1 (*Blastomyces* adhesin; formerly WI-1), a major surface protein that both promotes attachment to cells in the respiratory tract and modulates the host's immune response. Pulmonary blastomycosis, a chronic debilitating condition, is the usual form of the disease. Presenting signs include coughing, exercise intolerance and dyspnoea. The extent of the infection, which may be limited to the lungs and associated lymph nodes, is largely determined by the immune competence of the host. Although antibodies are produced, protective immunity depends on the cellular immune response. Many infections are subclinical, detectable only by seroconversion. In animals with inadequate cell-mediated immunity, there may be dissemination to skin, eyes and bones. The central nervous system and, in male dogs, the urogenital tract are occasionally affected. Primary cutaneous blastomycosis is uncommon (Wolf, 1979). The clinical presentation in disseminated disease relates to the distribution and severity of the lesions which are granulomatous or pyogranulomatous. Yeast cells are numerous in these lesions.

Itraconazole is the treatment of choice for dogs and cats. Amphotericin B may be combined with itraconazole if infection is severe. Animals should be monitored for possible nephrotoxic effects of treatment.

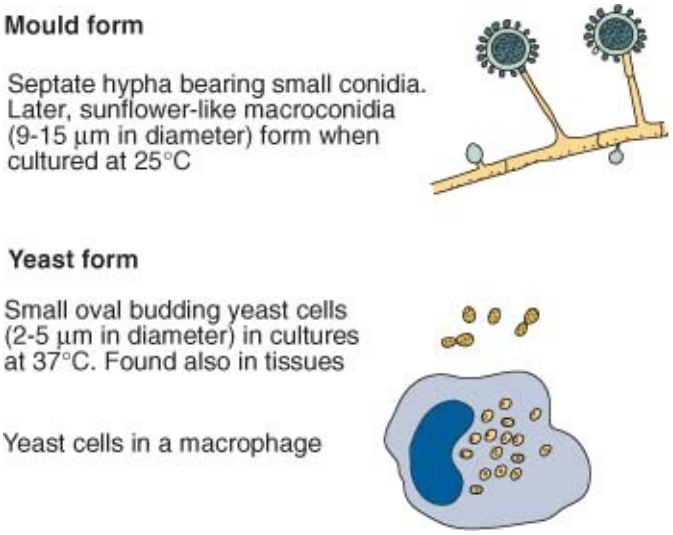
Histoplasma capsulatum

Three variants of *H. capsulatum* are recognized: *Histoplasma capsulatum* var. *capsulatum* (*H. capsulatum*), which can produce systemic histoplasmosis mainly in dogs and cats; *H. capsulatum* var. *farciminosum* (*H. farciminosum*) which causes equine epizootic lymphangitis; and *H. capsulatum* var. *duboisii*, a human pathogen limited to parts of equatorial Africa. The teleomorphs of these variants are members of the phylum *Ascomycota*, designated *Ajellomyces capsulatus*. The mould and yeast forms of *H. capsulatum* are shown in Fig. 46.2.

Usual habitat

Histoplasma capsulatum is found in soil, particularly when enriched with bird or bat faeces. Aerosols, following

Figure 46.2 The mould and yeast forms of *Histoplasma capsulatum*.



disturbance of soil beneath roosting sites, contain large numbers of infective propagules. *Histoplasma farciminosum* is a soil saprophyte.

Recognition and laboratory diagnosis

- When cultured at 25°C to 30°C on Sabouraud dextrose agar, the mould form grows as white to buff colonies with cottony aerial hyphae. Septate hyphae bear small conidia. In mature colonies, slender conidiophores produce tuberculate, sunflower-like macroconidia (9 to 15 µm in diameter).
- When cultured at 37°C on brain–heart infusion agar with added cysteine and 5% blood, yeast colonies are round, mucoid and cream-coloured.

Budding yeast cells are oval to spherical (2 to 5 µm in diameter).

- A commercially available nucleic acid probe can be used for identification of isolates (Stockman *et al*., 1993).
- Giemsa-stained smears of exudates or aspirates may be used for demonstrating yeast forms in macrophages.
- Histopathological examination of affected tissues reveals pyogranulomatous foci containing yeast forms. Molecular techniques can be used to detect the fungal DNA in histological sections (Ueda *et al*., 2003).
- A positive skin test, using histoplasmin, merely indicates exposure to the fungus.
- Using histoplasmin as antigen in an agar gel immunodiffusion test, two precipitin bands, H and M, can be identified with serum from affected animals. The reliability of this test for the diagnosis of the disease in animals is questionable.

Clinical infections

Histoplasmosis, which occurs in many countries, is endemic in the Mississippi and Ohio river valleys and in other areas of the USA. The dog and cat are the domestic species most often affected clinically. Epizootic lymphangitis occurs in *Equidae* in Africa, the Middle East and Asia.

Canine and feline histoplasmosis

Most infections in these species are asymptomatic. Following inhalation, microconidia are ingested by pulmonary alveolar macrophages, in which the yeast forms persist and replicate. Granulomatous lesions may be found in the lungs of both dogs and cats. Disseminated disease has been recorded in both species, probably associated with impaired cellmediated immunity. In dogs, ulcerative intestinal lesions are commonly encountered whereas intestinal involvement is rare in cats. Clinical signs in affected dogs include chronic cough, persistent diarrhoea and emaciation. Less frequently, peripheral lymphadenitis, ulcerative skin nodules, eye lesions, lameness and neurological dysfunction may be encountered. The clinical signs in cats relate mainly to pulmonary involvement and include dyspnoea, depression, fever and loss of weight. Disseminated histoplasmosis is invariably fatal. Itraconazole is the drug of choice on account of its low toxicity. Ketoconazole and amphotericin B can

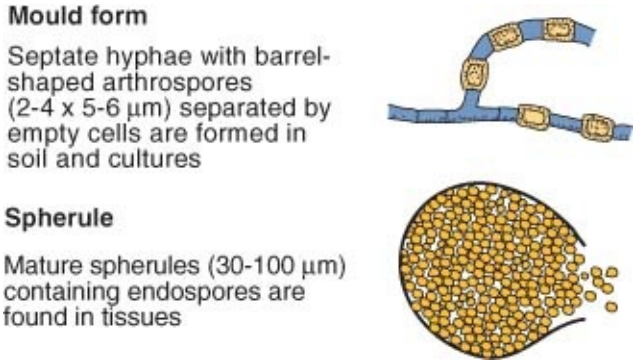
be used for treatment but animals should be monitored for signs of toxicity.

Epizootic lymphangitis

Epizootic lymphangitis, caused by *H. farciminosum*, is a contagious disease of *Equidae* which may have a high prevalence when animals are in close contact. Infection is usually acquired from environmental sources through minor skin abrasions on the limbs. However, primary ocular involvement and pulmonary involvement have also been recorded. Characteristic lymphocutaneous lesions, which resemble those of equine farcy (see Chapter 25), consist of ulcerated discharging nodules usually located along the course of thickened, hard, lymphatic vessels. Regional lymphadenopathy is often present. Yeast cells of *H. farciminosum* are found in large numbers in lesions, mainly within macrophages (Chandler *et al*., 1980). *Histoplasma farciminosum*, present in discharges, can be spread by biting insects and through contaminated grooming gear and harness.

In most countries where the disease is exotic, it is notifiable and a test and slaughter policy is implemented. If treatment is considered advisable, surgical

Figure 46.3 The mould form and spherule of *Coccidioides immitis*.



excision of skin lesions may be attempted in conjunction with sodium iodide therapy. Amphotericin B treatment has been recommended (al Ani, 1999).

Coccidioides species

The geophilic fungus *C. immitis* can infect a wide range of animal species including humans. Approximately 100,000 human cases occur in the USA annually and the fungus has been added to the select agent list of potential bioterrorism pathogens in the USA. Although grouped with the dimorphic fungi, *C. immitis* is biphasic rather than dimorphic because typical yeast forms are not produced. Large spherules containing endospores develop in tissues. The

spherule and mould forms of this fungus are shown in Fig. 46.3. Respiratory infections may follow inhalation of arthroconidia (arthrospores) produced from the mould form of the fungus in soil. Following inhalation, the arthrospore is stimulated by the presence of phagocytes, increased CO_2 and 37°C to differentiate into a multinucleated spherule which then reproduces by endosporulation. Upon maturation, the spherule ruptures releasing hundreds of endospores that can mature into new spherules in the surrounding tissues. The large size of the spherule may allow it to avoid engulfment by phagocytes. The tissue damage caused is thought to be the result of a combination of enzymatic activity and the host's immunological response. Systemic spread from pulmonary lesions has been described. *Coccidioides* species are members of the phylum *Ascomycota* but a teleomorphic stage has not been demonstrated.

Usual habitat

Coccidioides immitis grows in the soil of arid or semiarid low-lying areas of southwestern USA, northern Mexico and parts of Central and South America. Dust in these areas may be heavily contaminated with arthroconidia. Isolates from outside the endemic region of the San Joaquin Valley in California and formerly referred to as 'non-California' *C. immitis* have been found to be sufficiently different to warrant separate species status. The name *C. posadasii* has been proposed (Fisher *et al*., 2002).

Recognition and laboratory diagnosis

Because culturing of *Coccidioides* species is hazardous, it should be attempted only when stringent precautions are observed, including the use of a biohazard cabinet. Diagnosis is usually based on clinical findings and histopathology.

- When cultured on Sabouraud dextrose agar at 25°C to 30°C, colonies are shiny, moist and grey, becoming white and cottony. Thick-walled, barrel-shaped arthroconidia, separated by empty cells which undergo degeneration, are released following hyphal fragmentation (Fig. 46.3).
- The identity of suspect cultures can be confirmed using aqueous extracts in immunodiffusion tests with specific *C. immitis* antiserum.
- A commercially available nucleic acid probe can be used on cultures for identification (Stockman *et al*., 1993). *Coccidioides immitis* and *C. posadasii* can be distinguished by amplifying and sequencing the ribosomal

internal transcribed spacer (ITS) region (Tintelnot *et al*., 2007).

- The history may indicate that a suspect animal came from an endemic area. Radiographs of the thorax or affected limbs may detect lesions consistent with coccidioidomycosis.
- Spherules of *Coccidioides* species may be demonstrated in exudates or aspirates cleared with 10% KOH and may also be identified in stained tissue sections.
- Complement fixation, agar gel immunodiffusion, ELISA and latex agglutination tests can be employed to demonstrate rising antibody titres. The AGID test is highly specific but not sensitive.
- A positive skin test, using a filtrate of a mycelial culture (coccidioidin), is indicative of exposure to the fungus.
-)ntraperitoneal inoculation of mice with material from cultures may be necessary to demonstrate the formation of spherules *in vivo*.

Clinical infections

Since the occurrence of *Coccidioides* species is limited to defined arid regions of south-western USA, Mexico and Central and South America, most cases of coccidioidomycosis are encountered in animals from these areas. Although many animals from these regions become infected, relatively few develop clinical disease. The domestic species most often affected is the dog. The disease in dogs varies from asymptomatic to disseminated and fatal. Clinical coccidioidomycosis has also been described in horses, cats, llamas and marine mammals.

Canine coccidioidomycosis

In one prospective study, 70% of seropositive dogs had no evidence of clinical disease (Shubitz *et al*., 2005). Dogs with mild pulmonary coccidioidomycosis, which present with non-specific signs including cough, fever and inappetence, may recover spontaneously. Animals with extensive pulmonary lesions display persistent coughing, weakness, depression, fluctuating fever and loss of weight. Dissemination from pulmonary lesions, which is frequently related to immunosuppression, often results in osteomyelitis with lameness and radiological evidence of bone destruction as the condition progresses. Other tissues, including the skin, may be affected. Therapy with azole drugs, continued

for at least 6 months, may be effective but relapses can occur.

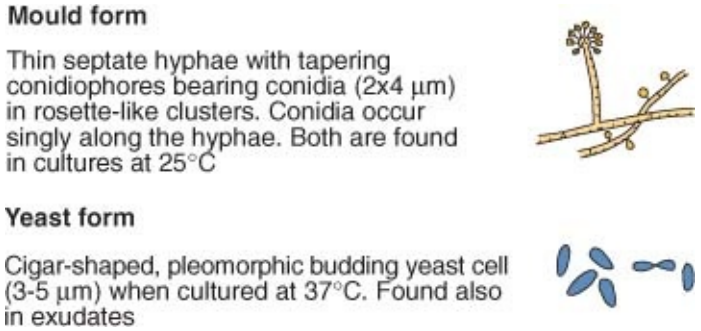
Equine coccidioidomycosis

Clinical signs of coccidioidomycosis in horses are nonspecific and include intermittent fever, abdominal pain, loss of weight and evidence of pulmonary and musculoskeletal involvement. Pulmonary disease, in which coughing may be the only presenting sign, occurs in about 60% of cases. Musculoskeletal pain, usually associated with osteomyelitis, is evident in about one-third of infected animals. Recurring superficial abscessation is also a feature. Thickening of the placenta, plaque-like lesions on the umbilical cord and nodules in the lungs of the foetus were recorded in a case of abortion caused by *C. immitis* (Langham *et al*., 1977). Treatment of disseminated coccidioidomycosis is usually unsuccessful.

Sporothrix schenckii

Sporothrix species belong to the phylum *Ascomycota*. *Sporothrix schenckii* is the pathogenic species and is widely distributed in the environment where it grows as a mould producing slender hyphae (1 to 2 µm in diameter) and conidiophores. The yeast and mould forms of this fungus are shown in Fig. 46.4. Infection occurs sporadically in horses, cats, dogs and humans. *Sporothrix schenckii* occurs worldwide and is particularly important in subtropical and tropical regions. Molecular studies suggest that *S. schenckii* is not a

Figure 46.4 The mould and yeast forms of *Sporothrix schenckii*.



single species but rather a complex of up to six putative phylogenetic species (Marimon *et al*., 2006).

Usual habitat

The fungus is saprophytic on dead or senescent vegetation such as rose thorns, timber, hay, straw and sphagnum moss.

Recognition and laboratory diagnosis

- When cultured on Sabouraud dextrose agar at 25°C, mould colonies grow rapidly and are white, becoming black or brown, wrinkled and leathery. Pear- shaped conidia are borne in a rosette pattern on slender conidiophores. In older cultures, conidia form singly on hyphae.
- When cultured at 35°C to 37°C on brain–heart infusion agar containing 5% blood, cream to tan yeast colonies develop within 3 weeks. The yeast cells, (2 to 3 × 3 to 5 μm), are cigar-shaped.
- Direct microscopic examination of exudates from feline lesions stained with methylene blue usually reveals large numbers of yeast cells. They are sparse in exudates from other animals.
- Histopathological examination of tissue sections, stained by the PAS or methenamine silver techniques, may reveal yeast cells.
- Fluorescent antibody or immunoperoxidase techniques applied to tissue sections allow specific identification of the yeast cells.

Clinical infections

Sporotrichosis is a chronic cutaneous or lymphocutaneous disease which rarely becomes generalized. Dissemination usually occurs in immunocompromized individuals. Sporadic cases are recorded in horses, cats, dogs, cattle, goats, pigs and humans. Infection is usually acquired from the environment as a result of the inoculation of spores beneath the skin or into wounds, where they develop as the yeast form. Infected cats carry the organism in the nasal and oral cavities and on their nails, facilitating transmission through biting and scratching. Epidemics have been reported in cats in Brazil (Schubach *et al*., 2008).

Equine sporotrichosis

Lymphocutaneous sporotrichosis is the most common form of the disease in horses (Blackford, 1984). Fungal spores usually enter through skin abrasions on the lower limbs. Nodules, which ulcerate and discharge a yellowish exudate, develop along the course of superficial lymphatic vessels. Subcutaneous oedema in the affected limb may result from lymphatic obstruction. Treatment with

inorganic iodides, administered in the feed, should continue for approximately 30 days after clinical recovery. Animals undergoing treatment should be monitored for signs of iodism. Itraconazole, fluconazole and voriconazole are effective for the lymphocutaneous form of sporotrichosis. Surgical excision of early lesions may be feasible.

Feline sporotrichosis

Nodular skin lesions occur most often on limb extremities, head and tail. Secondary nodules can develop along the course of lymphatics. Infection may be spread to other skin sites by grooming. Nodules ulcerate and discharge a seropurulent exudate. Following ulceration, extensive areas of underlying muscle and bone may be exposed (Dunstan *et al*., 1986). Large numbers of yeast cells in discharges from lesions in cats may pose a health hazard to humans handling affected animals (Zamri-Saad *et al*., 1990). Itraconazole, fluconazole and voriconazole are effective treatments for sporotrichosis.

Canine sporotrichosis

Sporotrichosis in dogs often manifests as multiple, ulcerated and crusted, alopecic, cutaneous lesions over the head and trunk. Lymphocutaneous involvement occasionally occurs but disseminated disease is rare (Scott *et al*., 1974). The treatment regime is similar to that for cats.

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Chapter 47

Zygomycetes of veterinary importance

Fungi in the phylum *Zygomycota* characteristically have broad (6 to 15 μm), coenocytic (relatively aseptate) hyphae and replicate asexually by producing sporangiospores within a sporangium. Fusion of gametangia from two different strains, resulting in the production of a thick-walled zygospore, is the mode of sexual reproduction. Zygospores are seldom formed in cultures except in the case of *Basidiobolus* species. The absence of septa allows nutrients to pass along hyphae, resulting in rapid growth. Septa may occasionally be observed at sites of hyphal damage and close to sporangia. The coenocytic hyphae are easily damaged and may become non-viable during sampling.

The phylum has two classes, the *Zygomycetes* and the *Trichomycetes*. Three orders in the class *Zygomycetes*, namely *Mucorales, Mortierellales* and *Entomophthorales*, are of veterinary importance. The pathogenic species *Mortierella wolfii* was formerly classified within the *Mucorales*, but has recently been placed in the newly created order *Mortierellales* which has a single family *Mortierellaceae*. Genera in the three orders containing potentially pathogenic species are indicated in Fig. 47.1. *Absidia corymbifera* has been renamed *Lichtheimia corymbifera*. Zygomycetes are widely distributed saprophytes, which can cause sporadic opportunistic infections. The term zygomycosis is applied to disease caused by infection with a member of the *Zygomycota*. Clinical disease has been described in a wide range of species including mammals, birds, fish, reptiles and amphibians. The term phycomycosis was formerly used to encompass infection by zygomycetes or by *Pythium insidiosum*, a fungal-like organism which produces opportunistic infections similar to those produced by zygomycetes (see Chapter 48). In general, more severe clinical disease tends to occur following infection with members of the *Mucorales* and *Mortierellales* than with members of the *Entomophthorales* which are associated with chronic diseases of the nasal mucosa and subcutaneous tissues.

Key points

- Broad aseptate hyphae (up to 15 μm diameter)
- Sporangiospores produced asexually
- Zygospores are the sexual spores
- Saprophytes, widely distributed in the environment
- Rapid growth
- Cause zygomycoses
- *Mucorales* and *Mortierellales*
  - *Lichtheimia* (*Absidia*), *Mucor, Rhizomucor* and *Rhizopus* are typical zygomycetes
  - *Mortierella* and *Saksenaea* species form spores only on nutrient-deficient media
  - Immunosuppression may predispose to infection
  - Mucormycoses are often systemic diseases
  - *Mortierella wolfii* associated with abortion and pneumonia in cattle
- *Entomophthorales*
  - Sporangium functions as a single conidium
  - Hyphae, sometimes septate, produced in animal tissues (up to 20 μm diameter)
  - Characteristic aggregates around hyphae
  - Granulomas caused by *Basidiobolus* species and *Conidiobolus* species; most common in horses

Figure 47.1 Genera of the zygomycetes which include species of veterinary importance.

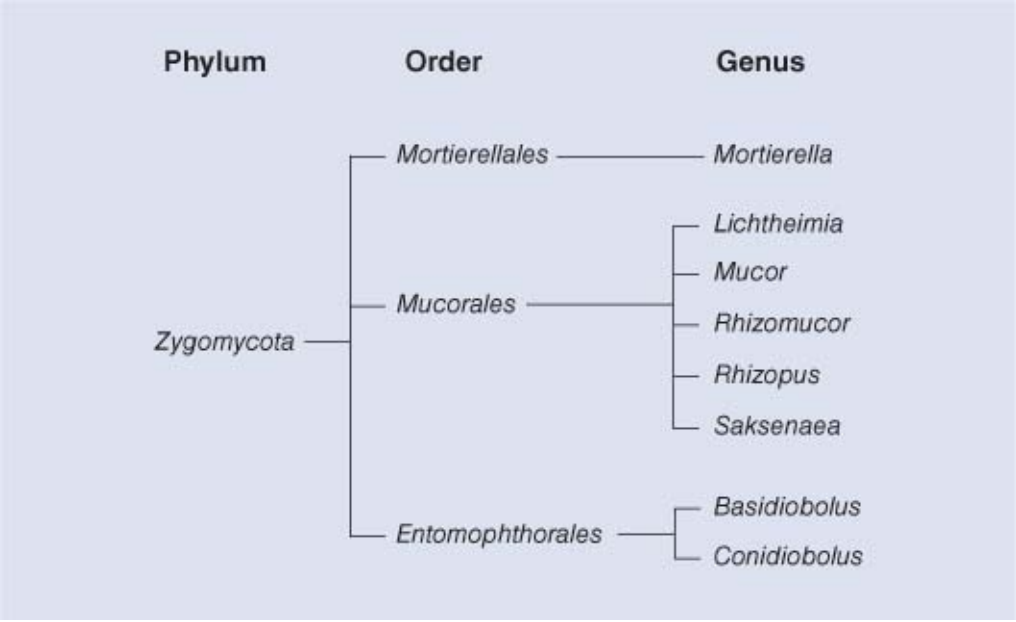
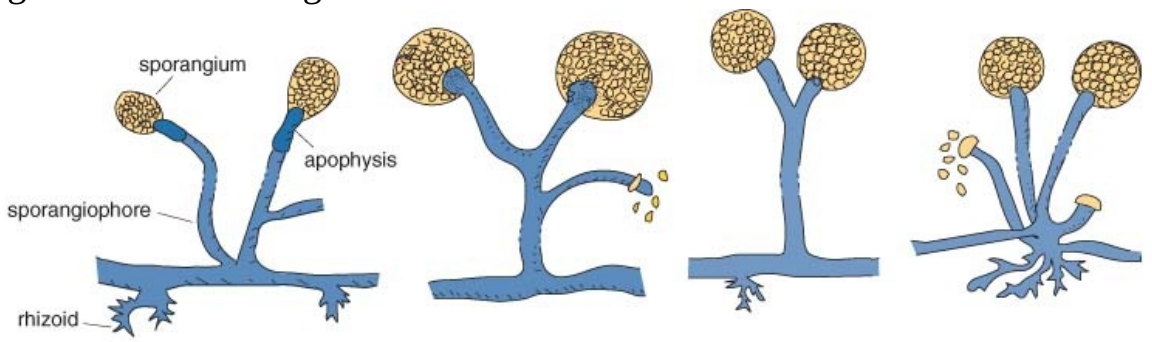


Figure 47.2 Morphological features of members of *Mucorales* which produce

sporangia on standard fungal media.



	Lichtheimia	*Mucor*	*Rhizomucor*	*Rhizopus*
Sporangia	Pear-shaped 20-120 μm	Round 50-300 μm	Round 60-100 μm	Round 40-350 μm
Sporangiophores	Branched	Branched	Branched	Branched
Location of rhizoids	Between sporangiophores	Absent	Few; between sporangiophores	Under sporangiophores
Apophyses	Present	Absent	Inconspicuous	Inconspicuous

Mucorales and *Mortierellales*

These fungi are commonly known as ‘pin’ or ‘bread’ moulds because their dark sporangia resemble pinheads and they are often found growing on stale bread. The morphological features of some members of the *Mucorales* are illustrated in Fig. 47.2. Several genera produce root-like rhizoids which allow anchorage to surfaces. Colonies grow rapidly on culture plates.

Strains associated with animal disease, which grow well at 37°C on Sabouraud dextrose agar, are susceptible to cycloheximide. Sporulation of two species, *Mortierella wolfii* and *Saksenaea vasiformis*, occurs only on media deficient in certain nutrients.

Mucormycoses, diseases caused by fungi belonging to the orders *Mucorales* and *Mortierellales*, are encountered sporadically worldwide. They often involve the gastrointestinal tract, the respiratory tract and associated lymph nodes. Clinical disease may be severe with dissemination to other organs on account of the angiotropism of these agents. Infection may be associated with immunosuppression.

Usual habitat

Members of the *Mucorales* and *Mortierellales* are saprophytes present in soil and vegetation and their spores are often airborne. Although *M. wolfii* has been

isolated from soil near silage and rotting hay, it is otherwise difficult to recover from environmental sources.

Differentiation of members of the *Mucorales* and *Mortierellales*

- Colonial morphology:

Growth of *Lichtheimia*, *Mucor, Rhizomucor* and *Rhizopus* species is rapid, filling the Petri dish with grey or brownish-grey fluffy colonies within a few days.

Mortierella wolfii has characteristic white velvety colonies with lobulated outlines. Colonies are about 5 cm in diameter after incubation for 4 days.

Saksenaea vasiformis produces rapidly growing colonies with a white downy appearance.

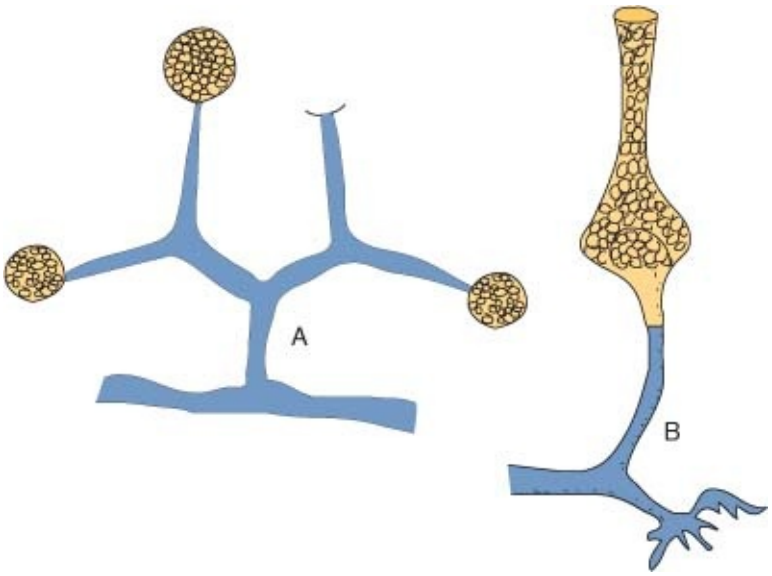
- Microscopic appearance:

Morphological features allow differentiation of the genera (Fig. 47.2).

Sporulation of *M. wolfii* and *S. vasiformis* can be induced by subculturing on to nutrient-deficient media such as hay-infusion agar. These two fungi have distinctive structural features (Fig. 47.3).

- Differentiation of species in the genera *Lichtheimia*, *Mucor, Rhizomucor* and *Rhizopus* is carried out in reference laboratories.

Figure 47.3 Morphological features of sporangiophores and sporangia of *Mortierella wolfii* (A) and *Saksenaea vasiformis* (B). Sporulation is induced by nutrient-deficient media.



Pathogenesis and pathology

Infection with these fungi is uncommon in healthy immunocompetent individuals. Factors that may predispose to infection include immunodeficiency, corticosteroid therapy, prolonged administration of broad-spectrum antibiotics and viral diseases such as panleukopenia and infectious peritonitis in cats (Ossent, 1987). Pathogenic species are thermotolerant, permitting them to grow at core body temperature. Infection may follow ingestion or inhalation of spores from contaminated environmental sources. Hyphae invade the mucosa, submucosa and local vessel walls, producing an acute necrotizing thrombotic vasculitis. Chronic lesions are usually localized and granulomatous.

Diagnostic procedures

- Apart from *M. wolfii*, which may produce abortion followed by acute pneumonia, members of the *Mucorales* rarely cause recognizable disease syndromes in animals.
- Specimens for laboratory examination should include tissues for both histopathology and culture. Cotyledons, abomasal contents from foetuses and uterine discharges should be collected from cases of abortion. Isolation of *M. wolfii* from autolysed tissues may be difficult.
- Staining of tissue sections by the PAS or methenamine silver techniques facilitates detection of aseptate hyphae.
- Fluorescent antibody methods have been used for identifying pathogens

such as *Lichtheimia (Absidia) corymbifera* (Jensen *et al*., 1990).

- Isolation is carried out on Sabouraud dextrose agar without cycloheximide. Cultures are incubated aerobically at 37°C for up to 5 days.
- Identification criteria for isolates:

Colonial morphology

Microscopic morphological features (Fig. 47.2).

- Serological tests such as agar gel diffusion have been developed but are of uncertain diagnostic value.
- Molecular techniques are being developed for the identification of isolates (Schwarz *et al*., 2006; Hata *et al*., 2008; Piancastelli *et al*., 2009).

Clinical infections

The zygomycoses of domestic animals are presented in Table 47.1. Laboratory procedures including isolation of the fungus and demonstration of hyphae in affected tissues are essential for the diagnosis of zygomycosis. The clinical signs, relating to the condition that predisposed to fungal invasion, may mask signs arising from the fungal infection. Irrespective of the location, mycotic lesions caused by members of the *Mucorales* and *Mortierellales* are less commonly encountered than those caused by *Aspergillus* species. Due to the sporadic nature of these conditions, prevention is difficult. Where possible, exposure to spores should be minimized by ensuring adequate ventilation and by exclusion of mouldy feedstuffs from the diet. Factors that predispose to infection should be avoided.

Table 47.1 Zygomycoses of domestic animals.

Fungal disease	Hosts	Clinical conditions
Mucormycosis	Cattle	Mesenteric and mediastinal lymphadenitis
		Abortion
		Pneumonia following abortion caused by *Mortierella wolfii*
		Oesophagitis and enteritis in calves
		Rumenitis, abomasal ulcers
		Cerebral mucormycosis
	Pigs	Enteritis in piglets
		Mesenteric and mandibular lymphadenitis
		Gastrointestinal ulcers
	Cats	Focal necrotizing pneumonia
		Necrotic enteritis
	Dogs	Enteritis
Entomophthomycosis	Horses	Cutaneous granulomas caused by *Basidiobolus* species

		Nasal granulomas caused by *Conidiobolus* species
	Dogs	Subcutaneous, gastrointestinal and pulmonary granulomas caused by *Basidiobolus* species
		Subcutaneous granulomas caused by *Conidiobolus* species
	Sheep	Nasal granulomas caused by *Conidiobolus* species

Mycotic abortion

The prevalence of mycotic abortion in cattle is influenced by climatic and other environmental factors. Reports from some regions suggest that fungi may be involved in 7% of bovine abortions (Knudtson and Kirkbride, 1992). Although *Aspergillus* species account for the majority of cases in many countries, *M. wolfii*, *Lichtheimia* species, *Mucor* species, *Rhizomucor* species and *Rhizopus* species have also been implicated and in some regions may predominate. Abortion, which usually occurs late in gestation, is often linked to the feeding of mouldy hay or silage. The location of lesions on cotyledons suggests haematogenous infection of the uterus, possibly from a pulmonary or enteric source. The cotyledons are enlarged and necrotic, and the intercotyledonary placental tissue is thickened and leathery. Vasculitis, associated with hyphal invasion, is demonstrable in sections of affected cotyledons. Occasionally, lesions may be observed grossly on the skin of aborted foetuses.

Abortion due to *M. wolfii*, an important cause of mycotic abortion in New Zealand, may be followed within days by an acute fibrinonecrotic fungal pneumonia (Carter *et al*., 1973). Because of the difficulty in isolating *M. wolfii* from autolysed tissues, abortion caused by this organism may be underdiagnosed (MacDonald and Corbel, 1981). Mycotic abortion in mares caused by *Lichtheimia corymbifera* has been reported.

Alimentary tract infections

Mycotic rumenitis in cattle may follow mucosal damage associated with ruminal lactic acidosis. The microscopic appearance of the causal fungi in ruminal lesions suggests that, in most cases, zygomycetes of the genera *Lichtheimia*, *Absidia* and *Mucor* are involved (Brown *et al*., 2007). Infarctions due to thrombosis, necrosis and haemorrhage are major features of the mycotic lesions. Extension of the inflammatory process through the ruminal wall results in fibrinous peritonitis. Zygomycotic abomasitis and ulceration in calves, which may follow neonatal infection, can also produce perforation and peritonitis. Acute gastrointestinal zygomycosis has been recorded in piglets (Reed *et al*., 1987).

Mycotic pneumonia

An acute fatal pneumonia of cows, which is caused by *M. wolfii* and occasionally follows abortion due to the fungus, is a well recognized syndrome in New Zealand (Carter *et al*., 1973). Chronic pneumonic lesions caused by other zygomycetes are encountered sporadically in cattle and other domestic species.

Entomophthorales

Basidiobolus and *Conidiobolus*, two genera in the *Entomophthorales*, are sometimes associated with opportunistic infections in animals. A unique feature of these fungi is the production of a single conidium which is forcibly discharged when mature. Molecular data tend to suggest that *Basidiobolus* species are more closely related to chytrid fungi than to *Entomophthorales*. *Basidiobolus ranarum* is the only pathogenic species in the genus and is limited in distribution to the warmer regions of the world. *Conidiobolus* species associated with disease include *C. coronata, C. incongruous* and *C. lamprauges*. The distribution of these species is also associated with warmer regions of the world.

Usual habitat

Basidiobolus species are saprophytes in soil and decaying fruit and vegetable material, and may be present in the faeces of amphibians, reptiles, insectivorous bats and marsupials (Speare and Thomas, 1985).

Conidiobolus species are saprophytes in soil and in decaying vegetation, particularly in rainforests.

Differentiation of the *Entomophthorales*

- Colonial morphology:

  Basidiobolus species are moderately fast-growing and form flat, smooth, yellowish grey colonies which become radially folded with a white powdery surface. They have an earthy odour similar to that of *Streptomyces* species.

  Conidiobolus species grow rapidly and produce flat, smooth, cream-coloured colonies which become radially folded and brownish with a white powdery surface. Discharged conidia adhere to the Petri dish lid.
- Microscopic appearance:

Basidiobolus species have broad (20 µm in diameter), mainly aseptate hyphae in which round, thick-walled zygospores (20 to 50 µm in diameter) form.

Conidiobolus species produce simple conidiophores which bear solitary, spherical conidia (10 to 25 µm in diameter). Germination of conidia results in the production of single or multiple hyphal tubes with secondary conidia.

- Differentiation to a species level is carried out in mycological reference laboratories.

Pathogenesis and pathology

Although not clearly defined, the route of entry of these fungi is probably through minor abrasions in the skin or nasal mucous membranes. Hyphal invasion of blood vessels is uncommon. Spread by lymphatics sometimes occurs (Hillier *et al*., 1994). Although disseminated disease is rare, it has been reported (Miller and Turnwald, 1984) in a dog infected with *B. ranarum* (syn. *B. haptosporus*).

Granulomatous lesions result from infection with these opportunistic pathogens. An eosinophilic deposit around individual hyphae (Splendore-Hoeppli phenomenon) may represent immune complex formation (Miller and Campbell, 1984).

Diagnostic procedures

- Specimens for laboratory examination should include biopsy or post-mortem tissues for histopathology and culture. Care should be taken to avoid excessive tissue disruption during sampling as the coenocytic hyphae are easily damaged and become non-viable.
- Fungal hyphae must be demonstrated in tissue sections. The thin-walled hyphae of *Basidiobolus* species are usually up to 20 µm in diameter whereas those of *Conidiobolus* species are up to 12 µm in diameter. There may be evidence of an eosinophilic cuff or sheath around individual hyphae (SplendoreHoeppli phenomenon).
- These fungi are thermotolerant and can be isolated on Sabouraud dextrose agar without added cyclohexamide after incubation aerobically at 37°C for up to 5 days.
- Identification criteria for isolates:

Colonial morphology

Microscopic appearance.

- For identification to a specific level, specimens should be sent to a reference laboratory.

Clinical infections

The entomophthomycoses of domestic animals are indicated in Table 47.1. *Basidiobolus* species cause cutaneous lesions in the horse and dog which resemble those associated with *Pythium insidiosum* (see Chapter 48). Infections with *Conidiobolus* species cause granulomas in horses (Humber *et al*., 1989; Zamos *et al*., 1996), sheep (Carrigan *et al*., 1992; Silva *et al*., 2007) and llamas (French and Ashworth, 1994). On rare occasions, *Conidiobolus* species cause pyogranulomatous and cutaneous lesions in dogs (Hillier *et al*., 1994).

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Chapter 48

Fungus-like organisms of veterinary importance

Three eukaryotic, fungus-like organisms, *Pythium insidiosum, Rhinosporidium seeberi* and *Lacazia loboi*, cause rare sporadic infections in animals which have contact with contaminated water. These organisms, which are found in either mycelial or unicellular forms in tissues, induce host reactions similar to those encountered in fungal infections. Pythiosis and rhinosporidiosis have been described in domestic animal species and in humans. *Lacazia loboi* is mainly a human pathogen although sporadic cases have been recorded in dolphins.

Pythium insidiosum

This fungus-like organism, also known as *Hyphomyces destruens*, is classified in the class *Oomycetes*, kingdom Chromista (kingdom Protista) It is found in aquatic environments and is an opportunistic animal pathogen whereas many other *Pythium* species are important as plant pathogens. Infection with *P. insidiosum* is rare in animals. Plant infections are essential for the propagation of the organism and the production of motile zoospores (Fig. 48.1).

Pythium insidiosum grows on a variety of laboratory media when incubated at both 25°C and 37°C. However, zoospores are produced only in water cultures. On solid media and in plant and animal tissues, the organism develops aseptate hyphae (4 to 10 μm in diameter) which are similar morphologically to those of the zygomycetes.

Key points

- *Pythium insidiosum*
  - Member of the kingdom Chromista
  - Found mainly in bodies of stagnant water
  - Genus contains important plant pathogens

– Grows on wide variety of media

– Motile zoospores may invade animal tissue with mild abrasions

– Causes cutaneous pythiosis in horses and gastrointestinal pythiosis in dogs

- *Rhinosporidium seeberi*

– Fungal-like organism of low pathogenic potential

– Found in stagnant water

– Does not grow on inert media

– Rhinosporidiosis, which occurs in horses, dogs and cattle, is characterized by nasal polyp formation

- *Lacazia loboi*

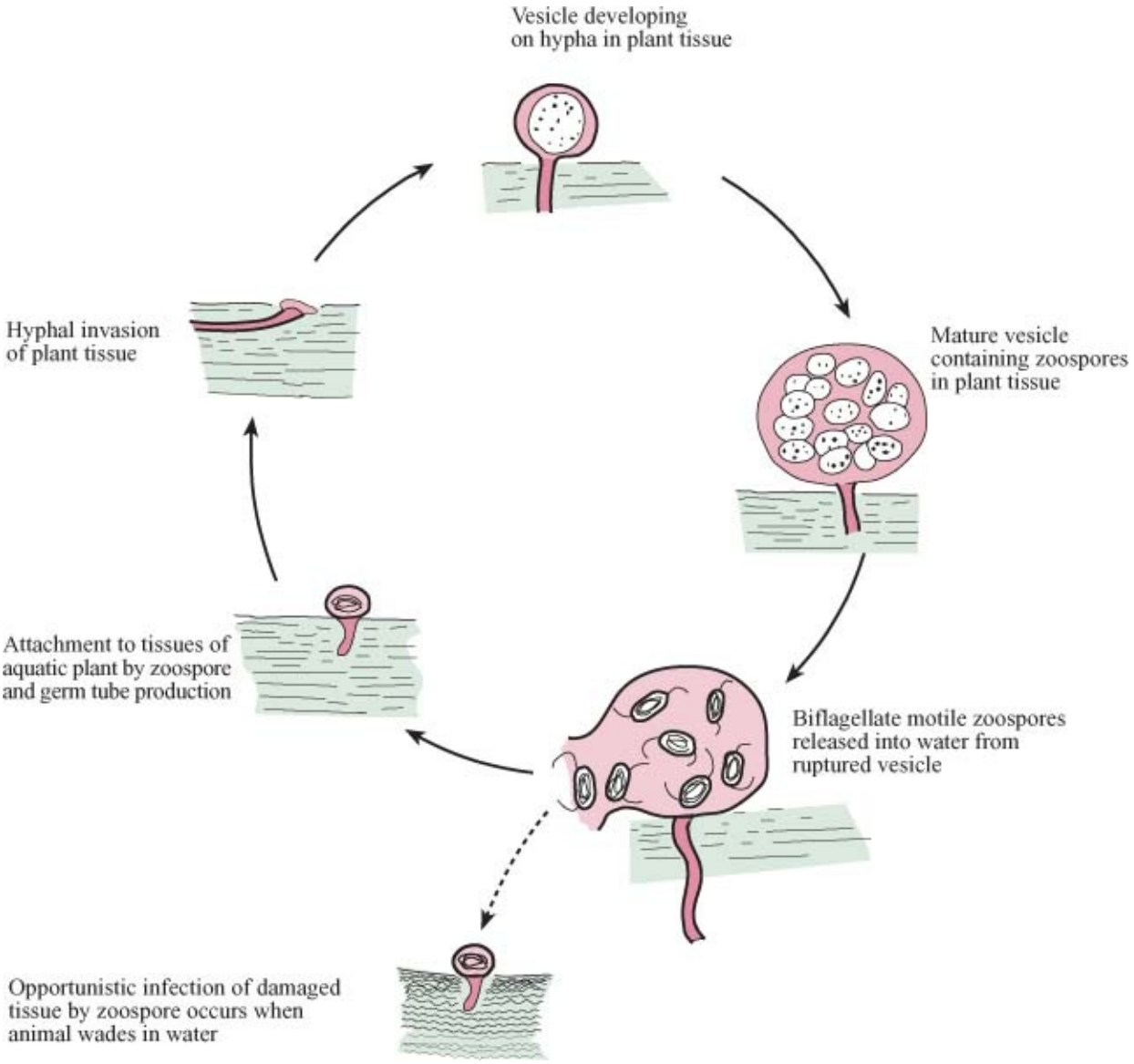
– Aquatic yeast-like organism

– Has not been cultured *in vitro*

– Causes cutaneous lesions in humans and dolphins

Pythiosis, characterized by granulomatous lesions in subcutaneous or intestinal tissues, has been reported in horses, dogs, calves, sheep and cats.

Figure 48.1 Stages in the life cycle of *Pythium insidiosum* in plant tissue. Sporadic invasion of animal tissue can occur at sites of minor trauma when animals are wading in water.



Usual habitat

Pythium insidiosum is usually found in stagnant inland waters and occasionally in soil.

Pathogenicity

Motile zoospores, which are apparently attracted by chemotaxis to wounds and abrasions on skin or intestinal mucosa, encyst on the exposed tissues. The encysted zoospores secrete a sticky material, possibly glycoprotein, which allows adhesion to tissues prior to invasion. Aseptate hyphae, which develop from germ tubes produced by the zoospores at body temperature, extend into the

tissues and may invade blood vessels, facilitating dissemination and producing thrombosis. Infection evokes a cellular immune response consisting mostly of eosinophils. Much of the tissue damage arising from the infection is thought to result from degranulation of eosinophils and mast cells.

Diagnostic procedures

- The nature and distribution of lesions and a history of access to stagnant water in regions where pythiosis occurs may suggest the disease.
- Specimens, including biopsy material and samples from cutaneous lesions in horses, should be delivered immediately to the laboratory. Samples for transportation should be washed in sterile distilled water and transported at ambient temperature.
- Tissue sections, stained by the PAS or methenamine silver methods, are used to demonstrate hyphal forms.
- Immunofluorescence or immunoperoxidase techniques can be used to identify *P. insidiosum* in tissue sections.
- Detection and identification of *Pythium insidiosum*has been achieved using a nested PCR assay (Grooters and Gee, 2002).
- Sabouraud dextrose agar, inoculated with material from lesions, is incubated aerobically at 37°C for 24 to 48 hours. The colonies, which are flat, whitish and radiating, may be up to 20 mm in diameter after 24 hours.
- Identification criteria for isolates:
  - Colonial morphology
  - Aseptate hyphae.
- Specific identification should be carried out in a reference laboratory. A DNA probe has been developed (Schurko *et al*., 2004).
- Serological tests such as agar gel diffusion and ELISA have been used for the diagnosis of pythiosis in affected animals (Grooters *et al*., 2002).

Clinical infections

Pythiosis is a rare, sporadic, non-contagious condition which occurs mainly in tropical and subtropical regions. It has been recorded in Australia, New Zealand, New Guinea, the Caribbean islands, and South, Central and North America. Although horses and dogs are the species most commonly infected, a few cases have been reported in calves (Miller *et al*., 1985).

Pythiosis in horses

Cutaneous pythiosis is the usual presentation in horses (Chaffin *et al*., 1992), although intestinal pythiosis has also been reported (Morton *et al*., 1991). Lesions usually occur on those parts of the body, particularly the limbs, which come in contact with water containing zoospores. Lesions are large, circular, granulomatous nodules which often ulcerate. Sinus tracts may develop exuding a sero-sanguineous discharge. Pruritus is marked. Necrotic yellowish coral-like masses (‘kunkers’ or ‘leeches’) can be removed intact from the granulomas. In addition to necrotic tissue, these masses contain eosinophils and hyphae of *P. insidiosum* (Mendoza *et al*., 1993). Bone involvement can occur in chronic disease. Surgical excision of lesions followed by immunotherapy has proved successful in some cases (Miller, 1981; Mendoza *et al*., 2003). Enteric pythiosis is characterized by stenotic fibrous gastrointestinal lesions.

Pythiosis in dogs

Canine infection most commonly involves the stomach and small intestine (Miller, 1985). Subcutaneous pythi-osis is less commonly encountered (Foil *et al*., 1984). Intestinal lesions are usually extensive when an affected animal is first presented for examination. Clinical signs include vomiting, weight loss, intermittent diarrhoea and palpable abdominal masses. Extension of the infection to the pancreas, mesenteric lymph nodes and bile ducts may occur. Cutaneous lesions, which occur on limbs, face or tail, are granu-lomatous nodules often with discharging sinus tracts. Surgical excision of lesions and long-term treatment with itraconazole may be beneficial.

Rhinosporidium seeberi

Rhinosporidium seeberi is a fungus-like organism which has not been cultured on inert media but has been maintained in monolayers of human rectal tumour cells (Levy *et al*., 1986). It belongs to a group of aquatic protistan parasites in the class *Mesomycetozoea*, kingdom Protozoa (kingdom Protista). Rhinosporid-iosis, a sporadic, non-contagious, pyogranulomatous infection of the skin or mucosa, has been recorded in horses, dogs, cattle, goats, waterfowl and humans. Cases occur predominantly in tropical and subtropical regions, particularly in Sri Lanka, southern India and Argentina.

Usual habitat

It is generally considered that stagnant water and possibly soil are the natural habitats of the organism.

Pathogenicity and pathology

Rhinosporidium seeberi is of low pathogenic potential and disseminated infection is rare. The life cycle of the organism is uncertain. Rhinosporidiosis presents most commonly as a chronic polypous rhinitis characterized by the presence of large sporangia (100 to 400 µm in diameter) in affected tissues. The sporangia, which have double-contoured cell walls with an outer chitinous layer and an inner layer of cellulose, contain up to 16,000 endospores (approximately 7 µm in diameter). Mature endospores can be stained by the PAS and methenamine silver methods. Several electron-dense bodies (1.5 to 2.0µm in diameter) containing DNA are present in the endospores.

The polyps, which may be sessile or pedunculated and up to 3cm in diameter, are composed of soft fibromyxomatous stromal tissue covered by epithelium. Mature sporangia may be detectable grossly in the stroma as minute white spots. Cellular response to sporangia is sparse except when they rupture. The release of endospores elicits a marked pyogranulomatous reaction (Easley *et al*., 1986).

Diagnostic procedures

- Nasal polyposis may suggest the presence of the condition.
- Specimens for laboratory examination should include biopsy material and scrapings from lesions.
- Cytological examination demonstrates a neutrophilic response and many endospores. Neutrophils form aggregates around endospores.
- Sporangia can be demonstrated histologically in tissue sections.

Clinical infections

Rhinosporidiosis, which is endemic in subtropical and tropical regions, has been encountered also in North America and Europe (Caniatti *et al*., 1998; Leeming *et al*., 2007 ; Miller and Baylis, 2009). Infection occurs through minor trauma in skin or mucous membranes. The reddish brown polyps in rhinosporidiosis may project from the nares and can occlude the nasal passages. Noisy breathing may

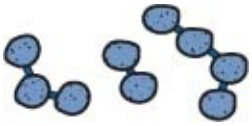
be exacerbated by exercise. Nasal discharge is usually present and epistaxis may occur. The uncommon cutaneous lesions may be single or multiple and sessile or pedunculated. Treatment by cryosurgery or electrocauterization is suggested to avoid excessive bleeding. Diamino-diphenylsulphone (dapsone) has proved beneficial although deleterious side effects, including haemolytic anaemia and thrombocytopenia, may occur in dogs. Recurrence of rhinosporidiosis may occur following treatment.

Lacazia loboi

Lacazia loboi, also known as *Loboa loboi*, is an unclassified yeast-like organism not yet cultured *in vitro*(Taborda *et al*., 1999). Phylogenetic studies have indicated that it is closely related to *Paracoccidioides brasiliensis*, a dimorphic fungal pathogen of humans, in the order *Onygenales*, phylum *Ascomycota* (Herr *et al*., 2001). It is probably an aquatic saprophyte and is capable of causing granulomatous cutaneous disease (lobomycosis or keloidal blastomycosis) in humans and dolphins. In skin sections stained by the PAS or methenamine silver techniques, large numbers of yeast - like cells (5 to 12 μm in diameter) are present in multinucleate giant cells. They replicate by budding and some remain attached to each other by narrow bridge-like structures forming short chains (Fig. 48.2).

Lobomycosis has not been reported in domestic animals. Human cases of the disease have been reported from tropical regions of South and Central America and affected dolphins have been found off the coast of Florida (Reif *et al*., 2006). Skin changes in dolphins range from white crusts to nodular or verrucose lesions which ulcerate easily and bleed (Bossart, 1984). Small lesions can be removed surgically. Lobomycosis in a dolphin has been treated successfully with miconazole.

Figure 48.2 Cells *of Lacazia loboi* as they appear in smears from lesions. These yeast-like cells often occur in short chains connected by short bridge-like structures.



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Chapter 49

Pneumocystis carinii

Pneumocystis carinii is a unicellular organism with a life cycle resembling that of a protozoal parasite. On the basis of significant genetic evidence it is classified in the family *Pneumocystidaceae*, phylum *Ascomycota*, in the kingdom Fungi (Pixley *et al*., 1991). Although the life cycle of *P. carinii* has not yet been fully determined, intrapulmonary infection probably involves asexual and sexual phases. The life cycle involves a thin-walled haploid trophic or vegetative form which can undergo binary fission or can enter into sexual reproduction through conjugation. Mating results in production of a diploid zygote and the initiation of sporogenesis. This is followed by transitional changes involving three consecutive sporocytic stages into a thick-walled cyst containing eight spores. Following their release, these ascospores enter a vegetative stage, completing the life cycle. A cell wall is present only in the cyst form of the organism. Because the main sterol in this thin cell wall is cholesterol rather than ergos-terol, this form of the organism is refractory to standard antifungal drugs. Recent studies suggest that benign colonization by *Pneumocystis carinii* in the lungs of immunocompetent mammals is relatively common, resulting in carriage and airborne transmission to susceptible in-contact animals of the same species (Chab é *et al*., 2004). In immunocompromized individuals, infection may result in pneumonia. Pneumocystosis in humans is often associated with immunosuppression following HIV infection. The organism has been found in a wide range of domestic, wild and captive mammalian species. Formerly, the genus was described as having a single species. Current molecular studies suggest that *P. carinii* is made up of a heterogeneous group of genetically isolated strains that have undergone functional and genetic adaptation to their respective host species over millions of years. Strains of *P. carinii* from different animal species exhibit a narrow host range and have distinct genetic and antigenic profiles (Peters *et al*., 1994). A trinomial nomenclature has been proposed whereby the name of a particular strain or ‘special form’ (*forma specialis*) is derived from the host species from which it originated. As an example, the name *P. carinii* f.sp. *equi*

would apply to horse strains. In recent years additional species have been formally described and proposed: *P. carinii* and *P. wakefieldiae* isolated from rats, *P. jirovecii* from humans, *P. murina* from laboratory mice and *P. oryctolagi* from Old World rabbits. The organism is difficult to culture *in vitro*.

Key points

- Member of the kingdom Fungi
- Distinct strains appear to be associated with particular animal species
- Difficult to culture *in vitro*
- Trophic, cyst and spore forms may be found in lungs of affected animals
- Pneumonia, which occurs only in immunosuppressed animals, occasionally affects horses and dogs

Usual habitat

The natural reservoir of *P. carinii* is not known but serological and molecular studies suggest a high prevalence of colonization in young, clinically normal mammals, probably as a result of airborne or transplacental transmission.

Pathogenesis and pathogenicity

The exact mode of transmission is uncertain but airborne spread is considered to be the most important route. In the animal body, the trophic form adheres in clusters to type 1 alveolar cells. Characteristic pathological findings, which are similar in all species, include diffuse pulmonary consolidation, marked thickening of alveolar septa and proteinaceous exudate in alveoli.

Diagnostic procedures

- Specimens for laboratory examination may include lung tissue and bronchoalveolar lavage fluid.
- Cytological and histopathological specimens are used for diagnosis. Giemsa-stained preparations can be used to demonstrate the different forms of the organism, whereas the methenamine silver method stains only the cyst form. Fluorescent-conjugated monoclonal antibody techniques are sensitive and specific.

- Immunocytochemical methods can be used for specific identification in tissue sections.
- The organism may be detected during electron microscopic examination of bronchoalveolar lavage fluid.
- The polymerase chain reaction is used in reference laboratories for DNA amplification (Peters *et al*., 1994 ; Ramos Vara *et al*., 1998).
- Serological tests, employed for epidemiological surveys, are not of diagnostic value.

Clinical nfections

Most cases of pneumonia caused by *Pneumocystis*species in domestic animals have been recorded in dogs, horses, rabbits, cats and pigs. The condition has been most extensively studied in young rabbits which provides a non-immunosuppressed model for *Pneumocystis carinii* pneumonia (Soulez *et al*., 1989). Hereditary immunodeficiency has been suggested as an explanation for the frequency of the disease in miniature dachshunds (Farrow *et al*., 1972). Arabian foals with combined immunodeficiency disorder are particularly susceptible (Perryman *et al*., 1978). Affected animals are afebrile and present with respiratory distress. Without treatment the disease may prove fatal. Trimethoprim-sulphamethoxazole, administered orally for 2 weeks, is usually effective.

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Chapter 50

Opportunistic infections caused predominantly by phaeoid fungi

A variety of saprophytic fungi which can infect traumatized tissues produce slowly progressive inflammatory lesions. Clinical cases are uncommon and generally represent exploitation of predisposing conditions, particularly devitalized tissues and immuno-suppression. Although lesions mainly involve the dermis or subcutis, they may be found elsewhere. In this chapter, discussion is limited to the saprophytic fungi which have been aetiologically implicated in phaeohyphomycosis and eumycetomas. Eumycetomas caused by *Sporothrix schenkii*, *Pythium insidiosum*, *Aspergillus* species and the zygomycetes are described in other chapters.

Phaeohyphomycosis is caused by species of phaeoid (dematiaceous, pigmented) fungi which produce dark-walled hyphae, pseudohyphae and yeast cells *in vivo*. Mycetomas are chronic subcutaneous infections caused by fungi (eumycetomas) or actinomycetes (actinomycetomas) and characterized by the presence of grains or granules. Eumycetomas may be caused by phaeoid or non-phaeoid fungi. The granulomatous lesions in eumycetomas are distinguished from the lesions of phaeohyphomycosis by the presence of granules composed largely of fungal mycelia in exudates. In some instances phaeohyphomycosis, especially when caused by infection with *Bipolaris specifera* or with *Exserohilum rostratum*, has been mistakenly categorized as eumycetoma (Chandler *et al*., 1980). True eumycetomas are rare in domestic animal species (Brodey *et al*., 1967; Lambrechts *et al*., 1991).

The pigmentation of phaeoid fungi is due to the presence of melanin in their hyphal walls. The dark coloration of granules occasionally found in eumycetomas also derives from melanin. Some phaeoid species such as *Bipolaris* species and *Scedoporium* species produce meagre amounts of melanin. In lesions caused by these species, their hyphae appear non-pigmented in tissue sections. Masson-Fontana silver stain can be used to demonstrate the presence of melanin. Melanin is thought to play a role in fungal penetration of tissues by

acting as an antioxidant aiding survival of the oxidative bursts produced by phagocytes and by protecting the fungal cell wall through the binding of host hydrolytic enzymes.

Key points

- Phaeoid (pigmented) fungi can infect traumatized tissue causing phaeohyphomycosis
- An uncommon manifestation of infection with both phaeoid and non-phaeoid fungi is eumycetoma formation
- The granulomatous lesions of phaeohyphomycosis and eumycetoma occur most frequently in subcutaneous tissues
- Sinus formation with serosanguineous discharges is a feature of superficial lesions
- Discharges from eumycetomas contain macroscopic granules composed of fungal elements. Granules formed by phaeoid fungi are black; those formed by other fungi lack pigmentation

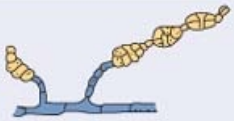
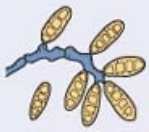
Usual habitat

The fungi implicated in phaeohyphomycosis and eumycetoma are found in soil and in plant material. Some are distributed worldwide and others are restricted to tropical and subtropical regions.

Clinical infections

The more important fungal species that have been isolated from subcutaneous phaeohyphomycosis in domestic animals are presented in Table 50.1. Some of these fungal species have also been isolated from the rare eumycetomas which have been confirmed in domestic animals. The domestic species most often affected by phaeohyphomycosis are cats, horses and cattle. Eumycetomas have been described in horses and also in dogs (Brodey *et al*., 1967). *Curvularia* and *Bipolaris* species have been recovered from granulo-matous lesions in the nasal cavity of cattle. *Scedosporium apiospermum* (anamorph of *Pseudallescheria boydii*) has been isolated from lesions of white line disease in horses.

Table 50.1 Dematiaceous fungi infrequently implicated in subcutaneous mycoses in domestic animals.

Fungus	Colonial appearance	Microscopic structure		Animals affected
Altemaria species	Colony matures within 5 days and has a greyish woolly surface; reverse is black	Conidiophores are septate and vary in length. Conidia are formed singly or in chains		Horses (*A. alternata)* Cats *(A. infectoria)*
Bipolaris spicifera	Colony matures in about 5 days and has a greyish brown surface; reverse is black	Conidiophores are elongate and bend at the attachment point of each conidium. Conidia are cylindrical with three to five septa		Cats, dogs, horses, cows
Curvularia species	Colony matures in 5 days and has a dark olive-green to brown or black surface; reverse is black	Conidiophores are simple or branched and bent at points of conidial formation. Due to the swelling of the central cell, conidia appear curved		Cows (*C. anomatum)* Dogs, horses *(C. geniculata)* Cats (*C. lunata)*
Exophiala jeanselmei	Colony requires up to 15 days to mature; surface is brown and skin-like becoming velvety; reverse is black	Initially, yeast-like budding cells may be present; conidiophores bearing conidia in clusters are produced later		Cats (*E. jeanselmei, E. spinifera)*
Exserohilum rostratum	Colony matures in 5 days and has a dark grey to black cottony surface; reverse is black	Conidiophores have a uneven appearance; conidia are fusiform with seven to eleven septa		Cows
Phialophora verrucosa	Colony matures in about 15 days. Surface is dark greenish-brown to black; reverse is black	Conidia are oval to round and accumulate at the apex of a phialide which has a cup-like collarette		Cats
Phoma glomerata	Colony matures within 5 days and is powdery or velvety and greyish brown; reverse is brown	A pycnidium, the asexual fruiting body, is dark and round with an opening. Conidia are borne on conidiophores inside the pycnidium		Goats
Scedosporium apiospermum (Pseudallescheria boydii)	Colony matures in 7 days, initially white, becoming grey or brown; reverse is white becoming greyish black	Both short and long conidiophores bear conidia which are oval with a flat base		Dogs, horses

Slowly enlarging subcutaneous granulomatous lesions, located mainly on the feet, limbs and head, are the most common presentations in both phaeohyphomycosis and eumycetomas. The lesions in eumycetomas are nodular. Ulceration and sinus tract formation with serosanguineous discharges are associated with both conditions. Lesions of phaeohyphomycosis in two horses

presented as black denuded skin plaques (Kaplan *et al*., 1975). *Alternaria* species were found to be the most common cause of nodular granulomatous fungal skin disease in cats in the United Kingdom (Miller, 2010). They have also been associated with nodular skin disease in horses (Genovese *et al*., 2001). The presence of distinct granules (either black or pale) in discharges distinguishes the lesions of eumycetomas from those of phaeohyphomycosis.

Systemic lesions caused by phaeoid fungi are extremely rare. Osteolytic phaeohyphomycosis caused by *Phialemonium obovatum* (Lomax *et al*., 1986) and *Scedosporium inflatum* (Salkin *et al*., 1992), now termed S. *prolificans*, has been recorded in dogs. Cerebral phaeohyphomycosis caused by *Cladophialophora bantiana* has been reported in dogs and a cat (Dillehay *et al*., 1987). A chronic granulomatous lesion in the abdominal cavity of a dog was described as a black grain eumycetoma (Lambrechts *et al*., 1991).

Diagnosis

- Suitable specimens for laboratory examination include fine-needle aspirates, punch biopsies and post-mortem tissue samples.
- Specimens are inoculated on to Sabouraud dextrose agar, with and without antimicrobial agents, and incubated aerobically at 25°C to 30°C for up to 6 weeks. Isolates should be subcultured to facilitate identification by a reference laboratory.
- Isolates are identified by colonial morphology and the microscopic appearance of fruiting structures (Table 50.1). However, a commercial DNA sequencing kit has shown some promise in the identification of dematiaceous fungi (Hall *et al*., 2004).
- Both PAS and methenamine silver techniques are used for demonstrating hyphae in tissue sections.
- The Masson - Fontana silver stain is used to demonstrate melanin in the hyphae of phaeoid fungi.
- A deposit of eosinophilic material around mycelial aggregates in eumycetomas, referred to as the Splendore-Hoeppli phenomenon, may be demonstrable in tissue sections.

Treatment

- Discontinuation of any form of immunosuppressive therapy (Swift *et al*., 2006).
- Surgical excision of lesions is effective (Beale and Pinson, 1990).
- Although antifungal therapy is usually ineffective, a combination of amphotericin B and 5-fluorocytosine may be beneficial.

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Chapter 51

Mycotoxins and mycotoxicoses

Mycotoxins, secondary metabolites of certain fungal species, are produced when toxigenic strains of these organisms grow under defined conditions on crops, pasture or stored feed. The acute or chronic intoxication following ingestion of contaminated plant material is termed mycotoxicosis. More than 100 fungal species are known to elaborate mycotoxins, and approximately 400 secondary metabolites with toxigenic activity are produced by these fungi. Many of these fungi belong to the genera *Penicillium, Aspergillus, Fusarium and Claviceps.*

Factors affecting mycotoxin production and the development of mycotoxicosis are presented in Fig. 51.1. For fungal growth and toxin production, a suitable substrate must be available along with moisture and optimal temperature and oxygen levels. Some mycotoxicoses have a high prevalence in particular geographical regions where agricultural practices favour their occurrence. Mycotoxicoses tend to be more common in developing countries where methods of harvesting and storage are inadequate and where regulations aimed at preventing the distribution and sale of suspect crops for human consumption or animal feed are not enforced. Toxigenic strains of fungi may grow preferentially on particular parts of a plant, some favouring the carbohydrate-containing seeds or kernels and others utilizing the cellulose substrate in fibrous stems or leaves.

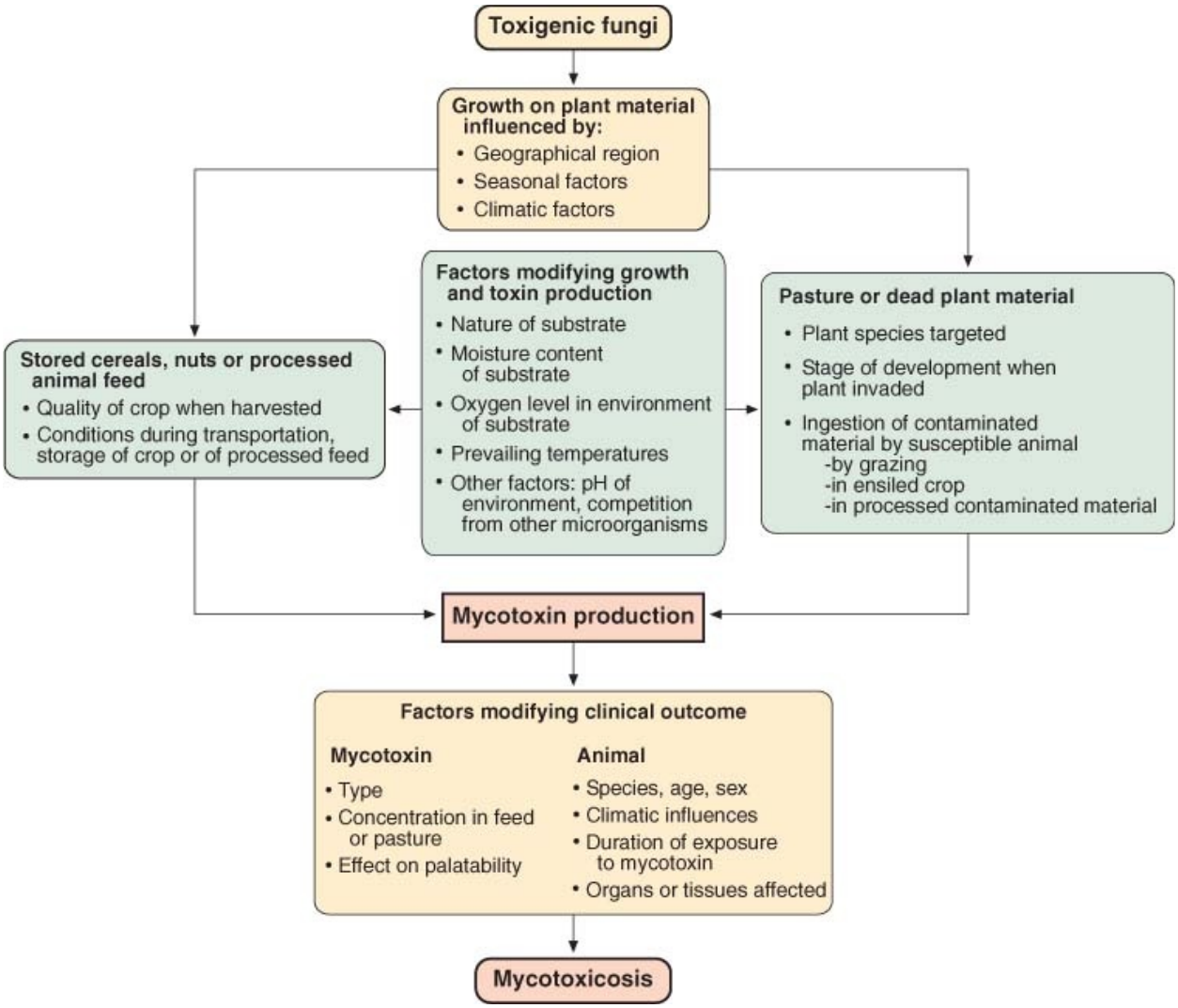
Mycotoxins are non-antigenic, low molecular weight compounds. Many are heat-stable, retaining toxicity following exposure to the processing temperatures used for pelleting and other procedures (Box 51.1). A particular mycotoxin may be produced by a number of fungal species. Moreover, some fungi can elaborate several mycotoxins which may differ in biological activities, producing complex clinical effects. Clinical diagnosis of the mycotoxicoses may also be complicated by the presence of a number of toxigenic species on a food source. The severity of clinical signs is influenced by the period of exposure to contaminated feed and the amount of mycotoxin ingested. Some batches of feed may be contaminated and mycotoxin may be unevenly distributed in a batch. Clinical evidence of the targeting of particular organs such as the liver or the central

nervous system is a feature of some mycotoxicoses. Immunosuppression, mutagenesis, neoplasia or teratogenesis may also result from exposure. Epidemiological and clinical features of mycotoxicoses are summarized in Box 51.2.

Key points

- Certain fungi elaborate metabolites (mycotoxins) in growing crops or stored feed under defined environmental conditions
- Mycotoxins, a diverse group of heat-stable, low molecular weight compounds, are non-antigenic
- Ingestion of contaminated plant material or contaminated crops may induce a characteristic disease process
- Susceptibility can vary with species, age and sex
- The effects of mycotoxins include immunosuppression, teratogenesis or carcinogenesis
- Diseases caused by mycotoxins (mycotoxicoses) are non-contagious, tend to be sporadic, seasonal and associated with certain batches of feed
- Diagnosis is based on clinical presentation and on demonstration of significant levels of a specific mycotoxin in feed or in animal tissues

Figure 51.1 Factors affecting mycotoxin production and manifestations of clinical disease.



Box 51.1 Characteristics of mycotoxins.

- Low molecular weight, heat-stable substances
- Unlike many bacterial toxins, non-antigenic; exposure does not induce a protective immune response
- Many are active at low dietary levels
- Specific target organs or tissues affected
- Toxic effects include immunosuppression, mutagenesis, teratogenesis and carcinogenesis
- Accumulation in tissues of food-producing animals or excretion in milk may result in human exposure

Mycotoxicoses of defined veterinary importance are presented in Table 51.1. The role of a number of mycotoxins considered to be involved in diverse clinical conditions in domestic animals is not yet clearly defined (Lomax *et al*, 1984;

Griffiths and Done, 1991). Many diseases with suspected links to mycotoxins are being re-evaluated as the toxic activities of new fungal metabolites are investigated (Fink-Gremmels, 2008).

Aflatoxicosis

Fungi associated with the production of aflatoxins, the mycotoxins which cause aflatoxicosis, include *Aspergillus flavus*, *A. parasiticus* and *A. nominus*. Ingestion of aflatoxins, which are difuranocoumarins elaborated by toxigenic strains of *Aspergillus* species, produces disease in a wide range of mammals, birds and aquatic species. Many substrates support the growth of aflatoxin production by toxigenic *Aspergillus* species. Maize, wheat, oats, barley, rice, peanuts and other nuts are among the crops that may be naturally contaminated by these mycotoxins. Fungal growth and mycotoxin production can proceed when the moisture content of a harvested crop or stored feed is close to 16% in a temperature range of 14°C to 30°C. In surveys of pet foods for the presence of aflatoxins, wild bird feed was found to be the most contaminated (Leung *et al*., 2006). All animal species are susceptible to aflatoxins but young animals and monogastric animals are more susceptible than mature ruminants.

Box 51.2 Epidemiological and clinical features of mycotoxicoses.

- Outbreaks usually seasonal and sporadic
- No evidence of lateral spread to in-contact animals
- Certain types of pasture or stored feed may be involved
- Clinical presentation is usually ill-defined
- Increased susceptibility to infectious disease may be evident
- Severity of clinical signs is influenced by the amount of mycotoxin ingested; recovery is related to duration of exposure
- Occurrence of vaccination failure may be increased
- Antimicrobial medication is ineffective
- Confirmation requires demonstration of significant levels of mycotoxin in feed or in tissues from affected animals

Although four prominent aflatoxins, namely B_1, B_2, G_1 and G_2, are particularly important in disease production, many more have been identified. Aflatoxin B_1, which is the mycotoxin most often encountered in disease

outbreaks and is reported to be particularly toxic for humans and animals, is hepatotoxic and carcinogenic. After absorption from the gastrointestinal tract, aflatoxins are metabolized by the liver to a range of toxic and nontoxic products. The liver is the principal organ affected by aflatoxins, resulting in hepatocellular necrosis and impaired liver function. In cattle, aflatoxin B1, which escapes rumen degradation, is converted into a hydroxylated form called aflatoxin M1, which is excreted in milk (Fink-Gremmels, 2008).

Toxicity relates to binding of metabolites to macro-molecules, especially nucleic acid and nucleoproteins. Consequently, the toxic effects include reduced protein synthesis, carcinogenesis, teratogenesis and aplasia of the thymic cortex leading to depressed cell-mediated immunity (Osweiler, 1990).

Clinical findings

Aflatoxicosis has been encountered worldwide in many domestic species. There is considerable variation in susceptibility between species and within age groups. Ducklings, turkey poults, calves, pigs and dogs are sensitive to toxic effects, whereas sheep and adult cattle are more resistant. Aflatoxicosis is uncommon in horses and goats. Subacute aflatoxicosis, associated with prolonged exposure to low concentrations of toxin, usually presents as slowly developing ill thrift and reduced growth rate. Immunosuppression, with increasing prevalence of endemic infection and inadequate responses to routine vaccination, may also be detected in affected groups of animals. This insidious form of aflatoxicosis is often of greater economic significance than acute forms of the disease associated with high concentrations of toxin in the diet. Acute aflatoxicosis has been recorded in birds and cattle. Ataxia, opisthotonos and sudden death are features of the acute disease in ducklings. Hepatopathy is a common finding and, in birds more than 3 weeks old, subcutaneous haemorrhages may be evident. Haemorrhagic diathesis, probably related to hepatopathy, is a characteristic of acute toxicity in chickens and turkeys. In turkey X disease, the first clearly defined outbreak of acute aflatoxicosis, widespread haemorrhages may have been due to the combined effect of aflatoxins and cyclopiazonic acid, which are often produced simultaneously by *A. flavus* (Robb, 1993). Acute aflatoxicosis in cattle may rapidly result in death (Cockcroft, 1995). In affected calves, blindness, circling, tenesmus, diarrhoea and convulsions have been recorded. Low levels of aflatoxins fed to young pigs depressed their cell-mediated and humoral immune responses and increased their susceptibility to infectious

agents. Clinical signs of aflatoxicosis in dogs include depression, anorexia, vomiting, diarrhoea, jaundice and epistaxis. Aflatoxins present in a diet which was fed for many months were identified as the cause of epidemic liver cancer in rainbow trout (Coppock and Jacobsen, 2009). Deaths in the human population in India following consumption of maize contaminated with aflatoxin have been reported (Moss, 2002).

Diagnosis

- Except in outbreaks of acute disease, clinical signs are vague. Epidemiological features and postmortem findings may be of diagnostic value. Aflatoxin may be demonstrated in tissues obtained at post-mortem.
- Carefully selected samples of suspect feed should be stored at−20°C until analysed.

Table 51.1 Mycotoxicoses of domestic animals.

Disease / Mycotoxins	Fungus / Crop or substrate	Species affected / Geographical distribution	Functional or structural effects / Clinical findings
Aflatoxicosis / Aflatoxins B_1, B_2, G_1, G_2	*Aspergillus flavus, A. parasiticus* / Maize, stored grain, groundnuts, soybeans	Pigs, poultry, cattle, dogs, trout / Worldwide	Hepatotoxicity, immunosuppression, mutagenesis, teratogenesis, carcinogenesis / Ill-thrift, drop in milk yield, rarely death from acute toxicity
Citrinin toxicosis / citrinin	*Penicillium citrinum, P. expansum, Aspergillus terreus* / Wheat, oats, maize, barley, rice	Pigs, cattle, poultry / Worldwide	Kidney lesions in pigs, haemorrhagic syndrome in cattle / Increased water consumption in pigs, dilute urine; multiple haemorrhages on mucosal surfaces in cattle
Cyclopiazonic acid toxicosis / Cyclopiazonic acid	*Aspergillus* species, some strains of *Penicillium camembertii* / Stored grain, meal	Pigs, poultry / Worldwide	Interference with ion transport across cell membranes / Weakness, food refusal, anorexia in pigs, weight loss in poultry
Diplodiosis / unidentified neurotoxin	*Diplodia maydis* / Maize cobs	Sheep, cattle, goats, horses / South Africa, Argentina	Neurotoxicity / Ataxia, paresis and paralysis in adults, perinatal deaths in lambs and calves
Ergotism / Ergotamine, ergometrine, ergocristine	*Claviceps purpurea* / Seedheads of ryegrass and other grasses, cereals	Cattle, sheep, deer, horses, pigs, poultry / Worldwide	Neurotoxicity and vasoconstriction / Convulsions, gangrene of extremities, agalactia, hyperthermia in hot climates
Facial eczema / Sporidesmin	*Pithomyces chartarum* / Pasture litter from ryegrass and white clover	Cattle, sheep, goats / New Zealand, Australia, South Africa, South America, occasionally USA and parts of Europe	Hepatotoxicity, biliary occlusion / Photosensitization, jaundice
Fescue toxicosis / Ergovaline	*Neotyphodium coenophialum* / Tall fescue grass	Cattle, sheep, horses / New Zealand, Australia, USA, Italy	Vasoconstriction / Dry gangrene in cold weather in cattle and sheep (fescue foot); hyperthermia and low milk yields (fescue summer toxicosis)
Fumonisin toxicosis / Fumonisins, especially B_1 and B_2	*Fusarium verticillioides*, other *Fusarium* species / Standing or stored maize	Horses, other *Equidae*, pigs / Egypt, South Africa, USA, Greece	Mycotoxic leukoencephalomalacia in horses; porcine pulmonary oedema / Neurological signs include weakness, staggering, circling, depression in horses; pulmonary oedema and hydrothorax in pigs
Mouldy sweet potato toxicosis / Derivative of 4-ipomeanol	*Fusarium solani, F. semitectum* / Sweet potatoes	Cattle / USA, Australia, New Zealand	Cytotoxicity producing interstitial pneumonia and pulmonary oedema / Respiratory distress, sudden death may occur
Mycotoxic lupinosis / Phomopsins A, B, C, D, E	*Diaporthe toxica* / Growing lupins with stem blight	Sheep, occasionally cattle, horses, pigs / Worldwide	Hepatotoxicity / Inappetence, stupor, jaundice, ruminal stasis, often fatal
Ochratoxicosis / ochratoxins A, B, C and D	*Aspergillus alutaceus*, other *Aspergillus* species, *Penicillium verrucosum*, other *Penicillium* species / Stored barley, maize and wheat	Pigs, poultry / Worldwide	Degenerative renal changes / Polydipsia and polyuria in pigs, fall in egg production in birds
Oestrogenism / Zearalenone	*Fusarium graminearum*, other *Fusarium* species / Stored maize and barley, pelleted cereal feeds, maize silage	Pigs, cattle, occasionally sheep / Worldwide	Oestrogenic activity / Hyperaemia and oedema of vulva and precocious mammary development in young gilts; anoestrus and reduced litter size in mature sows; reduced fertility in cattle and sheep

Patulin toxicosis / Patulin	*Penicillium expansum*, *Aspergillus* species / Rotting fruit especially apples, apple juice, mouldy bread	Cattle, sheep, pigs / Worldwide	Antibiotic-like effect on ruminal flora, acidosis; vomiting and anorexia in pigs / Poor food utilization in ruminants; weight loss in pigs
Slaframine toxicosis / Slaframine	*Rhizoctonia leguminicola* / Legumes, especially red clover, in pasture or hay	Sheep, cattle, horses / USA, Canada, Japan, France, The Netherlands	Cholinergic activity / Salivation, lacrimation, bloating, diarrhoea, rarely death
Sterigmatocystin toxicosis / Sterigmatocystin	*Aspergillus versicolor*, *A. flavus*, other *Aspergillus* species / Stored wheat flour, cereals, peanuts, dry beans	Cattle, poultry / Many countries	Hepatoxicity, enteric lesions / Drop in milk yield, dysentery
Tremorgen intoxications			
Perennial ryegrass staggers / Lolitrem B	*Neotyphodium lolii* / Perennial ryegrass	Cattle, pigs, poultry, sheep, horses, deer / USA, Australia, New Zealand, Europe	Neurotoxicity / Muscular tremors, incoordination, convulsive seizures, collapse
Paspalum staggers / Paspalinine, paspalitrems A, B and C	*Claviceps paspali* / Seedheads of paspalum grasses	Cattle, sheep, horses / New Zealand, Australia, USA, South America	Neurotoxicity / Muscular tremors, incoordination, convulsive seizures, collapse
Penitrem staggers / Penitrem A, verruculogen, other mycotoxins	*Penicillium crustosum* and other species, some *Aspergillus* species / Stored feed and pasture	Ruminants, other domestic animals / Probably worldwide	Neurotoxicity / Muscular tremors, incoordination, convulsive seizures, collapse
Aspergillus clavatus induced tremors / Unidentified neurotoxin	*Aspergillus clavatus* / Sprouted wheat, millers' malt culms	Cattle / China, South Africa, Europe	Neurotoxicity, degeneration of neurons / Frothing from mouth and knuckling of limbs when forced to move
Trichothecene toxicoses			
Deoxynivalenol toxicosis / Deoxynivalenol	*Fusarium graminearum*, *F. culmorum*, other *Fusarium* species / Cereal crops	Pigs, poultry / Countries with wet seasons and temperate climates	Neurotoxicity / Feed refusal, vomition, poor growth; teratogenic activity in rodents
T-2 toxicosis / T-2 toxin	*Fusarium sporotrichioides*, *F. poae*, other *Fusarium* species / Mouldy wheat and other cereals	Pigs, cattle, poultry / USA, some other countries	Cytotoxicity, immunosuppression, haemorrhage / Feed refusal in pigs; rumenitis in cattle; beak lesions and abnormal feather formation in chickens
Diacetoxyscirpenol toxicosis / Diacetoxyscirpenol	*Fusarium tricinctum*, other *Fusarium* species / Cereals	Cattle, pigs, poultry / North America, some other regions	Necrotic lesions, mucosal haemorrhages, vomition / Necrotic lesions in the alimentary tract, haemorrhages in the skin, depression
Stachybotryotoxicosis / Satratoxins, roridins, verrucarins	*Stachybotrys chartarum* / Stored cereals, straw, hay	Horses, cattle, sheep, pigs / Former USSR, Europe, South Africa	Cytotoxicity, coagulopathy, immunosuppression / Stomatitis, necrotic lesions in alimentary tract, haemorrhages
Myrotheciotoxicosis / Roridin	*Myrothecium verrucaria*, *M. roridum* / Ryegrass, rye stubble, straw	Sheep, cattle, horses / Former USSR, New Zealand, south-eastern Europe	Inflammation of many tissues, pulmonary congestion / Unthriftiness, sudden death

- Procedures for aflatoxin detection in feed and tissues include:
  – Immunoaffinity columns which are sometimes used for sample purification

to improve sensitivity.

– Thin-layer chromatography. Chromatograms are examined under UV light for the four main toxins on the basis of position and fluorescence. The fluorescence of aflatoxins B_1 and B_2 is blue and with G_1 and G_2 it is green.

– High-performance liquid chromatography.

– Ultra-performance liquid chromatography combined with mass spectrometry.

– Immunoassay techniques such as ELISA and radioimmunoassay procedures.

– Biological assays such as bile duct proliferation in ducklings, chick embryo bioassays, brine shrimp larvae tests and trout embryo bioassays.

Control and prevention

- The growth of fungal contaminants on stored feed should be limited by appropriate measures following harvesting and during storage.
- Batches of food for human and animal consumption may be monitored for aflatoxin contamination.
- Treatment with ammonia gas at high temperature and pressure has been used to detoxify contaminated batches of feed.
- Aflatoxins B_1 and G_1 are rapidly degraded by 2% ozone but aflatoxins B2 and G2 are more resistant and require treatment with 20% ozone for rapid degradation.
- Dilution of contaminated feed with uncontaminated supplies is no longer recommended and is illegal in the EU as a method to reduce aflatoxin concentration and minimize toxicity.
- Addition of hydrated sodium calcium aluminosilicate to feed is reported to reduce aflatoxin toxicity (Harvey *et al*., 1989).
- Glucomannan-containing polymers extracted from yeast cell walls have a high adsorptive capacity for binding different mycotoxins including aflatoxin B1 (Leung *et al*., 2006).

Citrinin toxicosis

Many *Penicillium* species including *P. citrinum*, *P. viridicatum*and *P. expansum* produce the mycotoxin citrinin. *Aspergillus terreus* and a number of other

Aspergillus species also produce the mycotoxin which is a potent nephrotoxin. Citrinin and ochratoxin A,which are often found together in stored cereal grain, can act synergistically. The toxic effects of citrinin include severe kidney damage, hepatic damage and immunosuppression as a consequence of damage to the immune system. Wheat, oats, maize, barley and rice are among the cereals which may contain citrinin.

In Scandinavia, a condition in pigs referred to as a porcine nephrosis syndrome has been attributed to consumption of barley contaminated with citrinin. Clinical features of this condition include increased water consumption and increased production of dilute urine containing protein. A haemorrhagic syndrome in cattle is associated with citrinin poisoning. In poultry, signs of citrinin intoxication include lethargy, decreased feed consumption, increased consumption of water and reduction in weight gain. Following feeding trials, dogs developed emesis, intussusception and renal failure. Natural citrinin toxicosis in dogs seems unlikely in view of the strong emetic effect of this mycotoxin.

Cyclopiazonic acid toxicosis

Many *Penicillium* species and a number of *Aspergillus* species, including *A. flavus*, produce cyclopiazonic acid. Some strains of *P. camembertii*, used in the production of gourmet cheese, also produce cyclopiazonic acid. This mycotoxin is an indole tetrameric acid which acts as a specific inhibitor of calcium-dependent ATPase and affects ion transport across cell membranes. The mycotoxin is produced by particular fungi growing on stored grain or meal. Weakness, anorexia and loss of body weight have been described in pigs consuming contaminated meal. In chickens, cyclopiazonic acid decreases feed conversion, interferes with weight gain and may cause mortality. Consumption of mouldy millet containing cyclopiazonic acid has been linked to kodua poisoning in humans, characterized by somnolence, nausea and tremors.

Diplodiosis

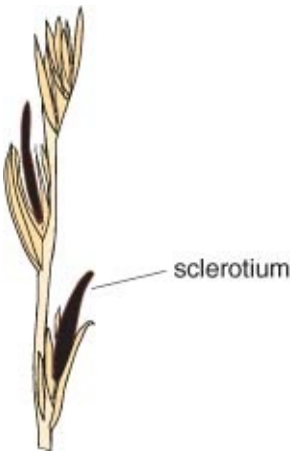
Ingestion of the toxin produced by *Diplodia maydis*, the corn cob rot fungus growing on maize cobs, causes a disease called diplodiosis in farm animals. Contaminated cobs fed to animals or ingested by animals grazing fields where maize has been harvested produce disease in cattle, sheep, goats and horses. The

condition has been recorded in southern Africa and Argentina. Growth of *D. maydis* on cobs over a number of weeks is necessary for toxin production. An interval of up to 2 weeks may elapse between ingestion of contaminated cobs and emergence of clinical signs. Prominent clinical signs include stiffness, incoordination, ataxia and paralysis. In some outbreaks of diplodiosis in cattle, the mortality rate may approach 40% (Odriozola *et al*., 2005). Signs of illness disappear when contaminated corn is removed from the diet. Exposure of pregnant ewes and cows during the second half of pregnancy results in stillbirths and neonatal deaths. Spongiform lesions may be found in a high percentage of the brains of affected lambs and calves; vacuolation of myelin sheaths and swelling of astrocytes have been recorded in adult cattle.

Ergotism

This disease, which occurs worldwide in many domestic animal species and in humans, follows ingestion of toxic levels of certain ergopeptide alkaloids found in the sclerotia of *Claviceps purpurea*. This fungal species colonizes the seed heads of ryegrasses and cereals such as rye and barley. Toxicity may be retained in silage (Hogg, 1991). Ovarian tissue of the seed is destroyed and replaced by fungal mycelium which enlarges, hardens and darkens to form a sclerotium, also referred to as an ergot (Fig. 51.2). An inscription on an Assyrian tablet dating from 600 BC refers to a ‘noxious pustule in the ear of grain’ thought to be a reference to ergot (Sanders-Bush and Mayer, 2006). Mature sclerotia, shed from seed heads in autumn and overwintering in the soil, germinate in the following spring. They produce stromata bearing perithecia in which asci, containing ascospores, develop. Windborne ascospores, forcibly discharged from the perithecia, germinate on suitable grasses and cereal plants to form a new generation of sclerotia. The most important ergopeptide alkaloids in the sclerotia are ergotamine, ergometrine and other derivatives of lysergic acid (Coppock and Jacobsen, 2009). These alkaloids have a number of pharmacological effects including direct stimulation of the adrenergic nerves supplying arteriolar smooth muscle and inhibition of prolactin secretion.

Figure 51.2 Sclerotia (ergots) of *Claviceps purpurea* in the seed head of growing ryegrass.



Clinical findings

Convulsive ergotism, an uncommon acute form of the disease, is occasionally observed in cattle, sheep and horses exposed to large doses of ergotamine. Signs include staggering, convulsive episodes and drowsiness. Smaller amounts of mycotoxin absorbed over relatively long periods result in persistent arteriolar constriction and endothelial damage. The effects of these changes, most noticeable in body extremities, are thrombosis and ischaemia. Swelling and redness of the extremities accompanied by lameness and stiffness are followed by terminal gangrene. There is a clear line of demarcation between non-viable and viable normal tissues. Cold ambient temperatures and muddy conditions under foot may contribute to the severity of the lesions. In chickens, dry gangrene of the comb, wattles and feet may develop.

In warm climates, hyperthermia may occur in cattle ingesting ergopeptide alkaloids (Ross *et al*., 1989). In pregnant sows, ergotism may present as poor mammary development and low litter sizes with premature births, low birth weights and high neonatal mortality due to starvation. Although ergopeptides may exert an oxytocin-like effect on the pregnant uterus, abortion is not a feature of ergotism.

Diagnosis

- Ergotism can often be diagnosed clinically. The presence of ergots in pasture grasses, in grasses used for silage or in grain provides supporting evidence.
- When dealing with suspect ground grains, extraction of alkaloids and their detection by chromatography may be necessary.

Prevention

- If ergots are confirmed in pasture, an immediate change to ergot-free pasture should take place.
- Regular grazing or topping to prevent seed-head formation in pasture grasses reduces the possibility of sclerotia formation.
- Grain containing ergots should not be fed to animals. Removal of ergots from small batches of grain can be achieved mechanically or by flotation methods.

Facial eczema

The saprophyte fungus *Pithomyces chartarum*, which grows on dead plant material at the base of growing pasture, causes facial eczema in grazing ruminants. Sheep, cattle and farmed deer are the species most severely affected by this mycotic disease. This economically important disease, which is seasonally common in New Zealand, also occurs in Australia, South Africa, South America and, to a limited extent, parts of Europe. In this disease, the skin lesions develop as a result of photosensitization following exposure to the hepatotoxin sporidesmin in the spores of the saprophytic fungus *Pithomyces chartarum*. The fungus sporulates prolifically on pasture litter during warm humid conditions in late summer or early autumn. Although most strains of *P. chartarum* isolated in New Zealand produce sporidesmin, a high proportion of non-toxigenic isolates are recovered in other countries (Collin and Towers, 1995).

Hepatobiliary lesions develop as a result of the accumulation and concentration of sporidesmin in the bile. Necrosis of biliary epithelium results in obstruction of intrahepatic ducts with cell debris and diffusion of toxin into the hepatic parenchyma producing damage to blood vessels and hepatocytes. The consequent atrophy, necrosis and fibrosis reduce the capacity of the liver to excrete phylloerythrin, a potent photodynamic compound formed from chlorophyll by enteric organisms, which is distributed to many tissues including the skin. The photodynamic activity of phylloerythrin when exposed to solar radiation produces skin lesions typical of the disease.

Clinical findings

There is a latent period of 10 to 14 days between ingestion of a toxic amount of sporidesmin and the development of photosensitization. In sheep, lesions develop in non-pigmented areas which are not covered by wool. The eyelids, muzzle and ears are inflamed and swollen. Serous exudation and scab formation may be followed by necrosis and sloughing of skin. Jaundice is usually present. In cattle, lesions are limited to areas of non-pigmented skin. Milk production may be severely reduced. Although mortality due to severe liver damage is limited, economic losses arising from debilitation may be considerable.

Diagnosis

- In ruminants, photosensitization accompanied by jaundice is suggestive of the disease.
- Environmental temperatures above 12°C along with heavy rainfall over a 48-hour period provide suitable conditions for the growth of *P. chartarum* on pasture and are likely to precipitate disease outbreaks.
- Counts of the characteristic spores of *P. chartarum* (Fig. 51.3) in pasture samples can be used for prediction of disease outbreaks. Pastures with high spore counts are toxic for grazing animals.
- Elevated serum liver enzymes such as gamma-glutamyl transferase are found in affected animals.
- Competitive ELISA techniques have been developed for field use. Sporidesmin may be detected in bile, urine, plasma or whole blood (Briggs *et al*., 1993).

Figure 51.3 Thick-walled spores of *Pithomyces chartarum* (10−20 × 20−30μm) with transverse and longitudinal septa.

Control and prevention

- Routine monitoring of pasture spore counts can be used to evaluate their safety for grazing.

- Spore formation by *P. chartarum* can be controlled by spraying pastures with benzimidazole fungicides.
- Accumulation of pasture litter can be controlled by pasture management techniques.
- Breeding programmes which select sheep resistant to the toxic effects of sporidesmin are employed in some countries.
- The administration of high doses of zinc to sheep and cattle by drenching with zinc oxide or zinc sulphate in drinking water, before ingestion of sporidesmin, has been shown to reduce sporidesmininduced liver damage. Zinc forms a stable mercaptide with reduced sporidesmin, removing it from the auto-oxidation cycle that leads to the generation of reactive oxygen radicals which cause cell damage and cell death (Smith and Towers, 2002). For large flocks where daily drenching is impractical, slow-release intraruminal boluses containing zinc, which can produce protection for up to 4 weeks, have been developed and are available commercially.

Fescue toxicosis

Tall fescue grass, *Festuca arundinacea*, is a perennial grass adapted to a wide range of soil types and climatic conditions. It is common in pastures in large areas of the USA and also grows in Australia and New Zealand. Two diseases are associated with tall fescue grass, summer fescue toxicosis and fescue lameness.

Summer fescue toxicosis

This condition occurs in cattle when ambient temperatures are high, typically above 30²C. High environmental temperatures induce hyperthermia in animals grazing tall fescue pasture contaminated with the endophytic fungus *Neotyphodium coenophialum*. Clinical signs associated with the hyperthermia include dyspnoea, inappetence and hypersalivation. Affected cattle move into wet areas and seek shade. Reduction in prolactin levels and reduced milk yield are features of this condition. In addition to agalactia, prolonged gestation, weak neonates and thickened placentae have been associated with this toxicosis. The mycotoxin ergovaline has been implicated in the aetiology of summer fescue toxicosis (Fink-Gremmels, 2008). The hyperthermia observed in this condition is attributed to the peripheral vasoconstriction caused by the mycotoxin which

shares some common features with ergot alkaloids. Other aspects of this condition are attributed to ergovaline's action as a dopamine receptor agonist.

Fescue lameness

Unlike summer fescue toxicosis, fescue lameness is associated with late autumn or early winter. Clinical signs of fescue lameness resemble ergotism in herbivores. Severe lameness is followed by gangrene and sloughing of the extremities, especially digits. Lesion development is attributed to the vasoconstrictive action of ergovaline produced by *Neotyphodium coenophialum*. The vasoconstrictive activity of ergovaline is exacerbated by low ambient temperatures. There are suggestions that a number of mycotoxins may be involved in the development of fescue lameness.

Fumonisin toxicoses

Fumonisins are produced by a number of *Fusarium* species, notably *Fusarium verticillioides* and *F. proliferatum* and at least ten other species. By interfering with sphingolipid synthesis and metabolism, these mycotoxins affect animals and humans in different ways. Horses and pigs are more susceptible to the toxic effects of fumonisins than cattle, sheep and poultry. Through their interference with folic acid metabolism, fumonisins have been associated with neural tube defects in humans (Coppock and Jacobsen, 2009). Fumonisin B1 has been linked to oesophageal cancer in people living in regions of South Africa, China, Italy and the USA (Bennett and Klich, 20032. This mycotoxin causes leukoencephalomalacia in horses, pulmonary oedema and hydrothorax in pigs, and hepatotoxic and carcinogenic effects in rats.

Mycotoxic leukoencephalomalacia

Fusarium species are commonly found growing on mouldy maize affected by rain or when stored with a high moisture content. Because most strains of the fungus do not produce toxin, the presence of the fungus is not indicative of fumonisin production. At least six fumonisins, namely B1, B2, B3, B4, A1 and A2, are known but fumonisin B1 is the most frequent cause of toxicity in animals, especially horses (Čonková et al., 2003). Ingestion of mouldy maize cobs containing the mycotoxin fumonisin B_1, which is produced by *Fusarium*

verticillioides, is responsible for sporadic neurological disease, mainly in horses, donkeys and mules. The disease has been reported from Egypt, South Africa, the USA and Greece. Neurological signs, which relate to liquefactive necrosis of the white matter in the cerebrum, include inability to swallow, weakness, staggering, circling and marked depression. Mania, described in some cases, may be due to hepatic failure. Fumonisin B1, when fed to horses at concentrations greater than 10 μg/g of feed, is lethal (Ross *et al*., 1991).

Porcine pulmonary oedema

Fumonisins B1 and B2 are associated with fatal pulmonary oedema in pigs. This condition, which can result from consumption of fumonisin B1 at dietary concentrations above 100 ppm, appears to be caused by pulmonary hypertension resulting in pulmonary oedema and hydrothorax. Chronic exposure to fumonisins is associated with hepatotoxicosis.

Mouldy sweet potato toxicity

Acute interstitial pneumonia in cattle has been attributed to eating mould-damaged sweet potatoes (*Ipomoea batatas*) in the USA, New Zealand and Australia. Phytoalexins, metabolites formed in sweet potatoes in response to structural damage, are metabolized by *Fusarium solani* and *F. semitectum* to lung oedema factor, 4-i pomeanol. This factor is converted by microsomal enzymes in pneumocytes to toxic products which damage the cells (Hill and Wright, 1992). Dyspnoea is the principal clinical sign. Death may occur within 10 hours of the onset of signs.

Mycotoxic lupinosis

Lupin seeds contain toxic alkaloids which can produce neurological disturbance in herbivores. This plant toxicosis is distinct from the mycotoxicosis associated with ingestion of peptide mycotoxins called phomopsins produced by *Diaporthe toxica*, the fungal cause of stem blight in lupins. *Diaporthe toxica*, a saprophytic fungus, produces stem damage and also grows on dead lupin material and affects pods and seeds. Summer rain is conducive to fungal growth. Cattle and sheep grazing stubble of lupin crops are susceptible to the mycotoxins produced by the fungus.

Five mycotoxins, A, B, C, D and E, are produced by *Diaporthe toxica*, and pathological changes attributable to consumption of phomopsins include hepatosis, muscle injury and kidney damage. The disease is important in Australia, New Zealand, South Africa and parts of Europe.

Acute lupinosis presents as hepatic encephalopathy with stupor, stumbling and recumbency preceding death. Surviving animals may develop jaundice and photosensitization. A skeletal myopathy associated with phomopsin toxicity has been reported in Western Australia (Allen *et al*., 1992). In sheep, oral administration of zinc is reported to reduce the severity of liver damage caused by phomopsins.

Ochratoxicosis

A group of related isocoumarins, referred to as ochratoxins, are produced by numerous toxigenic strains of *Aspergillus* species and *Penicillium* species. In temperate climates, *P. verrucosum* is associated with production of these mycotoxins; in tropical climates A. *alutaceus* and related species produce ochratoxins. Numerous *Aspergillus* species have the ability to produce these mycotoxins in stored grain with a high moisture content. Although ochratoxins are found principally in cereal grains such as barley, oats, rye, wheat and maize, they have been detected in animal by-products and also in fresh fruit, grape juice and wine (Moss, 2008).

Four ochratoxins, A, B, C and D, have been described and ochratoxin A is the most common and the most toxic member of the group. Pigs, poultry and dogs are the species most often affected by these mycotoxins. Ruminants have a high tolerance to these compounds. Ochratoxin A is a heat-stable, potent nephrotoxin which is also immunosuppressive and carcinogenic. Many of the biological effects of ochratoxin A relate to interference with protein synthesis.

In pigs, inappetence, depression, weight loss, polydipsia and polyuria are features of this mycotoxic nephropathy. Dogs are reported to be extremely sensitive to ochratoxin A (Puschner, 2002). Ochratoxicosis in poultry is characterized by renal disease, hepatic damage and immunosuppression. Affected poultry have a depressed growth rate, reduced egg production and poor quality eggs.

Balkan endemic nephropathy, a chronic progressive nephritis, described in the human population in regions of Romania, Bulgaria and a number of Balkan

countries, shows many common features with the degenerative renal lesions caused by ochratoxin A in pigs. However, the aetiology of this human nephropathy is not yet determined.

Mycotoxic oestrogenism

A number of *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. equiseti* and *F. crookwellense* growing on mouldy maize, maize stubble, oats, barley, wheat, rye and rice produce oestrogenic substances, notably zearalenone. Pasture levels of zearalenone may be sufficient in some countries to cause reproductive problems in sheep and cattle (Smith and Towers, 2002). Zearalenone is a potent, non-steroidal oestrogen and both it and its metabolites can bind to oestrogen receptors inducing a syndrome in animals referred to as hyperoestrogenism (Fink-Gremmels, 2008). A secondary metabolite of zearalenone, α-zearalenol, has been marketed as a growth-promoting agent. While still available in some countries, it was banned by the European Union in 1989.

The majority of mycotoxins exert either a direct or indirect toxic effect on cells, tissues or organs. Although described as a mycotoxin, zearalenone does not conform to the usual description of a fungal toxin either in its molecular structure or in its biological activity. Its undesirable effects on fertility in some species, however, and its ability to induce pathological changes in some reproductive organs place it in a separate category. Although pigs are considered to be the most sensitive animal species to zearalenone, young female calves are also susceptible. Clinical signs of hyperoestrogenism are observed infrequently in ruminating cows.

Pigs, particularly prepubertal gilts, are commonly affected by oestrogenism. The condition, sometimes erroneously called vulvovaginitis, develops about a week after ingestion of contaminated feed. Vulval oedema and hyperaemia, hypertrophy of mammary glands and uterus and, occasionally, vaginal and rectal prolapse are features in gilts. In sows, anoestrus, pseudopregnancy, infertility and reduced litter size with small weak piglets may suggest oestrogenism (Long and Diekman, 1986).

Low conception rates have been recorded in cattle and sheep with oestrogenism. Less frequently, affected cattle may present with vaginal discharge, nymphomania and abnormal mammary development in prepubertal

heifers. Zearalenone can be excreted in milk, posing a public health risk. As the mycotoxin can be detected by chromatography, isolation and identification of the specific fungus may not be required. Oestrogen activity in feed may be assayed by injection of extracts into sexually immature mice, which develop uterine hypertrophy when extracts are positive. An ELISA technique has been developed for detecting zearalenone in pasture samples and ovine urine.

Patulin toxicosis

Although first used as an antimicrobial agent more than 60 years ago, patulin was later reclassified as a mycotoxin. Currently, patulin is considered of greater importance in human medicine than in veterinary medicine. This mycotoxin is produced by a number of fungi including *Penicillium*, *Aspergillus* and *Byssochlamys* species. Patulin has been detected in rotten fruit, apple juice and mouldy bread. *Penicillium expansum*, the blue mould that causes soft rot of apples, pears and other fruit, is recognized as one of the most common producers of this mycotoxin. Patulin is commonly present in apple juice and surveys indicate that from 21% to 100% of samples analysed contained this mycotoxin (Moss, 2008). However, unlike the majority of mycotoxins, patulin, a polyketide, is a relatively unstable molecule. While it is stable in the weakly acidic conditions of apple juice, it does not survive the fermentation into cider products.

Because patulin has broad antimicrobial activity against Gram-positive and Gram-negative bacteria and protozoa, it has a negative effect on ruminal microflora (Fink-Gremmels, 2008). Intoxication associated with patulin ingestion by cattle may result in tremors, ataxia and recumbency. The antimicrobial effects of patulin alter the functioning of rumen microflora, and the consequences of these changes, which are distinctly different from typical mycotoxins, may result in acidosis, poor feed utilization, loss of body weight and diarrhoea with undigested fibres in the faeces.

Experimental studies in dogs indicate that patulin affects the lungs and gastrointestinal tract. Diarrhoea, lethargy, pulmonary haemorrhage and pulmonary oedema were the predominant clinical signs observed. When fed to young pigs, clinical signs included vomiting, salivation, anorexia and weight loss.

Slaframine toxicosis

The phytopathogen *Rhizoctonia leguminicola* causes black-patch disease when growing on red clover, alfalfa, soya bean and other legumes. Transmission of the fungus seems to be seed-borne and it survives seasonal growth cycles in plant tissues and on plant debris. On red clover seeds, the fungus may survive for up to 2 years. High temperatures accompanied by high humidity favour fungal growth.

Rhizoctonia leguminicola produces two indolizidine alkaloids, slaframine and swainsonine. Horses are particularly sensitive to the action of slaframine, and profuse salivation develops within hours of consuming contaminated plant material (Wijnberg *et al*., 2009). Cattle, sheep and goats are also susceptible to slaframine toxicity. Other clinical signs include excessive lacrimation, bloating, diarrhoea and polyuria. In hay, silage or forage which contains contaminated clover, toxin remains active for several months. Slaframine intoxication has been described in many regions of the USA and in Canada, Japan, France and The Netherlands. Recovery usually follows removal of contaminated material from the animal's diet and mortality is uncommon.

Sterigmatocystin toxicosis

As a precursor in the synthetic pathway for aflatoxins, sterigmatocystin is considered to be capable of causing liver damage and to be carcinogenic. Although similar in many respects to aflatoxins, sterigmatocystin is considered to be much less toxic. Sterigmatocystin-producing fungi include *Aspergillus versicolor, A. flavus, A. niduellus, A. terreus* and *Bipolaris sorokiniana*. This mycotoxin has been found in stored wheat flour, cereals, peanuts, dry beans, cheese and green coffee.

Dysentery, decreased milk production and deaths occurred in dairy cattle following ingestion of food contaminated with sterigmatocystin-producing strains of *A. versicolor* (Coppock and Jacobsen, 2009).

Tremorgen intoxications

Tremorgens, a heterogeneous group of mycotoxins, produce neurological effects including muscular tremors, ataxia, incoordination and convulsive seizures

following ingestion. Signs often develop after strenuous exercise or excitement. Recovery usually follows within hours of removal from contaminated pasture or withdrawal of contaminated feed. Most tremorgens produce their neurological effects without obvious morphological tissue changes. Unidentified neurotoxins of *Aspergillus clavatus* can cause neuronal degeneration and focal gliosis (Gilmour *et al*., 1989).

Perennial ryegrass staggers

This neurotoxic condition of grazing livestock, which occurs in the late spring and summer, causes muscular tremors, ataxia and other neurological signs in sheep, cattle, horses and farmed deer. Perennial ryegrass staggers occurs sporadically in North and South America, Australia, New Zealand and parts of Europe. This disease occurs only in pastures in which perennial ryegrass or hybrid ryegrasses are the predominant grasses present. *Neotyphodium lolii*, an endophytic fungus growing on perennial ryegrass (*Lolium perenne*), produces a number of mycotoxins called lolitrems. These tremorgenic neurotoxins, especially lolitrem B, are responsible for the clinical signs observed in ryegrass staggers. The tremorigenic neurotoxins are indole diterpene alkaloids and include paxilline, lolitrem B and a number of other compounds (Smith and Towers, 2002; Fink-Gremmels, 2008). Fungal invasion and the amount of lolitrem B in infected plant material increase to toxic levels as the temperature rises in late spring, whereas toxic levels decline as the temperature falls later in the year. Growth of *N. lolii* is largely concentrated in older leaf sheaths, flower stalks and seeds. Consequently, clinical signs usually develop in late summer or early autumn when growth of pasture grasses declines. Infected plants do not exhibit signs of fungal infestation. Animals fed on contaminated hay or silage may develop clinical signs.

Perennial ryegrass staggers may affect many animals in a flock or herd but deaths are rare and recovery is gradual if animals are removed from contaminated pasture. Neurotoxic tremorgens may cause incoordination by interfering with neuronal transmission in the cerebral cortex; histological lesions relating to the activity of these neurotoxins have not been reported.

Paspalum staggers

Tremorgens present in the sclerotia of *Claviceps paspali*, growing on the seed heads of paspalum grasses, produce incoordination and ataxia in cattle, sheep

and horses grazing contaminated grasses. The life cycle of *C. paspali* resembles that of *C. purpurea*. Paspalum staggers is caused by the indole terpenoids paspalinine and paspalitrems A, B and C. These mycotoxins share some common features with paxilline and lolitrems, implicated in perennial ryegrass staggers. The clinical signs observed in paspalum staggers are similar to those that occur in perennial ryegrass staggers. Death is rare but may occur from respiratory failure during sustained seizures. Recovery is gradual after animals are removed from contaminated pasture. Control may be achieved by topping of pastures to prevent development of paspalum seed heads.

Penitrem staggers and related conditions

A number of neurological diseases, often collectively referred to as penitrem staggers, are attributed to mycotoxins produced by many *Penicillium* species and also by *Aspergillus* species growing on pasture plants or stored feed. Tremorgenic mycotoxins associated with diseases in dogs are usually produced during food spoilage (Leung *e t al*., 2006). Tremorgens produced by *Penicillium* species include penitrems A, B and C, verruculogen and roquefortine. Penitrem A, the most toxic tremorgen mycotoxin associated with *Penicillium* species, is produced by *P. crustosum*. Roquefortine is produced by *P. roqueforti* and can be produced by many other *Penicillium* species including *P. crustosum*. Tremorgenic mycotoxins associated with *Aspergillus* species include fumitremorgen A and B produced by *A. fumigatus* and flavus tremorgen produced by *A. flavus*.

In livestock, the clinical signs produced by these mycotoxins are similar to those observed with ryegrass staggers. Two tremorgenic mycotoxins of clinical importance in dogs are penitrem A and roquefortine (Puschner, 2002). Mouldy cheese, bread and decaying organic matter are the most likely sources of these mycotoxins. Clinical signs, which occur soon after ingestion of toxic food by dogs, include weakness, muscle tremors, rigidity, seizures and recumbency. A body temperature greater than 40°C has been observed in some affected dogs. Induced emesis and gastric lavage is recommended when marked toxicity is evident.

Trichothecene toxicoses

The trichothecene mycotoxins constitute a large and diverse group of

sesquiterpenoid compounds produced by species of *Fusarium, Myrothecium, Cephalosporium, Stachybotrys, Trichoderma* and *Trichothecium*. The term trichothecene derives from trichothecin, one of the first members of the family identified (Bennett and Klich, 2003). On the basis of their molecular structure, the trichothecenes are grouped as non-macrocyclic and macrocyclic compounds. *Fusarium* is the major genus associated with the production of non-macrocyclic compounds. The macrocy- clic trichothecenes are produced by *Myrothecium, Stachybotrys* and *Trichothecium* species. Trichothecenes inhibit protein synthesis in eukarytotic cells. They inhibit mitochondrial functions and induce apoptosis. These mycotoxins have radiomimetic effects in tissues, and immunosuppression results from sublethal doses of trichothecenes.

Because of the complex nature of the mycotoxins involved and the marked differences in clinical presentation observed in avian and mammalian species to individual trichothecene mycotoxins, specific names are not usually assigned to diseases attributed to nonmacrocyclic trichothecene-related diseases. Broader terms such as food refusal and emetic syndrome are sometimes applied to these conditions. In contrast, diseases associated with macrocyclic trichothecenes produced by *Myrothecium, Stachybotrys* and *Trichothecium* species are more clearly defined and have been assigned specific names.

A number of toxigenic fungi involved in the production of non-macrocyclic trichothecene toxicoses can produce more than one mycotoxin. Accordingly, a number of mycotoxins may contribute to syndromes attributed to a single mycotoxin produced by a particular toxigenic fungus. Another difficulty with this group of diseases is that the mycotoxins responsible for some syndromes associated with trichothecene toxicoses are not yet specified. The syndromes associated with toxigenic *Fusarium* species in pigs and other animals include feed refusal, emesis, anorexia, mucosal haemorrhages and necrotic skin lesions. *Fusarium*species are often found on barley, maize, rye, wheat and mixed cereals in regions of the world with temperate climates.

Deoxynivalenol toxicosis

Production of deoxynivalenol is associated with *Fusarium graminearum, F. culmorum* and a number of other *Fusarium* species growing on maize, barley, wheat and other cereals. Deoxynivalenol is one of the most common mycotoxins found in cereals. Wet seasons and warm weather promote growth of *Fusarium* species on feed components.

Feed contaminated with deoxynivalenol is unpalatable for many farm animals, especially pigs. Even low levels of this mycotoxin in feed result in food refusal, vomiting, digestive disturbances and weight loss in pigs. The potent emetic activity of this mycotoxin (its synonym is vomitoxin) is evident in pigs, dogs and cats but ruminants are comparatively resistant to its activity. Decreased feed conversion has been reported in poultry fed grain contaminated with deoxynivalenol (Čonková *et al.*, 2003). In addition to its toxic effects on the gastrointestinal tract in many species, deoxynivalenol is reported to have teratogenic activity in rats (Coppock and Jacobsen, 2009). Because more than one trichothecene mycotoxin may be present in contaminated feed, the clinical syndromes associated with deoxynivalenol toxicity are not clearly established.

T-2 toxicosis

Fusarium species associated with production of T-2 toxin include *F. sporotrichioides* and *F. poae*. During World War II, a serious disease in the Russian population, alimentary toxic aleukia, was associated with the consumption of mouldy wheat contaminated with toxigenic *Fusarium* species. Clinical signs included mucosal lesions, toxic effects on the bone marrow, thrombocytopenia and haemorrhages. Although the clinical consequences of consumption of T-2 toxin are not clearly established in animals, cytotoxic activity, immunosuppression and toxic effects on the bone marrow have been observed in pigs and cats. Feed refusal of T-2 toxin-contaminated feed occurs with pigs but not with cattle. When administered to rodents in feeding trials, T-2 toxin induced cytotoxicity, immunosuppression and CNS signs. Poultry are susceptible to the direct toxic effects of T-2 toxin. Beak lesions, abnormal feather formation in chickens, a drop in egg production, tissue haemorrhages and increased susceptibility to infectious diseases have been recorded in poultry of different ages (Coppock and Jacobsen, 2009). Clinical signs observed in cats fed contaminated rations included vomiting, dysentery, ataxia and dehydration (Puschner, 2002). Severe irritation of the upper digestive tract resulting in haemorrhagic rumenitis has been observed in cattle consuming T-2 toxin in contaminated feed. Although the clinical spectrum of T-2 toxicity is not uniform in animals, cytotoxicity and interference with bone marrow activity, resulting in thrombocytopenia and haemorrhage, are a common outcome.

Diacetoxyscirpenol toxicosis

Production of diacetoxyscirpenol is associated with *Fusarium tricinctum* and a number of other *Fusarium*species growing on cereals. This mycotoxin, which is one of the most potent trichothecene toxins, is an epithelial necrotizing agent. Cellular depletion and necrosis of lymphoid organs, and haemorrhages in the skin, oral cavity, intestine and major organs, are attributed to the action of this toxin. The clinical syndrome associated with diacetoxyscirpenol in cattle includes mucosal haemorrhages, salivation and depression. In pigs, haemorrhagic intestinal lesions and emesis are attributed to the activity of diacetoxyscirpenol. The toxic effects of this mycotoxin in dogs include depression of bone marrow activity and associated haematological changes. Oral lesions involving the beak and tongue have been described in poultry consuming diacetoxyscirpenol in their diet. Toxic effects included inhibition of protein synthesis.

Stachybotryotoxicosis

The mycotoxins produced by *Stachybotrys chartarum,* macrocyclic trichothecenes, include satratoxins, roridins and verrucarins. Some isolates produce a range of additional substances with unspecified biological activity. *Stachybotrys chartarum* growing on straw, hay or grain produces these mycotoxins which are cytotoxic and exert a radiomimetic effect on tissues. These toxins inhibit DNA and protein synthesis. Stachybotryotoxicosis has been reported in the former Soviet Union, parts of Europe and South Africa. Horses, cattle, sheep and pigs are susceptible and fungal spores produced by *S. chartarum* are toxic for humans following contact, ingestion or inhalation (Coppock and Jacobsen, 2009). Clinical signs of toxicity in animals include diarrhoea, dysentery, haemorrhages on nasal and oral mucosae and epistaxis. Agranulocytosis is a feature of the intoxication, and immunosuppression predisposes animals to opportunistic infections. Horses appear to be particularly susceptible to the mycotoxins and, in addition to haemorrhages, myositis is reported in affected animals. Chronic exposure to the mycotoxins may have fatal consequences, resulting from a combination of haemorrhage and septicaemia.

Myrotheciotoxicosis

Sudden deaths in sheep and cattle have been attributed to the mycotoxin roridin produced by *Myrothecium roridum* and *M. verrucaria* growing on ryegrass, white clover plants in pasture or stored feed. Post-mortem findings related to the

mycotoxin included abomasitis, hepatitis and pulmonary congestion. Prolonged exposure to sublethal amounts of roridin may cause weight loss and unthriftiness.

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Chapter 52

Pathogenic algae and cyanobacteria

Algae are saprophytic eukaryotic organisms which are widely distributed in the environment especially in water. Many contain chlorophyll. Infrequently, some species of algae have been implicated in disease of domestic animals (Table 52.1). Colourless eukaryotic algae belonging to the genus *Prototheca* can invade tissues causing cutaneous and disseminated disease in a number of species and mastitis in cattle. Green algae belonging to *Chlorella* species have been associated with tissue invasion in ruminants on rare occasions. The prokaryotic cyanobacteria (formerly known as blue-green algae) produce potent toxins which can affect hepatic and neurological function.

Prototheca species

Prototheca species, widely distributed, saprophytic, colourless algae, are related to green algae of the genus *Chlorella*. It is thought that *Prototheca* species may be achlorophyllous descendents of *Chlorella* species. *Prototheca zopfii* has been associated with disseminated protothecosis in dogs and with mastitis in cows. Three biotypes of *P. zopfii* have been described (Roesler *et al*., 2003). Subsequent studies based on sequence analysis of the 18S rRNA gene have led to the proposal that *P. zopfii* should be reclassified as genotypes 1 (biotype 1) and 2 (biotype 2), and a new species *P. blaschkeae*(biotype 3) (Roesler *et al*., 2006). The majority of bovine protothecal mastitis cases are caused by *P. zopfii* genotype 2 (Möller *et al*., 2007). Cutaneous protothecosis in cats and dogs is caused by *P. wickerhamii*. *Prototheca*species grow aerobically forming yeast-like colonies on Sabouraud dextrose agar and on blood agar. During asexual reproduction, 2 to 16 sporangiospores develop within a sporangium (Fig. 52.1). The sporangiospores are released through a split which develops in the sporangial wall. In cultures, the sporangiospores of *P. zopfii* are larger than those of *P. wickerhamii*(Pore, 2005).

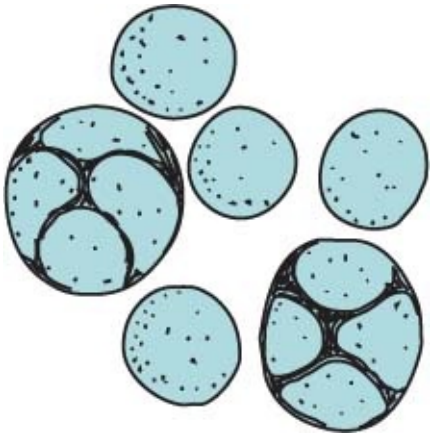
Key points

- *Prototheca species*
  - Eukaryotic colourless algae
  - Widely distributed in sewage and organic matter
  - *P. wickerhamii* causes cutaneous infections in cats and dogs
  - *P. zopfii* causes disseminated disease in dogs and mastitis in dairy cattle
- *Chlorella* species
  - Eukaryotic green algae
  - Morphologically similar to *Prototheca* species but contain chloroplasts
  - Associated rarely with lymphadenitis in ruminants
- Cyanobacteria
  - Prokaryotic photosynthetic organisms
  - Form 'algal' blooms on fresh-water surfaces
  - Produce potent hepatotoxins and neurotoxins affecting fish, birds and mammals

Table 52.1 Algae and cyanobacteria implicated infrequently in opportunistic infections or intoxications of domestic animals.

Agents	Methods of disease production	Clinical effects
*Protothecas*pecies	Tissue invasion	Skin lesions, mastitis
*Chlorella*species	Tissue invasion	Lymphadenopathy
Cyanobacteria	Toxin production	Hepatomegaly, photosensitization, neurological disturbance

Figure 52.1 The cells and two sporangia, containing sporangiospores, of *Prototheca zopfii*.



Infections due to *Prototheca* species are opportunistic. Organisms can enter tissues at sites of minor trauma in skin and mucous membranes or through the teat canal. Some outbreaks in cattle have been associated with the use of

contaminated intramammary products.

Diagnostic procedures

- Suitable specimens for laboratory examination include milk samples and biopsy or post-mortem tissues. An indirect ELISA has been described for the detection of antibodies in serum and whey (Roesler *et al*., 2001).
- Methenamine silver or PAS techniques can be used to demonstrate algal cells and sporangia in histological sections of granulomatous lesions.
- Immunofluorescent techniques are used to identify *P. zopfii* and *P. wickerhamii* in tissues.
- The organisms grow on blood agar and Sabouraud dextrose agar without cyclohexamide. Organisms may be isolated from contaminated specimens on prototheca isolation medium with added phthalate and 5-fluorocytosine (Pore, 2005). Culture plates are incubated aerobically at 35°C to 37°C for 2 to 5 days.
- Carbohydrate assimilation test kits for differentiating *Prototheca* species are available commercially. *Prototheca wickerhamii* assimilates trehalose but not 1-propanol, whereas *P. zopfii* assimilates 1-propanol but not trehalose.
- Identification criteria for isolates:
  - Colonial morphology
  - Microscopic appearance of sporangiospores
  - Carbohydrate assimilation tests.
- Molecular methods are available for the identification of *P. zopfii* (Onozaki *et al*., 2009).

Clinical infections

Although *Prototheca* species are commonly present in the environment, infections in animals are infrequent. Suppression of cell-mediated immunity may be a factor predisposing to disseminated disease (Migaki *et al*., 1981).

Cutaneous protothecosis in cats

A cutaneous form of protothecosis, caused by *P. wick-erhamii*, is the only manifestation of the disease reported in cats (Dillberger *et al*., 1988). Large, firm, discrete nodules occur on limbs and feet. Similar lesions have been described on the nose and ears and at the base of the tail. Microscopically the

granulomatous lesions, located in the dermis, contain multinucleate giant cells with engulfed organisms. Surgical excision of skin lesions is the most effective method of treatment. Ketoconazole therapy is often ineffective.

Disseminated protothecosis in dogs

Infection with *P. zopfii* probably occurs through the intestinal mucosa as dissemination is often preceded by haemorrhagic colitis (Migaki *et al*., 1981; Stenner *et al*., 2007). Affected dogs may present with protracted bloody diarrhoea along with signs of neurological or ocular disturbance. There may be progressive weight loss and debility. Treatment of disseminated protothecosis is usually unsuccessful. At post-mortem, granulomatous lesions in which protothecal cells may be demonstrated are found in skeletal muscles, brain, liver, kidneys, eyes and cochlea. In addition to disseminated protothecosis, a cutaneous form caused by *P. wickerhamii* has been recorded (Ginel *et al*., 1997).

Protothecal mastitis in cows

Prototheca zopfii can cause chronic progressive pyogranulomatous lesions in bovine mammary glands and associated lymph nodes. Indurative mastitis may affect a number of quarters. Because of their intracellular location, protothecal cells may be difficult to eliminate from the glands. Although the organisms are excreted intermittently in milk, they may not be demonstrable in samples, and some cases of the disease may be overlooked (Spalton, 1985). *Prototheca zopfii* can persist in the tissues throughout a dry period and may be excreted during the next lactation. Treatment is unsuccessful. Affected cows should be culled because they are potential sources of infection and their milk yields are permanently reduced. Disseminated protothecosis has been recorded in cattle on rare occasions (Taniyama *et al*., 1994).

Chlorella species

Green algae cause disease in ruminants on rare occasions. *Chlorella* species are morphologically similar to *Prototheca* species. However, they are photosynthetic, possessing chloroplasts containing green pigment which imparts colour to infected tissues. The organisms have been recovered from liver and associated lymph nodes of sheep (Zakia *et al*., 1989) and, in Australia, from cattle with lymphadenitis (Rogers *et al*., 1980). Disseminated chlorellosis has

been described in a dog (Quigley *et al*., 2009).

The cyanobacteria

The cyanobacteria are prokaryotic photosynthetic organisms found worldwide in fresh and marine water and in soil. Blue-green 'algal' blooms may form when conditions allow rapid replication of cyanobacteria. They may occur in water enriched with phosphates or nitrogen, when its temperature is between 15°C and 30°C, its pH is neutral or alkaline, and wind disturbance is minimal (Carmichael, 1994). In these circumstances, domestic or wild animals drinking contaminated water are likely to be exposed to toxin released from the organisms (Lopez-Rodas *et al*., 2008). More than 40 species of cyanobacteria are known to produce potent hepatotoxins or neurotoxins. Selected cyanobacteria, presumed to be toxigenic, are listed in Box 52.1. *Microcystis aeruginosa* is the species most often incriminated in episodes of poisoning. Some species such as *Anabaena flos-aquae* can generate both hepatotoxin and neurotoxin.

Cyanobacterial toxicoses

Toxins of the cyanobacteria, their modes of action and their clinical effects are presented in Table 52.2. Although death may occur within a short time after ingestion of a lethal dose of toxin, the dose-response curve is relatively steep and animals can ingest nearly 90% of a lethal dose without noticeable effects. The severity of intoxication depends on the degree of exposure and the toxin concentration in contaminated water. Birds and ruminants are usually more susceptible to the toxins than monogastric animals.

Box 52.1 Toxigenic cyanobacteria.

- *Microcystis aeruginosa*
- *Anabaena flos aquae*
- *Planktothrix rubescens*
- *Oscillatoria species*
- *Aphanizomenon species*
- *Nodularia species*
- *Cylindrospermum species*
- *Cylindrospermopsis species*
- *Nostoc species*
- *Lyngbya species*

Table 52.2 Toxins of cyanobacteria, their modes of action and clinical effects.

Toxins	Mode of action	Clinical effects
Microcystins and nodularins	Hepatotoxic; inhibition of protein phosphatases	Hepatomegaly and hepatoencephalopathy; photosensitization; raised serum liver enzyme levels; severe toxicity results in intrahepatic haemorrhage and death from hypovolaemic shock
Anatoxina	Neurotoxic; post-synaptic cholinergic agonist; mimics the activity of acetylcholine	Involuntary muscular contractions, convulsions; severe toxicity results in death
Anatoxina(s)	Neurotoxic; anti-acetylcholinesterase activity	Similar to the effects of anatoxin-a; hypersalivation
Saxitoxins and neosaxitoxins	Blockade of signal transmission in motor neurons	Flaccid paralysis; death from respiratory failure

Clinical signs

Clinical signs, which relate to the types of toxin ingested, may be diverse. Hepatotoxic effects, which may develop within hours of exposure, include muscle tremors, dyspnoea, blood-stained diarrhoea and coma (Kerr *et al*., 1987). Hepatomegaly may be detectable. Photosensitive dermatitis has been recorded in horses and ruminants. Signs of neurotoxicosis, which may develop within minutes of ingesting toxin, include hypersalivation, clonic convulsions, rigor and cyanosis (Gunn *et al*., 1992). Death may occur rapidly after the onset of clinical signs.

Diagnosis

- There may be a history of access to contaminated water with an ‘algal’ bloom(James *et al*., 1997; Puschner *et al*., 2008).
- The mouth or legs of affected animals may be stained green.
- Samples of bloom should be examined microscopically for the presence of cyanobacteria.
- Toxin must be demonstrated in the bloom or in stomach contents by chemical, biological or immunoassay techniques in a reference laboratory.
- There may be histopathological evidence of hepatotoxicosis.
- Serum concentrations of bile acids and liver enzymes may be elevated (Carbis *et al*., 1995).
- Other possible sources of intoxication should be considered in the differential diagnosis.

Treatment

- Affected horses and ruminants, removed from the source of toxin, should be housed out of direct sunlight.
- Emetics administered to recently exposed dogs may aid recovery.
- Activated charcoal slurry or ion-exchange resins may be used for adsorbing toxins from the gastrointestinal tract.
- Although atropine reduces the anti-acetylcholinesterase activity of anatoxin-a(s), no therapeutic antagonist is effective against anatoxin-a or the saxitoxins.

Control

- Access of animals to contaminated water must be restricted.
- Companion animals should not be fed fish from contaminated waters.
- Growth of cyanobacteria can be controlled in small bodies of water by the addition of copper sulphate. However, treatment of an ‘algal’ bloom with algicides results in the liberation of toxins from dead cells into the water.

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Chapter 53

Antifungal chemotherapy

Fewer than 200 species of fungi are capable of causing infection in otherwise healthy humans and animals. A number of infections arise from tissue invasion by opportunistic fungal pathogens such as *Candida albicans, Cryptococcus neoformans* and *Aspergillus fumigatus*. These opportunistic infections usually occur in hosts which are debilitated or immunosuppressed. Prolonged administration of antibacterial drugs or immunosuppressive agents predisposes humans and animals to opportunistic fungal infections.

Based on the initial site of infection, fungal infections can be classified into a number of broad groups: superficial mycoses, subcutaneous mycoses and systemic mycoses. Superficial mycoses are limited to the skin, other keratinized structures including hair and nails and also mucous membranes. Subcutaneous mycoses are infections that involve the dermis, subcutaneous tissues and occasionally adjacent structures. Systemic mycoses are infections that usually originate in the lungs and spread to many other organs. Mycotoxicoses constitute an important group of diseases which result from the ingestion of fungal toxins that have been preformed in stored food or standing crops.

Factors that predispose to opportunistic fungal infections include alteration of the normal microbial flora as a consequence of prolonged antibacterial therapy, primary or secondary immunodeficiency, immunosuppression following corticosteroid therapy, administration of antineoplastic drugs, acute viral infections, some neoplastic conditions and exposure to high infectious doses of fungal spores in confined spaces. Advanced AIDS is an important predisposing factor for fungal infections in the human population. It is unrealistic, therefore, to expect antifungal therapy alone to clear infections from the systems or tissues of immunosuppressed animals or from animals with primary or secondary immunodeficiency diseases. Accordingly, the host’ s immune status should be considered carefully when designing antifungal treatment regimes.

Although there are fewer antifungal drugs currently available than antibacterial drugs, major advances in antifungal chemotherapy have taken place in recent

years. There are four major classes of antifungal agents: allylamines, azoles, echinocandins and polyenes. Other antifungal compounds include griseoful-vin, flucytosine, iodides and morpholines. Some of these compounds, such as allylamines, can be used both topically and systemically while other antifungal drugs such as amorolfine and ciclopirox olamine are used topically.

The toxicity of many antifungal drugs used in past decades limited their therapeutic use in humans and animals. The availability of newer, less toxic, antifungal compounds which selectively inhibit fungal growth has resulted in a range of antifungal drugs with fewer side effects and more predictable antifungal activity.

The modes and sites of action of antifungal drugs range from compounds that interfere with fungal cell synthesis to drugs that inhibit mitosis of fungal cells. Prolonged treatment with antifungal drugs is often required to ensure clinical recovery. The susceptibility of pathogenic fungi to antifungal compounds is not always predictable. Some fungal pathogens are naturally resistant to chemotherapeutic drugs while other fungal species gradually develop resistance to antifungal drugs. In contrast to the spread of high-level multidrug resistance encountered in bacteria, antifungal drug resistance usually develops slowly.

Fungal pathogens and related organisms that cause disease in animals, measures to prevent infection and treatment options are summarized in Table 53.1. Classification of antifungal drugs and their modes and sites of action are summarized in Table 53.2.

Antifungal drugs

Allylamines

These antifungal drugs, which have both fungistatic and fungicidal activity, are used for treating a range of fungal pathogens, especially infections with dermatophytes. Two compounds in this category of synthetic drugs, naftifine and terbinafine, are used therapeutically. The former is used as a topical cream for dermatophyte infections, the latter in tablet and topical formulations. Allylamines inhibit the activity of squalene epoxidase, an enzyme required for the production of ergosterol, the principal sterol in the membrane of fungal cells. Decreased synthesis of ergosterol and accumulation of squalene produce a toxic effect on the fungal pathogen.

Terbinafine, a lipophilic drug, is reported to become concentrated in the dermis, epidermis, adipose tissue and nails. It has a broad spectrum of activity which includes dermatophytes, *Aspergillus* species, some dimorphic fungi and yeasts. In the human population, few side effects have been reported. There are limited data on the clinical use of terbinafine in dogs and cats but its efficacy against dermatophytes is of particular relevance in the treatment of ringworm. Terbinafine has been used successfully for the treatment of systemic aspergillosis in avian species. Although primary resistance to terbinafine has been observed, dermatophyte resistance to this antifungal drug is reported to be rare.

Griseofulvin

Although griseofulvin was formerly used extensively for the treatment of dermatophyte infections, it has been superseded by safer and more effective antifungal drugs. This orally administered drug has a spectrum of activity limited to dermatophytes. Griseofulvin, a fungistatic drug, exerts its effect by binding to microtubular proteins and interfering with microtubule formation. As a consequence of its activity, mitosis of fungal cells is inhibited. In addition, inhibition of nucleic acid synthesis may occur. Deposition of griseofulvin occurs in keratin precursor cells and, when these cells differentiate, the drug remains bound to keratin rendering the cells resistant to fungal invasion. Griseofulvin is used for treating dermatophyte infections in large and small animals. Treatment for several weeks may be required and should continue for up to 2 weeks after clinical recovery. Because of its teratogenic effects, griseofulvin is contraindicated in pregnant animals, especially queens and mares.

Some species of dermatophytes develop resistance to griseofulvin. Data relating to griseofulvin resistance are limited and the molecular basis of resistance is uncertain.

Azoles

Two chemically different groups of azole compounds, imidazoles and triazoles, are used therapeutically for their fungistatic activity (Table 53.2). The antifungal activity of all azole compounds derives from their ability to inhibit fungal cytochrome 14-α-demethylase. Inhibition of this enzyme, which is involved in the conversion of lanosterol to ergosterol, leads to depletion of ergosterol and accumulation of 14-α-methylsterols in the fungal cell membrane. These changes

disrupt fungal cell membrane activities and the functioning of membrane-bound enzyme systems. As a consequence of membrane damage, nutrient transport and chitin synthesis are impaired and fungal growth ceases. The fungistatic action of azole compounds, which takes place over several generations of fungi, becomes clinically evident at a slow rate and, accordingly, prolonged treatment regimes are required to ensure clinical recovery.

In contrast to the early azole drugs such as ketoconazole, fluconazole and itraconazole have a much greater affinity for fungal cell membranes than mammalian cell membranes and these triazoles produce fewer side effects. In recent years, fluconazole and itraconazole are being used in preference to ketoconazole for the treatment of fungal infections in humans and animals. Both itraconazole and fluconazole have wider spectra of activity than ketoconazole. Fluconazole is effective against dermatophytes, *Candida* species, *Cryptococcus neoformans, Histoplasma capsula-tum, Coccidioides immitis and Sporothrix schenckii*. It is moderately effective against *Blastomyces dermatitidis* but ineffective against *Aspergillus* species and zygomycetes. Itraconazole has a broader antifungal spectrum than fluconazole and, in addition to the fungal pathogens against which fluconazole is effective, itra-conazole is active against dematiaceous fungi and *Aspergillus* species. In common with fluconazole, itraconazole is ineffective against zygomycetes. Voriconazole, a new broad-spectrum triazole developed to overcome the resistance of fungal pathogens to fluconazole and itraconazole, is effective against yeasts, dimorphic fungi and *Aspergillus* species. It is the drug of choice for the treatment of invasive aspergillosis (Segal, 2009). Its spectrum of activity extends to the agents of phaeohyphomycoses but it is not effective against zygomycetes. Because of the risk of teratogenicity, azole drugs are contraindicated in pregnant animals.

Table 53.1 Fungal pathogens and related organisms which cause disease in animals, usual sites of infection, measures to prevent infection and therapeutic options*.

Fungal pathogens / Associated disease	Species affected, clinical conditions	Presenting clinical signs	Control measures; treatment options	Comments
***Aspergillus* species**				
Aspergillus species, mainly *A. fumigatus* / Aspergillosis	Brooder pneumonia in newly hatched chickens resulting in high mortality	Somnolence and inappetence	High standard of hygiene and fumigation of incubators	Yellowish nodules are present in the lungs and airsacs
	Mature birds: captive penguins, raptors and psittacine birds; lesions in lungs and airsacs	Dyspnoea and emaciation	Amphotericin B, itraconazole, voriconazole, terbinafine	Yellowish nodules in the lungs and airsacs resemble avian tuberculosis; surgical removal of *Aspergillus* granulomas from the upper respiratory tract may be a treatment option
	Mycotic abortion in cattle; occurs sporadically	Cows may abort without evidence of systemic infection; aborted foetuses may have raised cutaneous plaques resembling ringworm	Avoidance of poor quality fodder harvested in wet seasons which may be associated with the occurrence of abortion	Infection, which reaches the uterus haematogenously, causes placentitis leading to abortion late in gestation
	Canine nasal aspergillosis, encountered mostly in dolicocephalic breeds	Persistent profuse sanguinopurulent discharge, often unilateral; sneezing and epistaxis; increased radiolucency of turbinate bones	Treatment through tubes inserted surgically into the frontal sinuses and nasal chambers with itraconazole or voriconazole together with systemic treatment; other suitable antifungal agents which can be used systemically include fluconazole and caspofungin	Treatment for at least 6 weeks may be required
	Guttural pouch mycosis in horses	Epistaxis, dysphagia and laryngeal hemiplegia; postauricular swelling and unilateral nasal discharge	Surgical intervention to control serious haemorrhage may be required in some instances; topical treatment with antifungal drugs such as itraconazole; oral administration of potassium iodide may be effective in some cases	Prolonged treatment with antifungal drugs may be required. Potential toxicity of prolonged treatment and cost may determine the duration of oral or systemic treatment
Dermatophytes				
Microsporum species, *Trichophyton* species / Dermatophytosis (ringworm)	*M. canis, M. gypseum,* and *T. mentagraphytes* in dogs and cats; *T. verrucosum* in cattle; *T. equinum* in horses; skin lesions and alopecia	Dogs and cats: areas of alopecia, scaling and broken hairs Cattle: oval areas of alopecia with greyish crusts on face and around eyes Horses: lesions usually confined to girth strap and saddle region	Affected dogs and cats should be isolated, bedding should be burned; grooming equipment should be disinfected; local lesions may be treated with clotrimazole, miconazole, itraconazole or terbinafine; extensive lesions may require oral treatment with itraconazole, fluconazole, terbinafine or griseofulvin. Individual lesions in cattle can be treated with miconazole, clotrimazole or terbinafine; extensive lesions may require oral treatment with griseofulvin. In horses, treatment with clotrimazole, miconazole or other azole compounds is usually effective; affected horses should be isolated and grooming equipment disinfected	Azole compounds are usually more effective than griseofulvin. Both azole compounds and griseofulvin are contraindicated in pregnant animals. An inactivated *M. canis* vaccine for use in cats may be a beneficial control measure. Horses and cattle can be vaccinated with attenuated fungal cultures
Dimorphic fungi				
Blastomyces dermatitidis / Blastomycosis	Main hosts, dogs and humans; lesions in lungs with metastases to skin and other tissues	Coughing, exercise intolerance	Itraconazole is the treatment of choice for dogs and cats; ketoconazole is also effective; if infection is severe, amphotericin B should be combined with itraconazole	Treatment should continue for at least 2 months and be maintained until active disease subsides
Histoplasma capsulatum / Histoplasmosis	Dogs, cats and humans are susceptible; pulmonary lesions with metastases to other organs	Chronic cough, persistent diarrhoea, emaciation	Itraconazole is effective at an early stage of infection; ketoconazole may also be effective; amphotericin B may be combined with itraconazole if infection is severe	Animals being treated with amphotericin B should be monitored for signs of toxicity
Histoplasma farciminosum / Epizootic lymphangitis	Horses, other *Equidae*; lesions usually involve the skin, lymphatic vessels and lymph nodes	Pyogranulomatous lesions usually develop on the limbs in the region of the hocks; infection often becomes chronic	Sodium iodide administered intravenously at weekly intervals for 4 weeks is reported to be effective in some instances; surgical excision of lesions combined with treatment with amphotericin B has been recommended by some workers but the benefit of such treatment is uncertain	In countries where the disease is exotic, it is notifiable and a test and slaughter policy is implemented
Coccidioides immitis / Coccidiodomycosis	Dogs, horses and humans are the main hosts; pulmonary lesions with metastases to bone and other tissues	Dogs with disseminated disease may have a persistent cough, weakness, loss of weight and sometimes lameness	Itraconazole, fluconazole or ketoconazole are used to treat canine infections; amphotericin B treatment may be required for severe infections	Long-term treatment may be required for osteomyelitis caused by *C. immitis*

Sporothrix schenckii / Sporotrichosis	Sporadic cases are reported in horses, dogs, cats and humans; nodular skin lesions, which ulcerate, develop along the course of lymphatic vessels	Lymphocutaneous sporotrichosis is the most common form of the disease in horses; nodular skin lesions may occur on the limbs, head and tail in dogs and cats	Itraconazole, fluconazole and voriconazole are effective for treating lymphocutaneous sporotrichosis. A saturated solution of potassium iodide, administered orally, has been used successfully for the treatment of sporotrichosis in humans and animals for many years. In animals, treatment should continue for at least 30 days after clinical recovery	Animals receiving potassium iodide treatment should be monitored for signs of iodism
Fungal species associated with mycotoxin production				
Aspergillus flavus, A. parasiticus, growing on maize, stored grain, ground nuts / Aflatoxicosis	Pigs, ducklings, turkey poults, pheasants, calves, sheep, dogs, trout; toxic effects of aflatoxins include hepatotoxicity, immunosuppression, teratogenesis and carcinogenesis	Ill thrift, hepatocellular necrosis may lead to haemorrhagic diathesis, especially in avian species; acute disease in calves can result in nervous signs and death	Crops harvested during wet seasons should be excluded from the diets of animals; mouldy feed should not be fed to young animals; addition of hydrated sodium calcium aluminosilicate to feed is reported to reduce aflatoxin toxicity. Specific treatment cannot be recommended; symptomatic treatment may be of limited benefit in some species	Because of the risk of excretion in milk, feedstuffs containing aflatoxins should not be fed to dairy cows
Aspergillus alutaceus, other *Aspergillus* species, *Penicillium verrucosum*, other *Penicillium* species growing on stored feed / Ochratoxicosis	Pigs, poultry; degenerative renal changes	Polydipsia, polyuria and immunosuppression in pigs; fall in egg production in birds, renal and hepatic damage accompanied by weight loss	Mouldy barley, maize and wheat should be excluded from pig and poultry diets; poultry are especially susceptible to the toxic effects of ochratoxins	Ochratoxin A, when present in the tissues of food-producing animals, is of public health concern
Claviceps purpurea growing on the seedheads of ryegrass and other grasses and cereals / Ergotism	Cattle, sheep, pigs, deer, horses, poultry; neurotoxicity and vasoconstriction	Convulsions, gangrene of extremities, agalactia, hyperthermia in hot climates	Regular grazing or topping to prevent seedhead formation in pasture grasses; grain containing ergots should not be fed to animals	Human illness, associated with ingestion of rye, wheat and millet contaminated with ergot alkaloids, has been reported in a number of countries
Fusarium graminearum, F. culmorum and other *Fusarium* species growing on stored maize, barley and pelleted cereal feeds / Oestrogenism	Pigs, cattle, sheep; oestrogenic activity	Hyperaemia and oedema of vulva and precocious mammary development in young gilts; infertility in sows, cows and ewes	Contaminated feed should be replaced without delay	Zearalenone, produced by *F. graminearum*, can be excreted in milk, posing a possible public health risk

Fungal species associated with mycotoxin production				
Fusarium verticillioides growing on standing maize or stored maize / Leukoencephalomalacia in horses; pulmonary changes in pigs	Horses, mules and donkeys are susceptible; neurotoxicity, which results from liquefactive necrosis of the white matter of the cerebrum; hepatic necrosis may also occur	Inability to swallow, weakness, staggering, marked depression, recumbency; death may occur within 72 hours of clinical signs becoming evident	Mouldy maize should be excluded from the diets of *Equidae*. Specific treatment is not available	Fumonisin B1, produced by *F. verticillioides*, causes pulmonary oedema in pigs
Diaporthe toxica growing on *Lupinus* species and causing stem blight; seeds, pods and stubble may be involved / Mycotoxic lupinosis	Sheep, occasionally cattle, horses and pigs; hepatotoxicity	Stupor, jaundice, inappetence, ruminal stasis; often fatal	Lupins should be grazed in early summer, not in the autumn; lupins sprayed with fungistatic agents are less likely to have fungal growth; lupin crops should be inspected regularly so that fungal growth can be detected before grazing commences; *Lupinus* species resistant to fungal growth have been developed	Skeletal muscle myopathy has been observed in sheep grazing infected lupin stubble
Pithomyces chartarum growing on dead plant material at the base of the pasture, particularly ryegrass and white clover / Facial eczema	Sheep, cattle, goats and deer; hepatotoxicity and biliary occlusion	The mycotoxin involved, sporidesmin, causes severe damage to biliary epithelium leading to acute biliary obstruction and resulting in severe hepatic insufficiency, obstructive jaundice and photosensitization; the photodynamic agent is phylloerythrin, a normal metabolic product of chlorophyll which is retained in tissues because of the damaged liver and bile ducts	An outbreak of facial eczema can be predicted by monitoring the number of fungal spores in pasture samples; spraying pasture with fungicides inhibits germination of spores but cost limits this approach; oral administration of zinc compounds reduces the toxic effects of sporidesmin; drenching with zinc oxide, adding zinc sulphate to water supplies and use of intraruminal boluses containing zinc reduce sporidesmin-induced liver damage	Control measures are usually determined by the number of animals involved and the feasibility of implementing the control or treatment procedure. Cost is often a limiting factor in treatment and control
Fungal species associated with tremorgen intoxications				
Neotyphodium lolii growing on ryegrass pasture / Perennial ryegrass staggers	Cattle, sheep, horses, farmed deer; neurotoxicity	Muscular tremors, incoordination, convulsive seizures	Pasture management can be used to encourage the growth of other grass species; grazing management can reduce the risk of exposure	Recovery is rapid when animals are removed from contaminated pasture
Aspergillus clavatus growing on sprouted wheat and malt culms / Induced tremors	Cattle; neurotoxicity	Frothing from mouth, knuckling of limbs	Sprouted wheat and malt culms should be stored carefully to avoid fungal growth	Grain with a moisture content greater than 20% is liable to fungal spoilage
Claviceps paspali growing on seedheads of paspalum grasses / Paspalum staggers	Cattle, sheep, horses; neurotoxicity	Muscular tremors, incoordination, convulsive seizures	Topping pasture to prevent development of paspalum seedheads can prevent exposure to the mycotoxins	Access to bodies of water, especially rivers, or to steep slopes should be avoided until affected animals recover
Many *Penicillium* species, some *Aspergillus* species growing on stored feed and pasture / Penitrem staggers	Ruminants, other domestic animals; neurotoxicity	Muscular tremors, ataxia, convulsive spasms	Mouldy stored feed should not be fed to ruminants; immediate removal of grazing animals from pasture when clinical signs are observed	Severely affected animals may collapse and exhibit opisthotonos
Fungal species associated with trichothecene toxicoses				
Fusarium sporotrichoides, *F. graminearum* and other *Fusarium* species growing on cereals and straw / Haemorrhagic syndrome	Pigs, cattle, poultry; coagulopathy, immunosuppression	Necrotic skin lesions, haemorrhages	Mouldy feed should be excluded from diet	The mycotoxins involved have toxic effects on platelets
Myrothecium verrucaria and *M. roridum* growing on rye stubble, straw and white clover / Myrotheciotoxicosis	Sheep, cattle, horses; inflammation of many tissues, respiratory distress	Haemorrhagic gastroenteritis, immunosuppression, weight loss	Exclusion of mouldy feed from the animals' diet; symptomatic treatment may be beneficial in some affected species	In acute disease, sudden deaths may occur

Fungal species associated with trichothecene toxicoses				
Stachybotrys chartarum growing on stored cereals, straw, hay / Stachybotryotoxicosis	Horses, cattle, sheep, pigs; the mycotoxins are cytotoxic and have radiomimetic effects on tissues	Necrotic stomatitis, widespread petechial haemorrhages and blood-stained diarrhoea, panleukopenia, immunosuppression; death frequently results from a combination of haemorrhage and septicaemia	Contaminated feed should be excluded from the animals' diet	Horses appear to be particularly susceptible to the mycotoxins produced by *S. chartarum*
Fungi which convert non-toxic constituents of plants into toxic components				
A number of fungal species including *Aspergillus* species, *Penicillium* species and *Mucor* species, growing on hay and silage containing *Melilotus* species of clover (sweet clover) / Sweet clover poisoning	Cattle, sheep, horses; fungi growing on hay or silage containing sweet clover convert coumarin and melilotin to dicoumarol which interferes with blood coagulation in animals consuming such mouldy hay or silage	Dicoumarol produces hypoprothrombinaemia, resulting in prolonged clotting times, haemorrhage and severe anaemia	Mouldy hay or silage containing sweet clover should not be fed to animals. As part of the treatment regime, mouldy hay and silage should be removed from the animals' diet; blood transfusions and vitamin K given parenterally are used to restore blood coagulation which may require more than 24 hours to be effective	Internal or external haemorrhage may be evident clinically; trauma or surgical procedures may be followed by severe haemorrhage. Dicoumarol, which is not a mycotoxin, is a toxic factor produced by the activity of fungal species on non-toxic coumarin and melilotin, normal components of sweet clover and a number of grasses
Fungus-like organisms				
Lacazia loboi / Lobomycosis	Uncommon condition in humans and dolphins; granulomatous nodular skin lesions	In humans, lesions tend to occur on traumatized areas of skin including face, legs and feet; lesions in dolphins are similar to those in human skin	Surgical excision is the treatment used for localized lesions in humans; long-term treatment with clofazamine combined with itraconazole has been successful in a human patient	Long-term therapy is required in the treatment of lobomycosis

Prototheca species / Protothecosis	Cattle, dogs, cats; mastitis in cattle; enterocolitis in dogs; cutaneous lesions in cats	*P. zopfii* can cause progressive pyogranulomatous lesions in the bovine mammary gland; disseminated disease has been described in dogs and localized dermal lesions are reported in cats	Surgical excision may be successful for treating localized cutaneous protothecosis; amphotericin B has been beneficial in some instances; fluconazole, itraconazole and ketoconazole have been effective in individual animals but long-term treatment may be required	Immunodeficiency predisposes to protothecosis
Pythium insidiosum / Pythiosis	Horses, dogs, cats and cattle. Horses: granulomatous lesions, especially on the limbs, which ulcerate. Dogs: cutaneous and gastrointestinal lesions. Cats: facial swelling. Calves: facial ulcers and fistulous tracts	Non-healing wounds, ulcerated nodules with draining tracts; gastrointestinal pythiosis in dogs is characterized by vomiting, weight loss and sporadic diarrhoea	Three therapeutic methods may be considered, surgery, chemotherapy and immunotherapy. Radical surgery may be successful in some instances, especially in horses; treatment with sodium iodide, amphotericin B, itraconazole and terbinafine may be effective; reports suggest that injection of exoproteins and endoproteins extracted from *P. insidiosum* have aided recovery in horses	The prognosis is generally poor when lesions are extensive
Rhinosporidium seeberi / Rhinosporidiosis	Horses, cattle, dogs, cats and waterfowl; also affects humans; chronic disease involving the nasal mucosa and occasionally affecting the skin; often presents as a polypous rhinitis	Reddish brown polyps may project from the nasal cavity and occlude the nasal passages; noisy breathing may be exacerbated by exercise; cutaneous lesions may be single or multiple	Cryosurgery has been used to remove individual polyps but recurrence is common; diaminodiphenylsulphone (dapsone) has proved beneficial and resulted in a marked decline in recurrence of skin lesions; this drug is reported to inhibit maturation of sporangia of *R. seeberi*	An outbreak of ocular and nasal rhinosporidiosis has been reported in captive swans
Pathogenic yeasts				
Candida albicans / Candidiasis	Pups, foals, young pigs, kittens, chickens and turkeys; opportunistic infections may occur sporadically, usually associated with immunosuppression or with prolonged use of broad-spectrum antibacterial drugs; superficial infections limited to the mucous membranes of the oral cavity and the intestinal tract	Thrush of the oesophagus and crop of young birds leads to listlessness and inappetence; lesions in the intestinal tracts of mammals may result in diarrhoea, anorexia and dehydration; clinical signs generally relate to the extent and severity of lesions; mycotic mastitis may result from contamination of intramammary preparations	Prolonged antibacterial therapy should be avoided, especially in young animals; if severe candidiasis occurs, treatment with fluconazole and itraconazole is usually effective	In young mammals, candidiasis is usually associated with prolonged antibacterial therapy; in young birds, improved hygiene measures reduce the incidence of disease; particular attention should be given to the drinking water provided for chickens and poults
Pathogenic yeasts				
Cryptococcus neoformans / Cryptococcosis	Cats, dogs, cattle, horses, humans; nasal, cutaneous and neural forms of the disease occur in cats; disseminated disease occurs in dogs; mastitis in cows	Evidence of lesions in the upper respiratory tract of cats with sneezing and nasal discharge, polyp-like masses in the nasal cavity; neurological signs may be evident in dogs; cows with cryptococcal mastitis may have systemic signs together with a swollen quarter	Initial treatment with amphotericin B and flucytosine may be effective; fluconazole may be used as maintenance therapy if clinical signs of cryptococcosis persist	Many animals with cryptococcosis have an underlying immunosuppressive condition or have had prolonged treatment with corticosteroids
Malassezia pachydermatis / Associated with otitis externa and dermatitis	Dogs; otitis externa and seborrhoeic dermatitis	Dark pungent discharge from the ear canal, pruritis, head shaking and rubbing of ears; seborrhoeic dermatitis is characterized by pruritis, erythema with foul-smelling greasy exudate and matting of hair	Underlying causes of otitis externa should be identified and corrected. Bacterial and parasitic pathogens should be identified and treated appropriately. Shampoo containing miconazole and chlorhexidine may be effective for seborrhoeic dermatitis if predisposing factors are identified and corrected; ketoconazole shampoo is also reported to be an effective treatment	Immunosuppression and hypersensitivity conditions are among the factors that predispose dogs to infections with *M. pachydermatis*

Phaeoid fungi				
Alternaria species, *Bipolaris spicifera*, *Curvularia* species, *Exophiala jeanselmei*, *Exoserohilum rostratum*, *Phialophora parasitica*, *Phoma glomerata*, *Scedosporium apiospermum* and other fungal species / Phaeohyphomycosis	Cattle, horses, goats, dogs and cats; granulomatous lesions, usually in subcutaneous tissues	Subcutaneous granulomatous lesions mainly on the feet, limbs and head; ulceration and sinus tract formation with serosanguineous discharges are a feature of these lesions	Surgical excision of lesions may be effective in some instances; itraconazole combined with flucytosine may be effective; amphotericin B combined with surgical excision may be considered when lesions are accessible	Treatment for several months with antifungal drugs may be required
Zygomycetes				
Pathogenic genera include: *Lichtheimia, Mucor, Rhizomucor, Rhizopus, Mortierella, Saksenaea, Basidiobolus and Conidiobolus* / Zygomycoses	Cattle, pigs, cats, dogs, horses; abortion and rumenitis in cattle; enteritis in piglets; pneumonia in cats and enteritis in dogs; cutaneous granulomas in horses; nasal granulomas in horses and sheep caused by *Conidiobolus* species	Diseases caused by zygomycetes are uncommon in animals; clinical signs relate to tissue or system affected; *Mortierella wolfii* causes acute fatal pneumonia in cows following abortion caused by the fungus; this syndrome is described in New Zealand	Due to the sporadic nature of zygomycoses, the opportunity to treat affected animals with antifungal drugs rarely occurs; amphotericin B remains the drug of choice for zygomycoses	Factors which predispose to zygomycoses include immunodeficiency, corticosteroid therapy and prolonged treatment with broad-spectrum antibacterial drugs

*The safety of recently developed antifungal drugs should be considered carefully when treating young animals and pregnant animals as adverse reactions may occur in some species. Combinations of antifungal drugs should be used cautiously to avoid adverse effects.

Table 53.2 Classification of antifungal drugs and their modes of action.

Chemical class or biological activity of antifungal drug / Examples	**Mode of action**	**Comments**
Allylamines		
Naftifine Terbinafine	These antifungal drugs inhibit the activity of squalene epoxidase, an enzyme required for the production of ergosterol, the principal sterol in the membrane of fungal cells. Decreased synthesis of ergosterol and accumulation of squalene produces a toxic effect on the fungal pathogen	Allylamines have a broad antifungal spectrum and are particularly effective against dermatophytes. Terbinafine, a lipophilic drug, is reported to become concentrated in the dermis, epidermis and adipose tissue
Antimitotic antibiotic		
Griseofulvin	The first widely used orally administered antifungal drug, griseofulvin has a limited spectrum of action restricted to dermatophytes. This fungistatic antibiotic binds to microtubular proteins and interferes with microtubule spindle formation, thereby inhibiting fungal cell mitosis. It may also act as an inhibitor of nucleic acid synthesis	Although formerly a popular drug for the treatment of dermatophytosis in large animals, griseofulvin's place as an antifungal drug has been largely replaced by a range of more effective topical and synthetic antifungal agents
Azoles		
Imidazoles Clotrimazole Ketoconazole Miconazole Econazole Triazoles Fluconazole Itraconazole Voriconazole	The major effect of the imidazoles and triazoles is inhibition of 14-α-demethylase, a microsomal cytochrome P450 enzyme. Inhibition of this enzyme, which is involved in the conversion of lanosterol to ergosterol, leads to depletion of ergosterol and accumulation of 14-α-methylsterols in the fungal cell membrane. These changes disrupt cell membrane activities and the functioning of membrane-bound enzyme systems	Prolonged treatment regimes are required with azole drugs. Ketoconazole which has been used in animals for many years, is being replaced by itraconazole and fluconazole. Although effective against many fungal pathogens, fluconazole is not effective against *Aspergillus* species or zygomycetes. Itraconazole is active against *Aspergillus* species and highly effective against dimorphic fungi
Echinocandins		
Caspofungin	Echinocandins are semisynthetic lipopeptides which inhibit fungal 1,3-β-glucan synthase which is required for the synthesis of 1,3-β-glucan, a	Caspofungin has a limited spectrum of action. It is effective against *Aspergillus* species and most *Candida* species and it is also effective against

Micafungin Anidulafungin	major component of fungal cell walls. Since mammalian cells do not contain 1,3-β-glucans, these antifungal drugs are selectively toxic for fungi	*Pneumocystis carinii*. The antifungal spectrum of caspofungin, micafungin and anidulafungin appears to be similar
Fluorinated pyrimidine		
Flucytosine	This fluorinated pyrimidine enters fungal cells by the action of cytosine permease and is deaminated to 5-fluorouracil which is incorporated into RNA in place of uracil, with resulting impact on protein synthesis. In addition, further metabolism of this antifungal agent produces a potent inhibition of thymidylate synthetase which causes inhibition of DNA synthesis	Flucytosine has a narrow spectrum of antifungal activity. It is active against *Cryptococcus neoformans*, *Candida* species and some dematiaceous moulds. When used alone, drug resistance is an important cause of therapeutic failure. To overcome this problem, flucytosine is used in combination with amphotericin B
Iodides		
Potassium iodide Sodium iodide	Although sodium iodide and potassium iodide have been used for many years to treat fungal infections in animals, their mode of action is not well understood. Enhancement of immune responses against fungal pathogens has been suggested. A direct antifungal effect through interference with enzymes essential for fungal cell metabolism may contribute to the activity of iodides	Prolonged treatment with iodine compounds is often required to clear fungal infections. Sodium iodide has been used to treat sporotrichosis and also nasal aspergillosis in dogs. Following prolonged administration, there is a risk of iodism occurring in some animals
Morpholines		
Amorolfine	This antifungal compound, which is applied topically for the treatment of onychomycosis and dermatophytosis, is an inhibitor of sterol biosynthesis. Amorolfine's fungistatic activity correlates with the depletion of ergosterol which is essential for the functioning of the fungal cell membrane	The role of this antifungal agent for the treatment of dermatophyte infections in animals is not as well defined as in human medicine. Amorolfine is considered to be a highly effective antifungal agent for treating onychomycosis in human patients
Nucleoside-peptides		
Nikkomycin Z	This antifungal compound, which inhibits chitin synthase, interferes with the synthesis of fungal cell wall polysaccharides. It also potentiates the effect of flucytosine, a number of azole compounds and echinocandins	Although nikkomycin Z was shown to be highly effective against murine coccidioidomycosis, histoplasmosis and blastomycosis, further clinical trials are required to confirm its therapeutic effectiveness in fungal infections
Polyenes		
Amphotericin B	The polyenes are macrolide antibiotics which bind preferentially to sterols, especially ergosterol. Although there are hundreds of polyene antibiotics, amphotericin B is the only one with a level of toxicity that renders it suitable for systemic antifungal therapy in humans and animals. The antifungal activity of amphotericin B relates to its binding to fungal cell membrane sterol, primarily ergosterol. The consequences of this binding include disruption of the osmotic integrity of the fungal cell membrane with leakage of potassium ions and a variety of small molecules	Because parenteral administration of conventional micellar suspension of amphotericin B leads to undesirable toxic effects, three lipid-based formulations of the drug, namely liposomal amphotericin B, a lipid complex of amphotericin B and a colloidal suspension of the drug complexed with cholesterol sulphate, are available. These formulations appear to be less toxic than the micellar suspension
Pradimicin antibiotics		
Pradimicin BMS-181184	This water-soluble pradimicin derivative binds to the cell surface of yeast cells. Changes induced by this antifungal compound, which reacts with carbohydrate components in the target cell wall, include potassium leakage from yeast cells	Pradimicin derivatives appear to be selectively antifungal and have no affinity for cultured animal cells
Sordarins and azasordarins	Sordarin derivatives selectively inhibit fungal growth by blocking elongation factor 2 and disrupting protein synthesis	Sordarins are reported to have a broad antifungal spectrum with activity against dermatophytes, *Aspergillus* species, dimorphic fungi and *Pneumocystis carinii*
Substituted pyridone		
Ciclopirox olamine	This substituted pyridone alters membrane transport, damages the fungal cell membrane and interferes with metabolism of target cells by affecting mitochondrial electron transport processes during energy production	Ciclopirox olamine, which is applied topically, has broad-spectrum antifungal activity. It is fungicidal for dermatophytes, *Candida albicans*, *Malassezia* species and fungal pathogens causing onychomycosis

Resistance to azole antifungal drugs is a gradual process which involves several alterations in fungal strains subjected to antifungal drug pressure. In many of the isolates studied, multiple mechanisms of resistance were evident.

Echinocandins

This group of semisynthetic lipopeptides inhibit the synthesis of 1,3-β-glucan,a major component of many fungal cell walls. By acting as non-competitive inhibitors of 1,3-β-glucan synthase, echinocandins interfere with fungal cell division and also with cell growth. Because mammalian cells do not contain 1,3-β-glucan, echinocandins are selectively toxic for fungi in which glucans constitute the dominant cell wall component. A non - echinocandin lipopeptide which inhibits glucan synthase has shown good activity against yeasts and *Aspergillus* species *in vitro* and *in vivo*. Fungi such as *Cryptococcus neoformans*, which contain predominantly 1,6-β-glucans in their cell walls, are resistant to echinocandins. Three echinocandins, caspofungin, micafungin and anidulafungin, appear to have similar antifungal spectra.

Caspofungin, which has a limited spectrum of activity, is effective against *Candida* species, most *Aspergillus* species, *Pneumocystis carinii* and some fungi which cause phaeohyphomycoses. Its efficacy against dimorphic fungi is not predictable and it is not effective against *C. neoformans* or the zygomycetes.

Primary resistance to echinocandins among clinical isolates of *Candida* species and *Aspergillus* species appears to be uncommon. Because of their selective toxicity for fungal cell walls, few adverse reactions to echinocandins have been reported in the human population. Data relating to clinical trials with echinocandins in animals are limited but it is likely that these novel antifungal drugs will be of particular therapeutic value for treating aspergillosis and candidiasis in avian and mammalian species.

Flucytosine

This fluorinated pyrimidine is transported into susceptible fungal cells by the action of cytosine permease and there converted by the action of fungal cytosine deaminase to 5-fluorouracil. Later, 5-fluorouracil is converted to 5-fluorouridylic acid which then competes with uracil in the synthesis of RNA. This results in RNA miscoding and inhibition of DNA and protein synthesis.

Flucytosine has a narrow spectrum of activity which includes *Candida* species, *Cryptococcus neoformans* and a limited number of dematiaceous moulds.

Because resistance to flucytosine can develop quickly during treatment, this drug is used in combination with amphotericin B or fluconazole. Combination therapy is used for the treatment of cryptococcosis, especially in cats.

Primary and acquired resistance to flucytosine may result from absence, dysfunction or deletion of one or more enzymes which are not essential for fungal survival.

Iodides

Although sodium iodide and potassium iodide have been used for treating fungal infections for many years, their modes of action are not well understood. Enhancement of immune responses against fungal pathogens has been suggested as one antifungal mechanism. An alternative mode of action may relate to direct interference with fungal cell metabolism.

A high percentage of human patients with sporotrichosis recovered following oral administration of potassium iodide and relapses were rare (Arenas, 2005). Sodium iodide has been used successfully to treat cutaneous and lymphocutaneous sporotrichosis in animals. Prolonged treatment with iodine compounds is often required and may lead to a risk of iodism. To avoid residues in milk and meat, iodine compounds should be used carefully in food-producing animals.

Morpholines

Amorolfine, a morpholine derivative with antifungal activity, is used topically to treat dermatophytosis and onychomycosis. This antifungal compound inhibits ergosterol synthesis which is essential for the functioning of the fungal cell membrane. Other effects include squalene accumulation and disturbance of chitin synthesis. Amorolfine has a broad antifungal spectrum which includes dermatophytes, dimorphic fungi and other fungal pathogens associated with onychomycosis. It is inactive against *Aspergillus* species. Although considered to be a highly effective antifungal agent for treating onychomycosis in humans, this compound’s therapeutic activity in animals is not yet well defined.

Nucleoside-peptides

Chitin synthesis is inhibited by a number of nucleoside-peptides including nikkomycin Z. This antifungal compound inhibits chitin synthase, thereby

interfering with fungal cell wall synthesis. It is reported to potentiate the effect of flucytosine, some azole compounds and echinocandins. Nikkomycin Z has been highly effective in clinical trials for the treatment of murine pulmonary blastomycosis, histoplasmosis and coccidioidomycosis. Further clinical trials in naturally infected animals are required to confirm its therapeutic effectiveness against dimorphic fungal infections.

Polyenes

The polyenes are macrolide antibiotics which have a broad spectrum of activity against fungal pathogens. Many of these compounds are too toxic for therapeutic use but amphotericin B is suitable for antifungal therapy in humans and animals. In common with other polyenes, amphotericin B binds preferentially to sterols, especially ergosterol in the fungal cell membrane. Although amphotericin B binds to cholesterol, the principal membrane sterol of mammalian cells, it does so less avidly than to ergosterol. Binding of this antifungal drug to the fungal cell membrane alters its permeability resulting in leakage of sodium, potassium and hydrogen ions and cell death. Amphotericin B also disrupts the function of oxidative enzymes in target cells. The spectrum of activity of this antifungal drug includes pathogenic yeasts, dimorphic fungi, *Aspergillus* species and zygomycetes.

Intravenous administration of amphotericin B can result in nephrotoxicity. The toxic effect is related to the binding of the drug to the sterol-rich cell membranes in kidney tubules. Because of the problems associated with conventional amphotericin B use, new formulations with reduced toxicity and improved pharmacological properties have been developed. Three new formulations of the drug, namely a lipid complex of amphotericin B, liposomal amphotericin B and a colloid suspension of the drug, are available. These new formulations offer greater efficacy than conventional amphotericin B with reduced toxicity. Their high cost, however, has limited their selection for the treatment of systemic fungal disease in animals.

Development of secondary resistance to amphotericin B has not emerged as a major problem. Some isolates of *Candida* species may be relatively resistant to amphotericin B and resistance among *Aspergillus* species may be encountered occasionally.

Pradimicin antibiotics

Among the pradimicin antibiotics, pradimicin BMS-181184 has antifungal activity. This water-soluble pradimicin derivative binds to cell surface carbohydrate components of yeast cells. Changes induced by this antifungal compound include potassium leakage from yeast cells. Because it does not have affinity for cultured animal cells, this pradimicin derivative appears to have selective antifungal activity. Pradimicin A, a low molecular weight carbohydrate-binding agent with antifungal activity, also has selective inhibitory activity against human immunodeficiency virus (Balzarini *et al*., 2007).

Sordarins and azasordarins

This new class of antifungal drugs selectively inhibits fungal growth by blocking elongation factor 2 and disrupting protein synthesis. Sordarin derivatives have a broad antifungal spectrum which includes dermatophytes, *Aspergillus* species, dimorphic fungi and *Pneumocystis carinii*. When combined with amphotericin B, sordarins have a synergistic action against *Candida* species and *Aspergillus* species. In clinical trials using animal models, sordarins were effective against histoplasmosis, coccidioidomycosis and pneumocystosis. Toxicological data suggest that sordarins are relatively safe drugs but more extensive clinical trials are required to confirm their safety before they can be endorsed for general use.

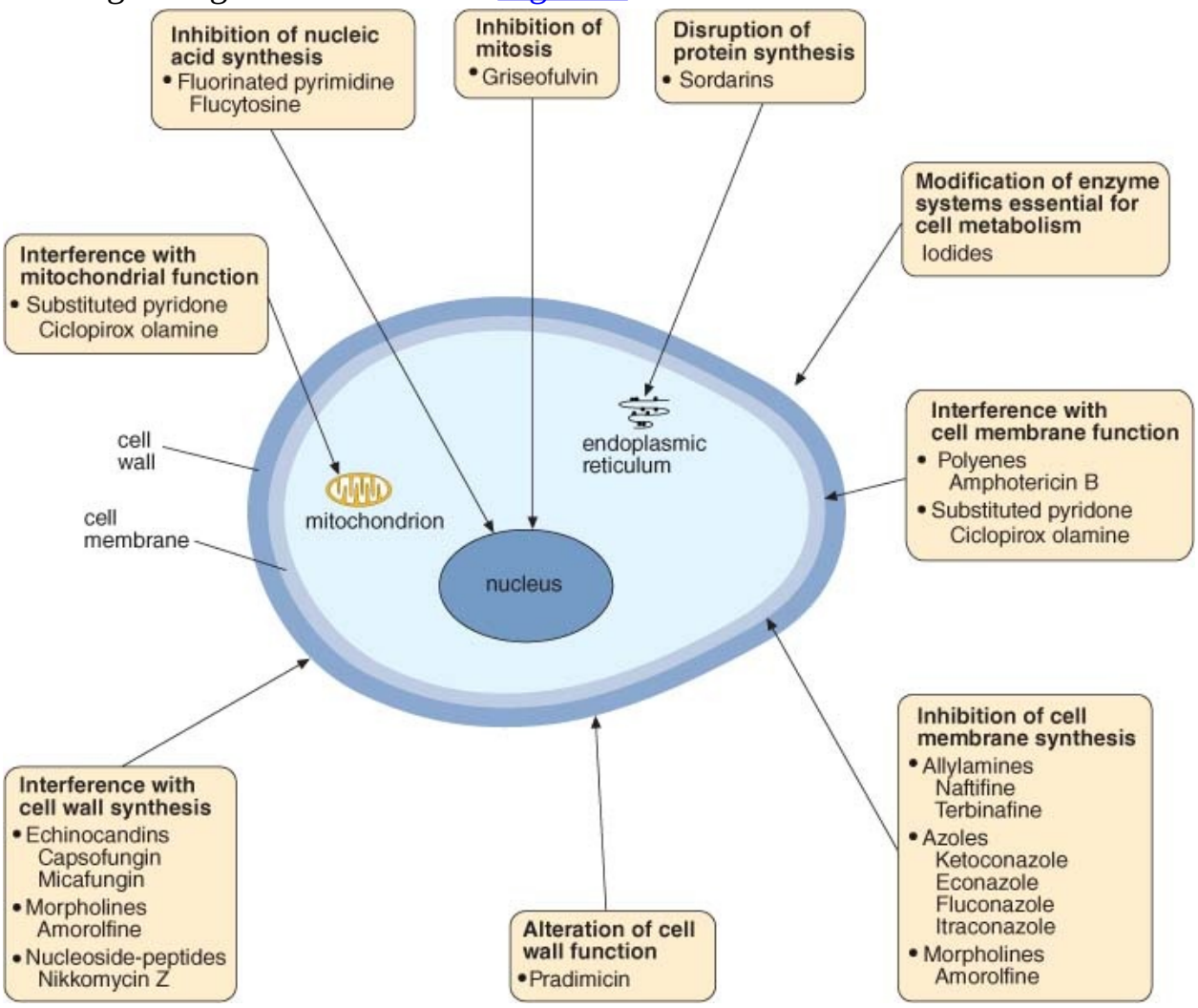
Ciclopirox olamine

This antifungal drug, a substituted pyridone, has a broad antifungal spectrum. Ciclopirox olamine alters membrane transport, cell membrane integrity and mitochondrial electron transport processes. In addition to its antifungal activity against dermatophytes and yeasts, including *Malassezia* species, it is active against some Gram-positive and some Gram-negative bacteria. It also has anti-inflammatory activity which may be beneficial in the treatment of some superficial fungal infections. This drug, which is used topically, penetrates through the epidermis into the dermis. It is especially effective for the treatment of onychomycosis in humans but long-term treatment is required to eliminate the fungal infection. Toxicity has not been reported when ciclopirox olamine is used topically.

Classification of antifungal drugs and their modes of action are presented in Table 53.2. The modes and

Figure 53.1 Modes and sites of action of antifungal drugs sites of action of

antifungal drugs are illustrated in Fig. 53.1.



Resistance to antifungal drugs

A recognized feature of many infectious agents is that they are either inherently resistant or become resistant to drugs that interfere with their replication or otherwise alter their metabolism. Fungal pathogens conform to this biological pattern of either possessing resistance to antifungal drugs or acquiring resistance as a consequence of sustained antifungal therapy. Primary resistance denotes natural resistance of a particular fungal genus or species. The resistance of zygomycetes to fluconazole is an example of such intrinsic resistance. Secondary resistance is the acquisition of resistance following the pressure of antifungal therapy. There are a number of ways whereby secondary resistance may develop. Mutation and selection account for much of the secondary

resistance that develops. A commonly observed type of secondary resistance is the replacement of a particular fungal pathogen with a more resistant strain or, in some instances, with a more resistant species. Alternatively, a single strain may include a resistant subpopulation which may be selected for by sustained antifungal therapy. Other changes in fungal cells which may lead to decreased susceptibility to antifungal drugs include transient gene expression resulting from antifungal therapy, and possession and expression of particular virulence factors. Much of the acquired resistance to antifungal drugs in the human population has been attributed to prophylactic therapy or to suboptimal dosage associated with prolonged treatment.

In contrast to the rapid emergence and spread of high-level multidrug resistance encountered with bacteria, antifungal resistance usually develops slowly and involves the emergence of resistant species or a gradual change in the cellular structures or functions which result in resistance to the antifungal drug. Unlike the resistance mechanisms observed for some antibacterial agents, there is no evidence at present that fungal pathogens are capable of breaking down or structurally modifying antifungal agents as a means of achieving resistance. Primary and acquired resistance to flucytosine is attributed to an absence, dysfunction or deletion of one or more enzymes which are not essential for fungal survival. Mechanisms of resistance to fluconazole include decreased binding activity to the P450 cytochrome, increased production of the target cytochrome and decreased accumulation of the drug in fungal cells due to reduced uptake or increased efflux mechanisms.

Resistance to amphotericin B is reported infrequently. Quantitative or qualitative change in the sterol content of the fungal cell may lead to resistance to this antifungal drug. Mechanisms of quantitative ergosterol change include a decrease in the amount of ergosterol in the cell due to inhibition of synthesis, replacement of ergosterol with episterol or other sterols and alterations in the ratio of sterols to phospholipids (Arikan and Rex, 2005). Resistance related to qualitative change may include reorientation or masking of ergosterol in the fungal cell membrane. It has been reported that previous exposure to azole compounds, which lead to depletion of ergosterol, may contribute to amphotericin B resistance in fungal cells.

Resistance to azole drugs may arise from qualitative or quantitative alterations in the target enzyme, 14-α-demethylase, decreased access of the drug to the enzyme or a combination of the two mechanisms. Quantitative modifications may arise from increased copy numbers of the enzyme leading to increased

synthesis of ergosterol. Azole resistance may occur due to changes in sterol or phospholipid composition in the fungal cell membrane resulting in decreased permeability to the antifungal drugs. In addition to decreased accumulation of the antifungal drug in the target cells, increased efflux mechanisms may contribute to azole resistance. Efflux pump systems have been implicated in fluconazole and itraconazole resistance in *Candida* species and in itraconazole resistance in *Aspergillus fumigatus*.

Resistance to flucytosine may arise due to loss of cytosine permease activity, a defect in cytosine deaminase activity or a decrease in the activity of uracil phosphoribosyl-transferase. When used alone for the treatment of cryptococcosis and candidiasis, drug resistance is an important cause of therapeutic failure. To overcome this problem, flucytosine is used in combination with amphotericin B.

Although clinical failures have been reported during treatment of fungal infections with terbinafine and naftifine, these failures were not attributed to the development of resistance but to host-related factors. Recent reports suggest that resistance to allylamines does occur but is rare.

Resistance to griseofulvin therapy has been reported but reliable data are not available. The molecular basis of resistance remains unclear.

Caspofungin, micafungin and anidulafungin have potent fungicidal activity against *Aspergillus* species and *Candida* species. An altered glucan synthesis enzyme complex with decreased sensitivity to inhibition by caspofungin has been demonstrated in laboratory-derived mutants of *Candida albicans*. These strains have point mutations in the *FKS1* gene which encodes an integral cell membrane protein. Mutations in another cell wall synthesis gene, *GNS1*, resulted in low-level resistance.

Antifungal therapy may not be clinically successful even though the drug selected is effective against the fungal pathogen. The host, the antifungal drug and the fungal pathogen influence many aspects of the treatment regime and, ultimately, the outcome. Of particular importance are the immune status of the host, the site and severity of the fungal infection, the activity of the drug at the site of infection and the dose and duration of therapy. Prolonged treatment regimes are required for many antifungal drugs such as azoles. The age and nutritional status of the host also influence the outcome of treatment. The development of antifungal resistance is usually gradual and multifactorial in nature. To avoid the development of secondary resistance, careful selection of the antifungal drug and proper dosing strategies should be implemented. When necessary, combination therapy which achieves synergy with the antifungal

drugs selected should be employed. A better understanding of the virulence factors of pathogenic fungi would allow the formulation of treatment regimes appropriate for the tissue or system invaded. Consideration of host factors should form part of the treatment strategy, as measures aimed at enhancing the host' s immune response to the invading pathogen may contribute to the therapeutic efficacy of antifungal drugs.

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Section V
Introductory Virology

Chapter 54

Nature, structure and taxonomy of viruses

The term ‘virus’ (Latin, *poison*) refers to members of a unique class of infectious agents which are extremely small, contain only one type of nucleic acid and have an absolute dependence on living cells for replication (Box 54.1). Viruses have some of the characteristics of living cells, such as a genome and the ability to adapt. Unlike living cells, however, they lack autonomy as they cannot capture and store free energy. Because of these limitations viruses are generally viewed as sub-cellular, non-living, infectious entities which only become part of a living system when they have infected host cells, a form of borrowed life (van Regenmortel, 2000). The genomes of the viruses that infect animals are considerably smaller than those of prokaryotic cells, ranging from about 2 kilobase pairs (kbp) to 800 kbp. In the majority of virus families, the nucleic acid is present as a single molecule; however, in some RNA virus families (e.g. *Reoviridae and Orthomyxoviridae*), the nucleic acid occurs in separate segments. Although the nucleic acid of viral genomes is usually linear, it is circular in some viruses such as parvoviruses. Genomes of both DNA and RNA viruses can be single-stranded or double-stranded. Particular characteristics of DNA and RNA viruses are presented in Tables 54.1 and 54.2 and in Figs 54.1 and 54.2.

Recently, an icosahedral DNA virus named Sputnik has been found in association with the large mimivirus, *Acanthamoeba polyphaga* mimivirus (APMV). Sputnik has been shown to multiply in the virus factory found in amoebae co-infected with APMV. The activities of Sputnik are deleterious to APMV and the term virophage has been assigned to this small ‘parasitic’ virus (La Scola *et al*., 2008). Two unique types of infectious agents, which are structurally less complex than viruses, are recognized, viroids and prions. Viroids are composed of naked RNA and prions are proteinaceous infectious particles which are devoid of demonstrable nucleic acid.

In recent years, remarkable advances have been made in our understanding of the epidemiology, pathogenesis and control of viral diseases. However, as new viral diseases are recognized in the animal and human populations, fresh

challenges confront those engaged in virological research.

The origin of viruses

Viruses have evolved to a stage where they are considered to be among the most efficient and economic forms of microbial life. They can be categorized in three main groups on the basis of their nucleic acid composition: DNA viruses, RNA viruses and viruses that utilize both DNA and RNA for replication. Differences between these groups are significant and may be indicative of independent origins for each group. Although the origin of viruses is uncertain, and no fossil remains exist, four theories have been proposed to explain their evolution. They may have originated from primitive, pre-cellular RNA replicons, which evolved to become dependent on cells for replication. Alternatively, they may have arisen from segments of cellular nucleic acid which acquired the ability to replicate at the expense of the host cell. The third theory, the regressive theory of the origin of viruses, postulates that they arose from free-living organisms, which gradually lost genetic information until they became totally dependent on the biosynthetic pathways of their host cells. The study of the genomes of viruses and their host organisms supports the view that viruses are probably as ancient as cells and have co-evolved with them. Whole genomic sequencing studies indicate that all life forms and sub-cellular replicons share basic structural and functional motifs. In fact, viruses have mediated lateral gene transfers among cells and in this way have contributed to cellular change.

Box 54.1 Characteristics of viruses which can infect animals.

- Small infectious agents, ranging in size from 20 to 400 nm
- Composed of nucleic acid surrounded by a protein coat; in addition, some contain envelopes
- Contain only one type of nucleic acid, either DNA or RNA
- Unlike bacteria and fungi, viruses cannot replicate on inert media; viable host cells are required for replication
- Some viruses have an affinity for particular cell types

Table 54.1 Characteristics of the families of DNA viruses of veterinary importance.

Family	Virion size (nm)	Capsid symmetry	Envelope	Type of genome
Adenoviridae	70–90	Icosahedral	–	Linear, double-stranded DNA
Asfarviridae	175–215	Icosahedral	+	Linear, double-stranded DNA
Circoviridae	17–22	Icosahedral	–	Circular molecule of positive-sense or ambisense, single-stranded DNA
Herpesviridae	200–250	Icosahedral	+	Single molecule of linear, double-stranded DNA
Papillomaviridae	55	Icosahedral	–	Single molecule of circular, double-stranded DNA
Parvoviridae	18–26	Icosahedral	–	Single molecule of linear, positive-sense or negative-sense, single-stranded DNA
Poxviridae	300 × 200	Complex	+	Single molecule of linear, double-stranded DNA

Table 54.2 Characteristics of the families of RNA viruses of veterinary importance.

Family	Virion size (nm)	Capsid symmetry	Envelope	Type of genome
Arteriviridae	40–60	Icosahedral	+	Linear, single molecule of positive-sense, single-stranded RNA
Astroviridae	28–30	Icosahedral	–	Linear, single molecule of positive-sense, single-stranded RNA
Birnaviridae	60	Icosahedral	–	Two segments of linear, double-stranded RNA
Bornaviridae	90	Icosahedral	+	Linear, single molecule of negative-sense, single-stranded RNA
Bunyaviridae	80–120	Helical	+	Three segments of linear, negative-sense or ambisense single-stranded RNA
Caliciviridae	27–40	Icosahedral	–	Linear, single molecule of positive-sense, single-stranded RNA
Coronaviridae	120–160	Helical	+	Linear, single molecule of positive-sense, single-stranded RNA
Flaviviridae	40–60	Icosahedral	+	Linear, single molecule of positive-sense, single-stranded RNA
Orthomyxoviridae	80–120	Helical	+	Six to eight segments of linear, negative-sense, single-stranded RNA
Paramyxoviridae	150–300	Helical	+	Linear, single molecule of negative-sense, single-stranded RNA
Picornaviridae	30	Icosahedral	–	Linear, single molecule of positive-sense, single-stranded RNA
Reoviridae	60–80	Icosahedral	–	Ten to twelve segments of linear, double-stranded RNA
Retroviridae	80–100	Icosahedral	+	Diploid, linear, positive-sense, single-stranded RNA
Rhabdoviridae	180 × 75	Helical	+	Linear, single molecule of negative-sense, single-stranded RNA
Togaviridae	70	Icosahedral	+	Linear, single molecule of positive-sense, single-stranded RNA

Figure 54.1 Diagrammatic representations of families of DNA viruses of vertebrates. The genomes of these viruses are composed of either double-stranded (ds) or single-stranded (ss) DNA.

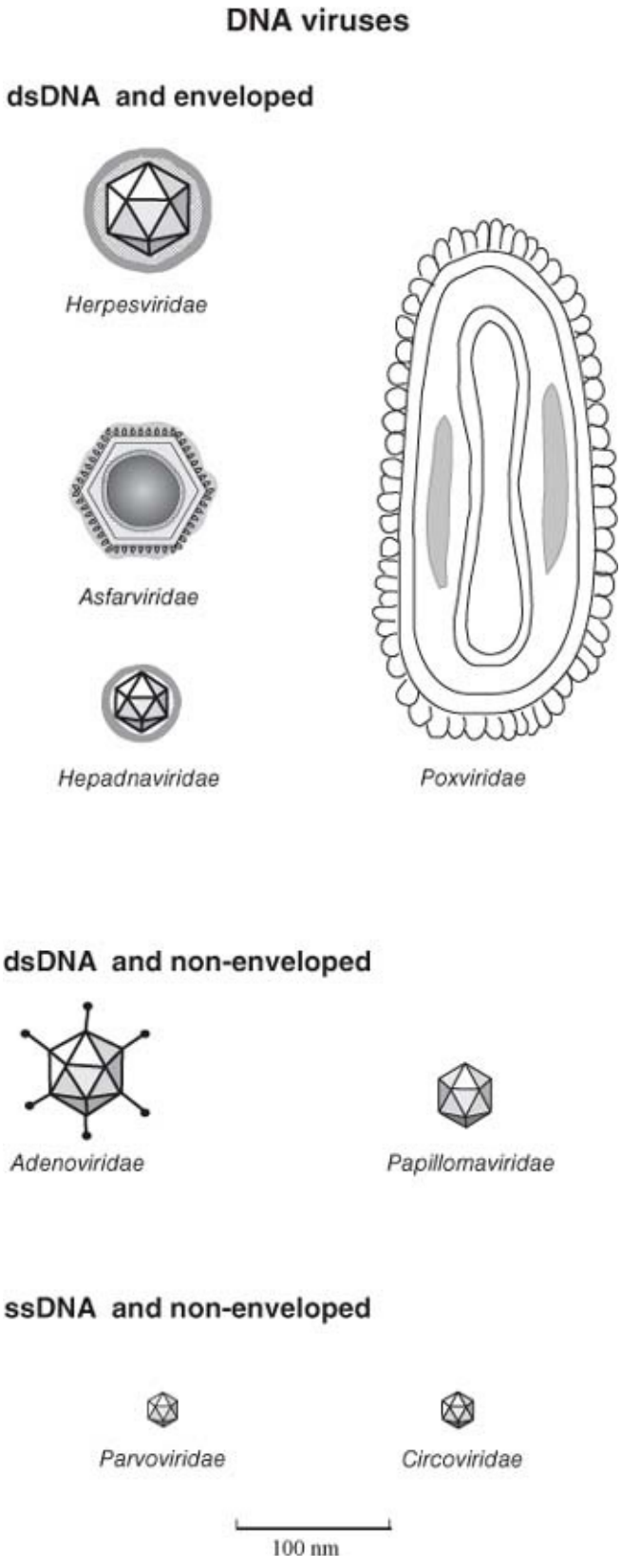
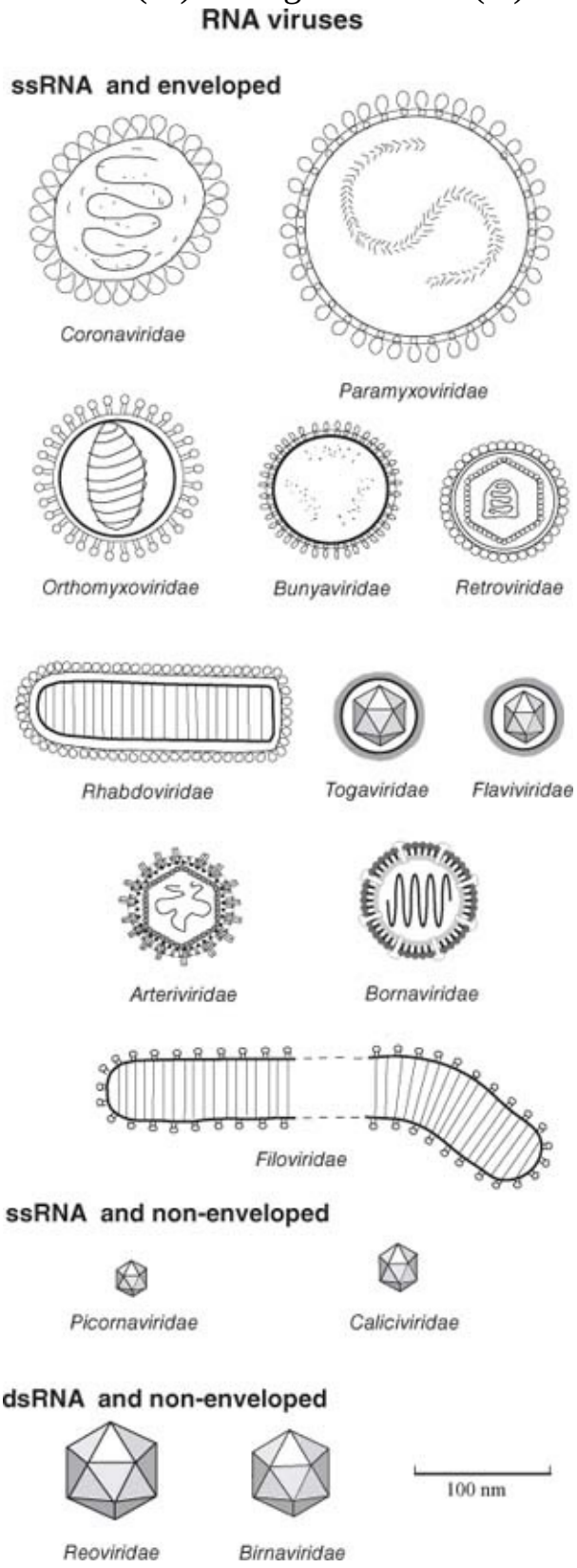


Figure 54.2 Diagrammatic representations of virions in families of RNA viruses

of vertebrates. The genomes of these viruses are composed of either double-stranded (ds) or single-stranded (ss) RNA.



Structure of viruses

A fully assembled infective virus is termed a virion. This term is not synonymous with the term virus. A virion can be considered solely in terms of its physical constituents or chemical composition. In contrast, a virus possesses not only these biophysical properties but also additional characteristics which become evident when it infects a susceptible host cell. The definition of a virus must, therefore, include the various functional activities and biotic interactions that make it a biological entity (van Regenmortel, 2000).

The fundamental component of the virion is a nucleoprotein core with the ability to infect host cells and replicate in them, thus ensuring continued survival. The genome of vertebrate viruses is enclosed within a shell of proteins called a capsid (Fig. 54.3). It is haploid except in retroviruses, in which it is diploid. The term nucleocapsid is used to describe the packaged form of the genome in the capsid. Each subunit of the capsid is composed of a folded polypeptide chain. Collections of these subunits constitute structural units or protomers which in turn comprise assembly units. The term capsomer or morphological unit is used to describe features such as protrusions seen on the surface of virus particles in electron micrographs. These often correspond to groups of protein subunits arranged about a local axis of symmetry. Capsids are therefore composed of multiples of one or more types of protein subunits. The orderly arrangement of similar protein–protein interfaces results in a symmetrical structure. Icosahedral and helical symmetries are the two types of capsid symmetry described in viruses (Fig. 54.4).

Closed-shell virions, isometric viruses, have structures based on icosahedral symmetry, a structural form which offers the maximum capacity and greatest strength for a given surface area. The icosahedron, one of the five platonic solids, has 20 equilateral triangles forming its faces, 30 edges and 12 vertices. At its simplest, a viral icosahedron has 60 identical structural units, three in each triangular face. Larger numbers of smaller units are accommodated on the triangular faces only in specific multiples of 60, represented by the formula $T = h^2 + hk + k^2$ where h and k are integers having no common factors and T is the triangulation number. For caliciviruses, $T = 3$ and for herpesviruses, $T = 16$. The structural units at each vertex form groups of five termed pentons, while those on the faces form groups of six called hexons. There are two-fold, three-fold and

five-fold axes of rotational symmetry which pass through edges, faces and vertices, respectively. Viruses with icosahedral symmetry are often not seen as icosahedrons; they may appear in electron micrographs as spheres or hexagons. Icosahedral capsids are generally assembled in the host cell prior to incorporation of the viral nucleic acid. Some viral preparations may contain capsids devoid of nucleic acid. The nucleic acid of double-stranded DNA viruses is condensed into a form suitable for incorporation into capsids by the action of cellular histones and basic virus-encoded molecules. The protective capsid of many RNA viruses is formed by the insertion of protein units between each turn of the nucleic acid helix, incorporating the RNA in the tubular package. As a result, the capsid protein helix coincides with that of the nucleic acid; the length of the helix is determined by the length of the RNA molecule. In RNA viruses, each capsomere consists of a single polypeptide molecule.

Figure 54.3 Diagrammatic representation of capsomers forming the protective icosahedral capsid surrounding the viral nucleic acid.

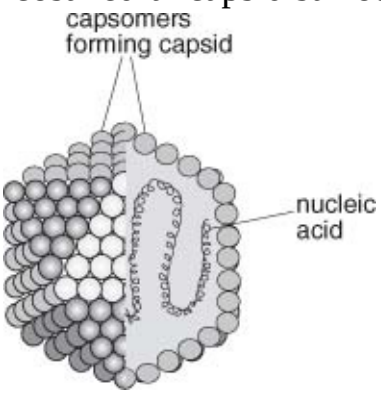
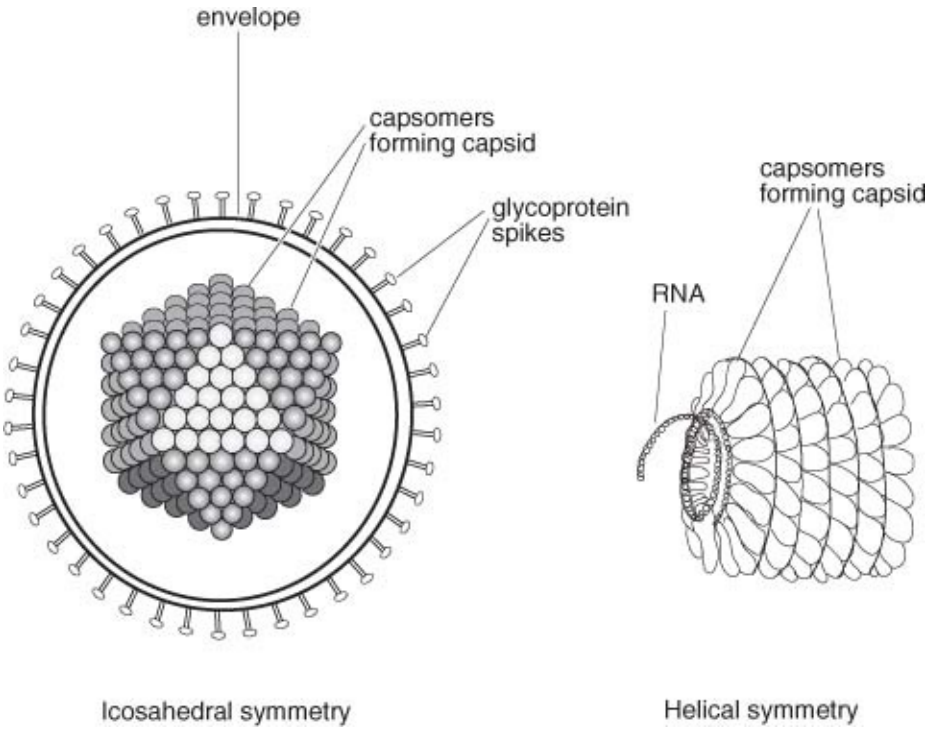


Figure 54.4 Diagrammatic representation of icosahedral and helical symmetry.



In many types of viruses, the nucleocapsid is covered by an envelope composed of a lipid bilayer and associated glycoproteins. The envelope is acquired when the nucleocapsid buds through a cellular membrane, usually the plasma membrane. In some viral infections, the envelope is acquired from the endoplasmic reticulum, the Golgi apparatus or the nuclear membrane. Proteins encoded by viral nucleic acid and integrated as glycoprotein into the appropriate membrane by the compartmentalization mechanisms of the host cell are an integral part of the viral envelope. These glycoproteins are associated with binding to receptors on host cells, membrane fusion, uncoating of the virion and destruction of receptors on host cells. A single envelope glycoprotein may have multiple functions. In most enveloped viruses, the envelope must be intact to maintain infectivity and treatment with lipid solvents such as ether or chloroform renders them non-infectious. Epitopes on envelope glycoproteins are often important for inducing protective immune responses in infected animals. Peplomers or spikes are knob-like projections from the envelope in certain viruses including coronaviruses, retroviruses, orthomyxoviruses, rhabdoviruses and paramyxoviruses These structures are formed from oligomers of surface glycoproteins. They often bind to cell receptors and, in addition, may have enzymatic activity. A layer of protein, termed matrix protein, is present between the nucleocapsid and the envelope in some enveloped viruses. This layer provides additional rigidity to the virion. Helical RNA viruses of animals are

enveloped.

Taxonomy of viruses

The sole experimental indication of the minute size of viruses at the end of the nineteenth century was their ability to pass through filters which retained bacteria. Information relating to viruses derived largely from studies on the diseases which they caused. Early classification systems were therefore based on their pathogenic effects and transmission patterns. In the 1930s, details of the structure and composition of viruses began to emerge. Subsequently, it was possible to group viruses on the basis of shared features of virions. During the 1950s and 1960s, several classification schemes were adopted. The International Committee on Nomenclature of Viruses (ICNV) was established in 1966 to develop a single universal taxonomic scheme. The successor to the ICNV, the International Committee on Taxonomy of Viruses (ICTV), established in 1973, developed and expanded the universal scheme in which virion characteristics are used to assign viruses to five main hierarchical levels, namely order, family, subfamily, genus and species (Tables 54.3 to 54.8). Virus orders are designated by the suffix -*virales*. In orders, phylogenetically related families are grouped together. Four orders containing viruses of animals have been defined thus far. These are the *Mononegavirales* comprising the families *Paramyxo-viridae, Rhabdoviridae, Bornaviridae* and *Filoviridae*; the *Picornavirales* comprising the families *Picornaviridae, If laviridae, Dicistroviridae, Marnaviridae* and *Secoviridae*; the *Herpesvirales* comprising the families *Herpesviridae, Alloherpesviridae* and *Malacoherpesviridae*; and the *Nidovirales* comprising the families *Coronaviridae, Arteriviridae* and *Roniviridae*. It has been proposed that a new order, *Retrovirales*, be created comprising the families *Retroviridae, Hepadnaviridae, Caulimoviridae, Pseudoviridae* and *Metaviridae*. Families are therefore designated by the suffix-*viridae*. Of more than 70 families currently recognized, about 22 contain viruses of veterinary importance. The suffix -*virinae* denotes a subfamily. Viral genera are designated by the suffix -*virus*. More than 280 genera are recognized. The criteria for defining a genus differ from family to family. The species taxon is regarded as the most important level in the classification of viruses. However, its definition and application have always been difficult and controversial. In 1991, the ICTV accepted the definition of a virus species proposed by van Regenmortel (1990) which states that ‘a virus species is defined as a polythetic class of viruses that constitutes a

replicating lineage and occupies a particular ecological niche’. This implies that a virus species is defined by a combination of multiple properties and characteristics; no single or unique property is essential for species definition. This comprehensive type of taxonomy is the Adansonian system of classification. In the present scheme of virus taxonomy, the primary delineating criteria are the type and nature of the genome, the mode and site of viral replication and the structure of the virion. Currently, more than 1,900 virus species are recognized by the ICTV, with the periodic inclusion of new species. In addition, international specialist groups monitor large numbers of strains and subtypes. These latter categories have become accepted for practical reasons such as vaccine development and the diagnosis of disease. Universal definitions or formal nomenclature for strains and subtypes of virus species are not recognized internationally.

Table 54.3 Families containing double-stranded DNA viruses of vertebrates.

Family	Subfamily	Genus	Type species
Adenoviridae		*Mastadenovirus*	*Human adenovirus C*
		Aviadenovirus	*Fowl adenovirus A*
		Atadenovirus	*Ovine adenovirus D*
		Siadenovirus	*Frog adenovirus*
Asfarviridae		*Asfivirus*	*African swine fever virus*
Herpesviridae	*Alphaherpesvirinae*	*Simplexvirus*	*Human herpesvirus 1*
		Varicellovirus	*Human herpesvirus 3*
		Mardivirus	*Gallid herpesvirus 2*
		Iltovirus	*Gallid herpesvirus 1*
	Betaherpesvirinae	*Cytomegalovirus*	*Human herpesvirus 5*
		Muromegalovirus	*Murid herpesvirus 1*
		Roseolovirus	*Human herpesvirus 6*
		Proboscivirus	*Elephantid herpesvirus 1*
	Gammaherpesvirinae	*Lymphocryptovirus*	*Human herpesvirus 4*
		Rhadinovirus	*Saimiriine herpesvirus 2*
		Macavirus	*Alcelaphine herpesvirus 1*
		Percavirus	*Equid herpesvirus 2*
Alloherpesviridae	*Unnamed*	*Ictalurivirus*	*Ictalurid herpesvirus 1*
		Salmonivirus	*Salmonid herpesvirus 1*
		Batrachovirus	*Ranid herpesvirus 1*
		Cyprinivirus	*Cyprinid herpesvirus 3*
Papillomaviridae		*Alphapapillomavirus*	*Human papillomavirus-32*
		Betapapillomavirus	*Human papillomavirus-5*
		Gammapapillomavirus	*Human papillomavirus-4*
		Deltapapillomavirus	*European elk papillomavirus*
		Epsilonpapillomavirus	*Bovine papillomavirus-5*
		Zetapapillomavirus	*Equine papillomavirus-1*
		Etapapillomavirus	*Fringilla coelebs papillomavirus*
		Thetapapillomavirus	*Psittacus erithacus timneh papillomavirus*
		Iotapapillomavirus	*Mastomys natalensis papillomavirus*
		Kappapapillomavirus	*Cottontail rabbit papillomavirus*
		Lambdapapillomavirus	*Canine oral papillomavirus*
		Mupapapillomavirus	*Human papillomavirus-1*
		Nupapapillomavirus	*Human papillomavirus-41*
		Xipapapillomavirus	*Bovine papillomavirus-3*
		Omikronpapillomavirus	*Phocoena spinipinnis papillomavirus*
		Pipapapillomavirus	*Hamster oral papillomavirus*
Poxviridae	*Chordopoxvirinae*	*Orthopoxvirus*	*Vaccinia virus*
		Parapoxvirus	*Orf virus*
		Avipoxvirus	*Fowlpox virus*
		Capripoxvirus	*Sheeppox virus*
		Leporipoxvirus	*Myxoma virus*
		Suipoxvirus	*Swinepox virus*
		Molluscipoxvirus	*Molluscum contagiosum virus*
		Yatapoxvirus	*Yaba monkey tumour virus*
		Cervidpoxvirus	*Deerpox virus W-848-83*

Table 54.4 Families containing single-stranded DNA viruses of vertebrates.

Family	Subfamily	Genus	Type species
Circoviridae		*Circovirus*	*Porcine circovirus-1*
		Gyrovirus	*Chicken anaemia virus*
Parvoviridae	*Parvovirinae*	*Parvovirus*	*Minute virus of mice*
		Erythrovirus	*Human parvovirus B19*
		Dependovirus	*Adeno-associated virus-2*
		Amdovirus	*Aleutian mink disease virus*
		Bocavirus	*Bovine parvovirus*

Table 54.5 Reverse transcribing DNA and RNA viruses of vertebrates (reversiviruses).

Family	Subfamily	Genus	Type species
Retroviridae	*Orthoretrovirinae*	*Alpharetrovirus*	*Avian leukosis virus*
		Betaretrovirus	*Mouse mammary tumour virus*
		Gammaretrovirus	*Murine leukaemia virus*
		Deltaretrovirus	*Bovine leukaemia virus*
		Epsilonretrovirus	*Walleye dermal sarcoma virus*
		Lentivirus	*Human immunodeficiency virus 1*
	Spumaretrovirinae	*Spumavirus*	*Simian foamy virus*
Hepadnaviridae		*Orthohepadnavirus*	*Hepatitis B virus*
		Avihepadnavirus	*Duck hepatitis B virus*

Table 54.6 Families containing double-stranded RNA viruses of vertebrates.

Family	Subfamily	Genus	Type species
Birnaviridae		*Avibirnavirus*	*Infectious bursal disease virus*
		Aquabirnavirus	*Infectious pancreatic necrosis virus*
Reoviridae	*Sedoreovirinae*	*Orbivirus*	*Bluetongue virus*
		Rotavirus	*Rotavirus A*
	Spinareovirinae	*Orthoreovirus*	*Mammalian orthoreovirus*
		Coltivirus	*Colorado tick fever virus*
		Aquareovirus	*Aquareovirus A*

The ease with which base sequencing can be carried out on viral nucleic acids has revolutionized approaches to virus taxonomy and phylogeny. Reference genome sequences are available for all viral taxa in databases such as GenBank (http://www.ncbi.nlm.nih.gov/GenBank), which permit substantial shortcuts to specific taxonomic placements. In addition, the use of statistical methods for comparing sequence similarities has contributed to studies on the evolution of viruses. The ICTV intends to reserve the hierarchical level or order solely for the recognition of phylogenetic relationships. Recently, interest has focused on the study of virus evolution in real time at the subspecies level. Particular attention has been given to the frequency of mutation in a number of RNA viruses whereby, on average, every progeny virus will differ by one mutation and, as a consequence of this, a given viral population will comprise a heterogeneous

assortment of mutants. This phenomenon has resulted in the development of the concept of viral quasispecies (Eigen, 1993). This concept envisages that each virus quasispecies exists as a genetically diverse, rapidly evolving population of virions, with non-identical but closely related mutant and recom-binant viral genomes represented by a complex mutant spectrum and a consensus (average) nucleotide sequence. The consensus sequence may not physically exist in the mutant spectrum. A viral quasispecies may be dominated by one or several master (dominant) sequences, which generally have a selective advantage and may or may not coincide with the consensus sequence. The population as a whole, the quasispecies, is the unit of selection and acts as a genetic pool which is subject to a continuous process of variation, competition and selection. The population is in dynamic equilibrium, with the expansive force of mutation balanced by the constraining force of selection. The survival of the quasispecies depends on the stability of the consensus sequence, the complexity of the information in the genome and the copying fidelity If an advantageous mutant appears, the original quasispecies will be substituted by a new one, characterized by a new consensus sequence and a new distribution of mutants (mutant spectrum). This concept is particularly important with regard to RNA viruses with larger genomes. Because a cellular proof-reading mechanism for RNA is lacking, error rates during replication of viral RNA are much higher than those in replicating viral DNA. As a result, non-lethal mutations tend to accumulate in the genome of RNA viruses. It has been estimated that RNA viruses can evolve up to one million times faster than DNA viruses. However, high polymerase error rates impose an upper limit on genome size due to the ‘error threshold’,a critical average error rate. Above this threshold a virus cannot maintain sequence integrity. As a result, the genomes of RNA viruses are restricted to about 30 kilobases or less, typically 5 to 15 kb. In contrast DNA viral genomes as large as 800 kb have been described.

Table 54.7 Families containing negative-sense, single-stranded RNA viruses of vertebrates.

Family	Subfamily	Genus	Type species
Bornaviridae		*Bornavirus*	*Borna disease virus*
Bunyaviridae		*Orthobunyavirus*	*Bunyamwera virus*
		Hantavirus	*Hantaan virus*
		Nairovirus	*Dugbe virus*
		Phlebovirus	*Rift valley fever virus*
Orthomyxoviridae		*Influenzavirus A*	*Influenza A virus*
		Influenzavirus B	*Influenza B virus*
		Influenzavirus C	*Influenza C virus*
		Thogotovirus	*Thogoto virus*
		Isavirus	*Infectious salmon anaemia virus*
Paramyxoviridae	*Paramyxovirinae*	*Respirovirus*	*Sendai virus*
		Morbillivirus	*Measles virus*
		Rubulavirus	*Mumps virus*
		Avulavirus	*Newcastle disease virus*
		Henipavirus	*Hendra virus*
	Pneumovirinae	*Pneumovirus*	*Human respiratory syncytial virus*
		Metapneumovirus	*Avian metapneumovirus*
Rhabdoviridae		*Lyssavirus*	*Rabies virus*
		Vesiculovirus	*Vesicular stomatitis Indiana virus*
		Ephemerovirus	*Bovine ephemeral fever virus*
		Novirhabdovirus	*Infectious haematopoietic necrosis virus*

In formal viral nomenclature, the names of families, subfamilies, genera and species are italicized. The first letter of each name is upper case. Prior to the 1998 meeting in San Diego of the ICTV, neither capital letters (with the exception of species names derived from place names) nor italics were used for species names. Lower case and plain script are always used for informal names of viruses. The informal designation is commonly used for virus species, whereas formal designations tend to be reserved for taxonomic references. Confusion may arise when the same informal designation is applied both to a family and to a genus. For example, coronavirus may refer either to all members of the family *Coronaviridae* or to those of the genus *Coronavirus only* Terms based on virus tropisms or modes of transmission are also used because of their convenience in categorizing viruses. Examples include enteric viruses, respiratory viruses, arboviruses and oncogenic viruses. The term ‘arbovirus’ relates to the fact that the virus is ‘arthropod borne’ Included in this category are viruses in the families *Togaviridae*, *Flaviviridae*, *Rhabdoviridae*, *Reoviridae*, *Asfarviridae* and *Bunyaviridae*. Oncogenic viruses, which have the potential to induce transformation of host cells, are found in the families *Retroviridae*, *Papillomaviridae*, *Adenoviridae* and *Herpesviridae*

Table 54.8 Families containing positive-sense, single-stranded RNA viruses of vertebrates.

Family	Subfamily	Genus	Type species
Arteriviridae		*Arterivirus*	*Equine arteritis virus*
Astroviridae		*Mamastrovirus* *Avastrovirus*	*Human astrovirus* *Turkey astrovirus*
Caliciviridae		*Vesivirus* *Lagovirus* *Norovirus* *Sapovirus* *Nebovirus*	*Vesicular exanthema of swine virus* *Rabbit haemorraghic disease virus* *Norwalk virus* *Sapporo virus* *Newbury-1 virus*
Coronaviridae	*Coronavirinae*	*Alphacoronavirus* *Betacoronavirus* *Gammacoronavirus*	*Alphacoronavirus 1* *Murine coronavirus* *Avian coronavirus*
	Torovirinae	*Torovirus* *Bafinivirus*	*Equine torovirus* *White bream virus*
Flaviviridae		*Flavivirus* *Hepacivirus* *Pestivirus*	*Yellow fever virus* *Hepatitis C virus* *Bovine viral diarrhoea virus 1*
Picornaviridae		*Enterovirus* *Hepatovirus* *Cardiovirus* *Aphthovirus* *Parechovirus* *Erbovirus* *Kobuvirus* *Teschovirus* *Avihepatovirus* *Sapelovirus* *Tremovirus* *Senecavirus*	*Human enterovirus C* *Hepatitis A virus* *Encephalomyocarditis virus* *Foot-and-mouth disease virus* *Human parechovirus* *Equine rhinitis B virus* *Aichi virus* *Porcine teschovirus* *Duck hepatitis A virus* *Porcine sapelovirus* *Avian encephalomyelitis virus* *Seneca Valley virus*
Togaviridae		*Alphavirus* *Rubivirus*	*Sindbis virus* *Rubella virus*

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Further reading

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Chapter 55

Replication of viruses

Unlike bacteria, which can grow on inert media, viruses are obligate intracellular parasites and can multiply only in host cells. This requirement arises from their limited genomic composition which obliges them to utilize various combinations of host cell transcriptional and translational machinery, organelles, enzymes and other macromolecules for replication. The effects of viral multiplication on host cells range from minor to major changes in cellular metabolism to transformation, persistent infection and cytolysis. Studies of viral reproduction are usually conducted in synchronously-infected cell cultures. In these cultures, cells are infected with an excess of virus particles to ensure that a single synchronous cycle of virus replication takes place. The duration of the cycle may range from 6 to 40 hours. Within hours of infection, an eclipse phase occurs, when virions are not detectable by methods such as virus assay or electron microscopy. After this eclipse phase, virions are demonstrable both intracellularly and extracellularly. The number of viral particles increases exponentially. Fully assembled virions are released from infected cells either by budding or by cytolysis. The number of virions released is largely dependent on the species of infecting virus and on the host cells and may approach many thousands.

The replicative cycle of a virus can be conveniently divided into a number of stages: attachment and entry into the cell, uncoating of viral nucleic acid, synthesis of virus-specific proteins, production of new viral nucleic acid and assembly and release of newly formed viruses from the host cell (Box 55.1). A virion must first attach to cell surface receptors in order to produce infection. Initial virus–cell interaction is a randomevent, which relates to the number of virus particles present and the availability of appropriate receptor molecules. Virus–cell interaction determines both the host range and the tissue tropism of viral species. Different viruses have evolved to the point where they can utilize a wide range of host cell surface proteins as receptors. Certain groups of surface molecules appear to be preferred as receptors including proteoglycans,

glycoconjugates with terminal sialic acid residues, integrins and the IgG superfamily of transmembrane proteins. Many of these surface molecules are highly conserved and are essential for fundamental cellular functions. Some viruses have more than one type of ligand molecule and they may bind to several cell surface receptors in sequential order during attachment. Several viruses undergo an initial, non-specific binding, which helps to concentrate virions at a cell surface, prior to binding to more specific receptors. Adenoviruses bind to a primary receptor via a knob-like protein structure on the end of the projecting fibres at the vertices of the capsid. As a result, the fibre becomes distorted, bringing the virus closer to the cell membrane and permitting the engagement of a second receptor, an integrin, at a site on the penton base. In the case of some species of virus, individual virions can detach and adsorb to another cell when infection of a particular host cell does not proceed. For orthomyxoviruses and paramyxoviruses, detachment of virus is mediated by viral neuraminidase, a receptor-destroying enzyme.

Box 55.1 Stages in virus replication.

- Attachment to a surface receptor on a susceptible host cell
- Entry into the cell
- Uncoating of viral nucleic acid
- Replication of viral nucleic acid and synthesis of virus encoded proteins
- Assembly of newly formed virus particles and release from host cell

Virus uptake or penetration is an energy-dependent process which can occur in a number of ways. Receptor-mediated endocytosis is a mechanism which is frequently used for cell entry by non-enveloped viruses. The most common endocytic pathway is the clathrin-mediated endocytic pathway, which is normally used by cells to internalize receptor-bound ligands, fluid, lipids and membrane proteins. This pathway involves pre-existing or induced clathrin-coated pits, which invaginate into the cell and are pinched off, forming endocytotic vesicles which contain the virus-receptor complex. The cage-like lattice formed around the vesicles by clathrin molecules breaks down soon after endocytosis and the vesicle becomes competent to fuse with an early endo-some. The increasing acidification of the endosome as it becomes a late endosome frequently leads to conformational change in the virus and helps to facilitate penetration into the cytosol. The envelopes of several viruses, such as orthomyxoviruses, rhabdoviruses and flaviviruses, fuse with the membranes of

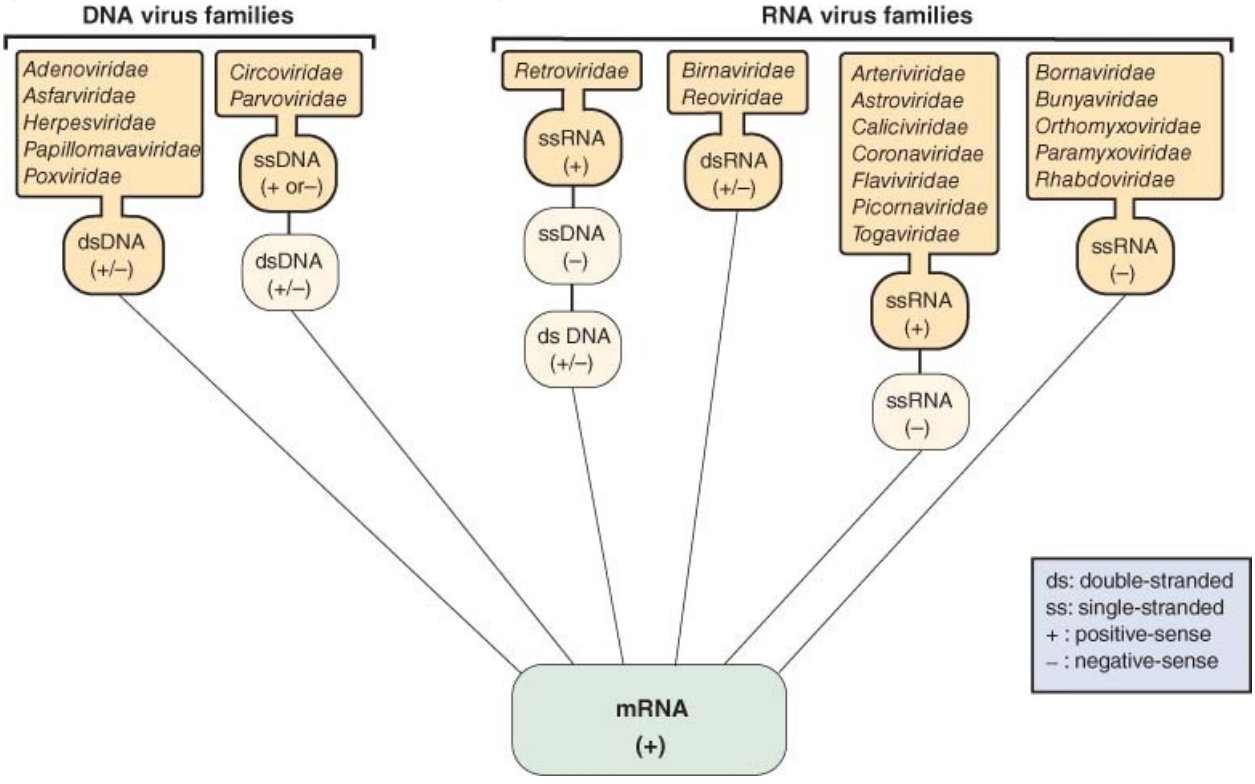
endosomes, releasing nucleocapsids directly into the cytoplasm. Eventual degradation of viral structures remaining in the late endosome occurs following fusion with a lyso-some. In rare instances, lysosomes may play a part in penetration. Alternative, endocytic pathways utilized by viruses include caveolae-mediated and raft-mediated pathways. A particular virus may use different endocytic pathways depending on cell type, virus multiplicity and conditions of growth. An alternative strategy, which may be used by enveloped viruses including paramyxoviruses, retroviruses and herpes-viruses, involves direct fusion of the viral envelope with the plasma membrane. This allows release of the nucleocapsid directly into the host cell cytoplasm. Mechanisms employed by non-enveloped viruses to penetrate into the cytosol through the cell membrane or through the limiting membrane of an organelle such as an endosome or a caveosome are incompletely understood but include: (a) puncture, which involves the direct introduction or translocation of the viral genome into the cytoplasm through channels or pores generated by the virus (picornaviruses) in the plasma membrane; (b) perforation, which involves transfer of the intact virus without lysis of the membrane (parvo-viruses); (c) lysis, such as occurs when adenoviruses are released from endosomes by acid-activated rupture of the limiting membrane.

Uncoating is the process whereby the viral genome is released in a form suitable for transcription and may involve partial or full disassembly. In the case of enveloped viruses, in which the nucleocapsid is discharged directly into the cytoplasm, transcription can usually proceed without complete uncoating. In non-enveloped viruses, uncoating is poorly understood but generally involves conformational changes, proteolytic enzyme activity, progressive loss of structural proteins and weakening of intermolecular interactions. In reoviruses, the genome may express all functions without complete release from the capsid. The uncoating of most other non-enveloped viruses proceeds to completion. Poxviruses are uncoated in two stages. The initial stage is mediated by host enzymes, with complete release of viral DNA from the core requiring virus-specified proteins. In some viruses which replicate in the cell nucleus, uncoating may be completed at the nuclear pore complex (NPC). For viruses that replicate in the nucleus, entry into the nucleoplasm may occur via the NPC, or alternatively the virus may wait until the nuclear membrane breaks down during cell division. The main limitation to transport via the NPC is size, with a functional pore diameter of 39 nm. As a result, only the smallest viruses such as parvoviruses can be transported intact, while in the case of larger viruses, a

subviral complex or nucleic acid is transported into the nucleus.

The synthesis of viral proteins by host cells, which is the central event in replication of viruses, requires the production of viral mRNA. Those DNA viruses, the majority of which replicate in the nucleus, can avail of host cell transcriptases to synthesize viral mRNA. Other viruses utilize their own enzymes to generate mRNA. A significant proportion of the genome of RNA viruses is devoted to encoding RNA-dependent RNA polymerases (RdRp), because these enzymes are not available in host cells. The polymerase enzymes function both as a transcriptase to produce viral mRNA and as a replicase to produce progeny viral RNA. Many viruses have evolved strategies which facilitate interference with the activity of cellular mRNA. Viruses direct the synthesis of either a separate mRNA for each gene or a polycistronic mRNA encompassing a number of genes. Eukaryotic cell protein-synthesizing mechanisms, however, translate only monocistronic messages. If a large precursor protein molecule is produced, cleavage into individual proteins is required and each family of viruses employs unique strategies for this purpose.

Figure 55.1 Families of DNA and RNA viruses of veterinary importance grouped according to genome types and pathways for messenger RNA synthesis (modified from Baltimore, 1971).



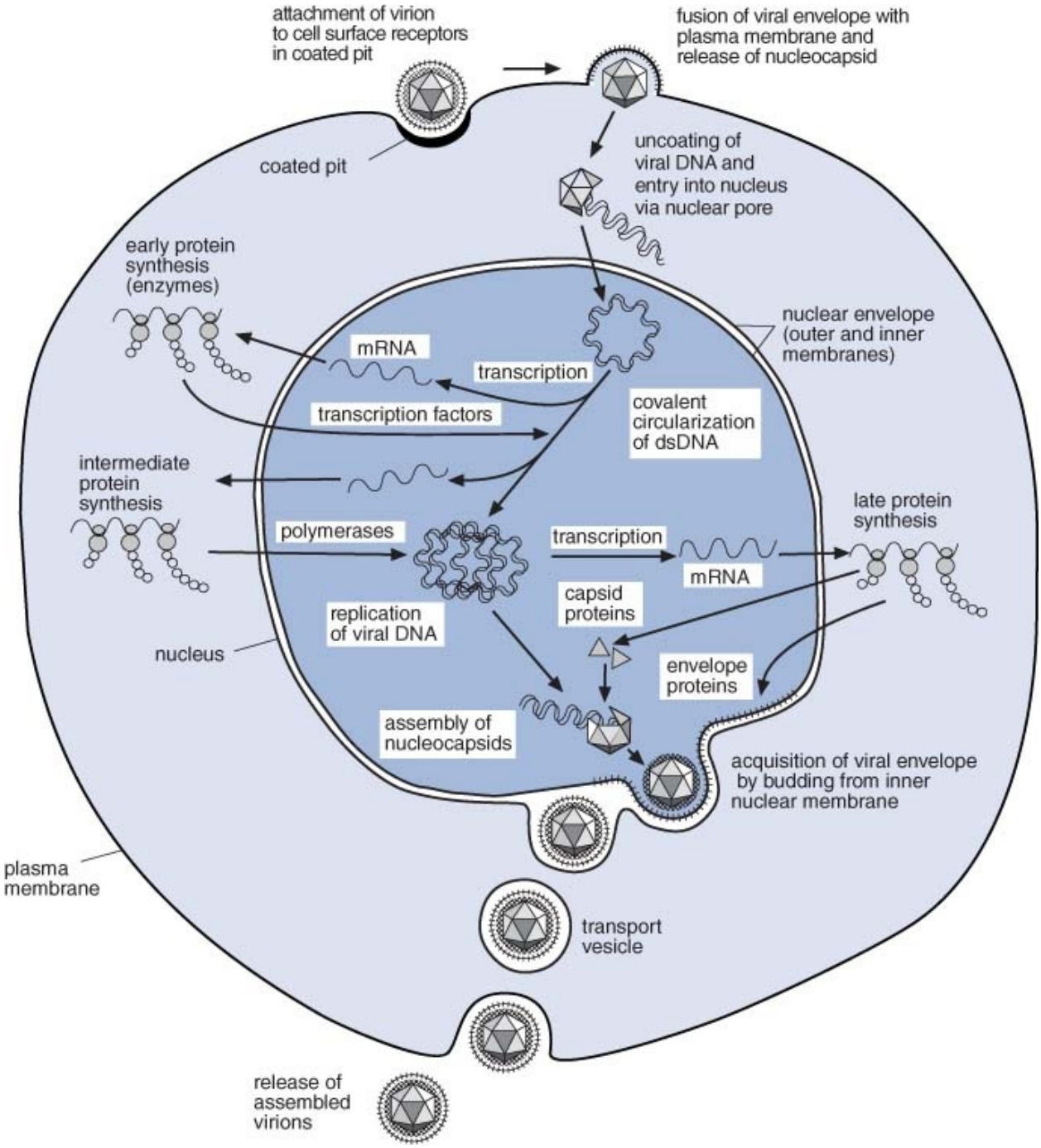
At a molecular level, the diversity of viral genome structures and replication strategies is marked. However, all viruses must express mRNA during the early stages of infection, so that the translational machinery of the cell can be harnessed to make viral proteins. Based on the nature of the genome and the pathways of mRNA synthesis, viruses of veterinary importance can be grouped into six classes (Baltimore, 1971). A seventh class has since been added as a result of the discovery of hepadnaviruses which are reverse-transcribing double-stranded DNA viruses. Central to this scheme is the designation of the genome of single-stranded RNA viruses as positive-sense or negative-sense nucleic acid (Fig. 55.1). In this context, the word ‘sense’ refers to the translated nucleic acid. The nucleic acid of positive-sense single-stranded RNA viruses is mRNA in sense and can be translated directly into virus protein.

In this chapter, specific viruses have been selected to illustrate the replicative mechanisms of both DNA and RNA viruses. However, individual viruses within these two groups frequently exhibit unique replicative methods. Although they have many similarities, the mechanisms involved in the replication of DNA and RNA viruses are complex intracellular events, which require separate consideration.

Replication of DNA viruses

Double-stranded DNA viruses, such as herpesviruses, papillomaviruses and adenoviruses, which replicate in the nucleus of the cell, have a relatively direct replication strategy as the starting material (dsDNA) can be recognized by the host cell machinery. The viral DNA is transcribed by cellular-DNA-dependent RNA polymerase (transcriptase) to form mRNA. In contrast, the single-stranded DNA viruses, parvoviruses and circoviruses, which also replicate in cell nuclei, must first utilize cellular DNA polymerase to synthesize double-stranded DNA. This is then transcribed to mRNA by cellular transcriptases. Because of this transcription requirement, the replication of parvoviruses, small ssDNA viruses encoding very few genes due to size constraints in the genome, is largely confined to rapidly dividing cells. Stages in the replication of a herpesvirus, an enveloped double-stranded DNA virus, are illustrated in Fig. 55.2. Replication of the DNA genomes of poxviruses and African swine fever virus takes place in the cytoplasm of the host cell, as the genomes of these viruses encode enzymes which enable them to replicate in the cytoplasm and, accordingly, are independent of enzymes encoded by the host cell nucleus.

Figure 55.2 Stages in the replication of a herpesvirus, an enveloped, double-stranded DNA virus.



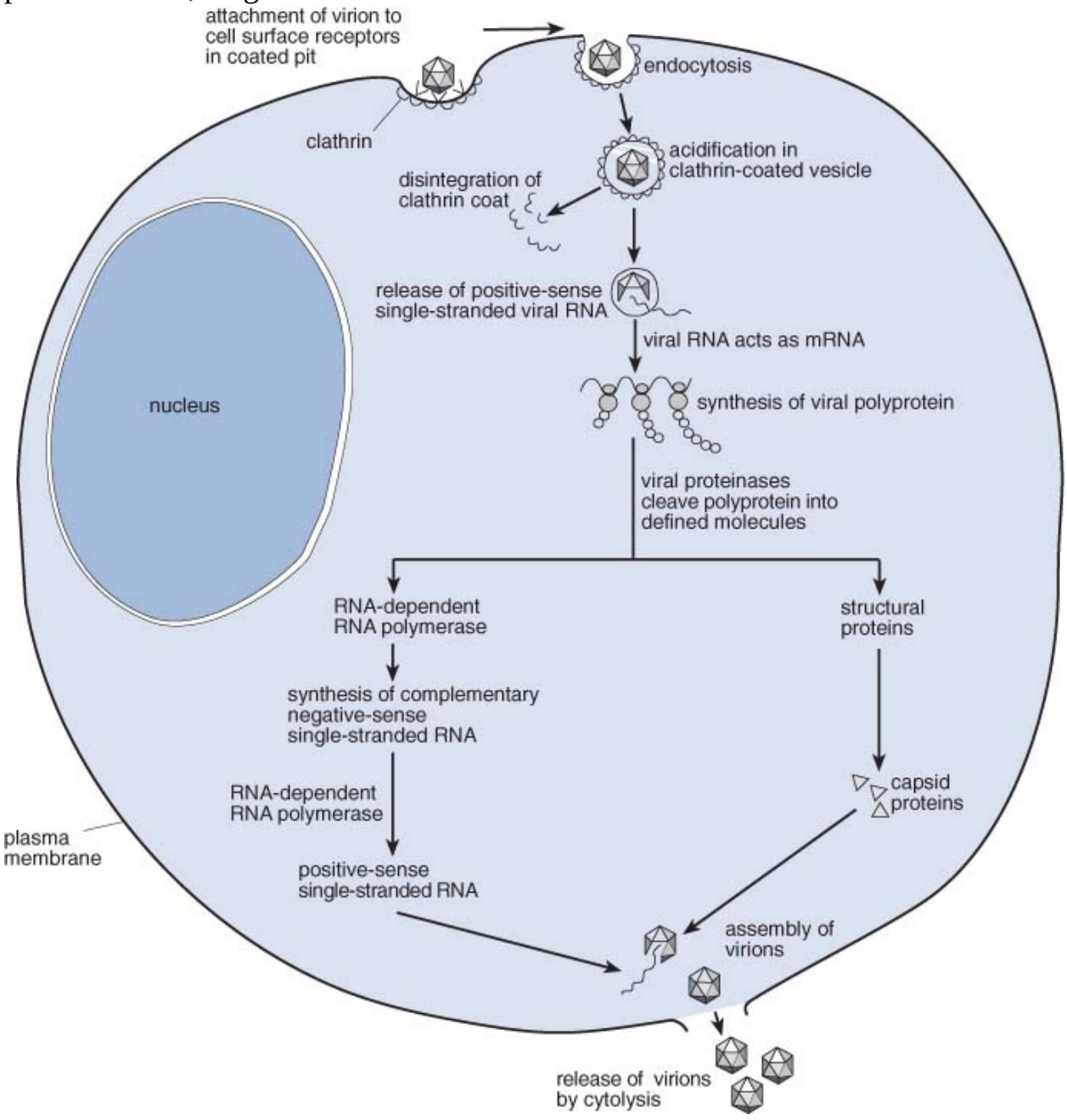
A defined temporal sequence of events occurs during replication and transcription of DNA viruses. Specified genes encode for early proteins, which include the enzymes and other proteins necessary for virus replication and, in some instances, for suppression of the synthesis of host cell proteins. Subsequently, replication of viral nucleic acid and transcription of the genes

which encode the late proteins occur. These late proteins (so called because they are translated following replication of viral DNA), which are also frequently transcribed from newly formed viral nucleic acid, are structural components synthesized late in the infection cycle. This temporal sequence is not clearly demonstrable in the replicative cycles of RNA viruses in which most of the genetic information is expressed contemporaneously. This early/late switch of genes in DNA viruses is considered to be an evolutionary adaptation to competition with the host cell for gene expression. As a result, early gene expression is modest whereas, incontrast, late gene expression occurs following genome replication when the increased gene copy numbers facilitate viral dominance of protein synthesis mechanisms within the host cell. Intermediate gene sets have been identified in poxviruses and herpesviruses.

Replication of RNA viruses

Most RNA viruses replicate in the cytoplasm. However, retroviruses, orthomyxoviruses and bornaviruses replicate in the nuclei of host cells. Reoviruses, orthomyxo-viruses and birnaviruses, double-stranded RNA viruses, have segmented genomes. Transcription occurs in the cytoplasm under the direction of a viral transcriptase. The negative-sense strand of each segment is transcribed to produce individual mRNA molecules. In contrast, the genomes of positive-sense, single-stranded RNA viruses can act directly as mRNA following infection (Fig. 55.3). The enzymes necessary for genome replication in these viruses are produced after infection by direct translation of virion RNA. Taking picornaviruses as an example, this RNA can bind directly to ribosomes and is translated to yield a single polyprotein, which is then cleaved to yield both functional and structural proteins. Because direct translation can occur, naked RNA extracted from positive-sense, single-stranded RNA viruses is infectious. The positive-sense, single-stranded RNA viruses utilize a number of different synthetic pathways during replication. In togaviruses, only about two-thirds of the viral RNA is directly translated during the first round of protein synthesis. Subsequently, full-length negative-sense RNA is synthesized, and from this a full-length positive-sense RNA destined for encapsidation and a one-third-length positive-sense RNA strand are formed. The genomes of caliciviruses, coronaviruses and arteriviruses also encode for mRNA which can be full length or shorter (referred to as subgenomic).

Figure 55.3 Stages in the replication of a picornavirus, a non-enveloped, positive-sense, single-stranded RNA virus.



Negative-sense single-stranded RNA viruses possess an RNA-dependent RNA polymerase. The naked RNA of these viruses, unlike that of the positive-sense single-stranded RNA viruses, cannot initiate infection, thus these viruses need to incorporate their own RdRp. After infection by the virion, the genomic RNA functions as a template for transcription of positive-sense mRNA and also for virus replication, utilizing the same polymerase. The positive-sense RNA

subsequently serves as the template for synthesis of negative-sense genomic RNA. Most singlestranded, negative-sense RNA viruses replicate in the cytoplasm of the cell. Notable exceptions are orthomyxoviruses and Borna disease virus, which replicate in the nucleus. Part of the segmented genome of some members of the *Bunyaviridae* is ambisense, utilizing a mixed replication strategy with features characteristic of both positive-sense and negative-sense single-stranded RNA viruses. Figure 55.4 illus-trates stages in the replication of a rhabdovirus, an enveloped, negative-sense, single-stranded RNA virus.

Figure 55.4 Stages in the replication of a rhabdovirus, an enveloped, negative-sense, single-stranded RNA virus.

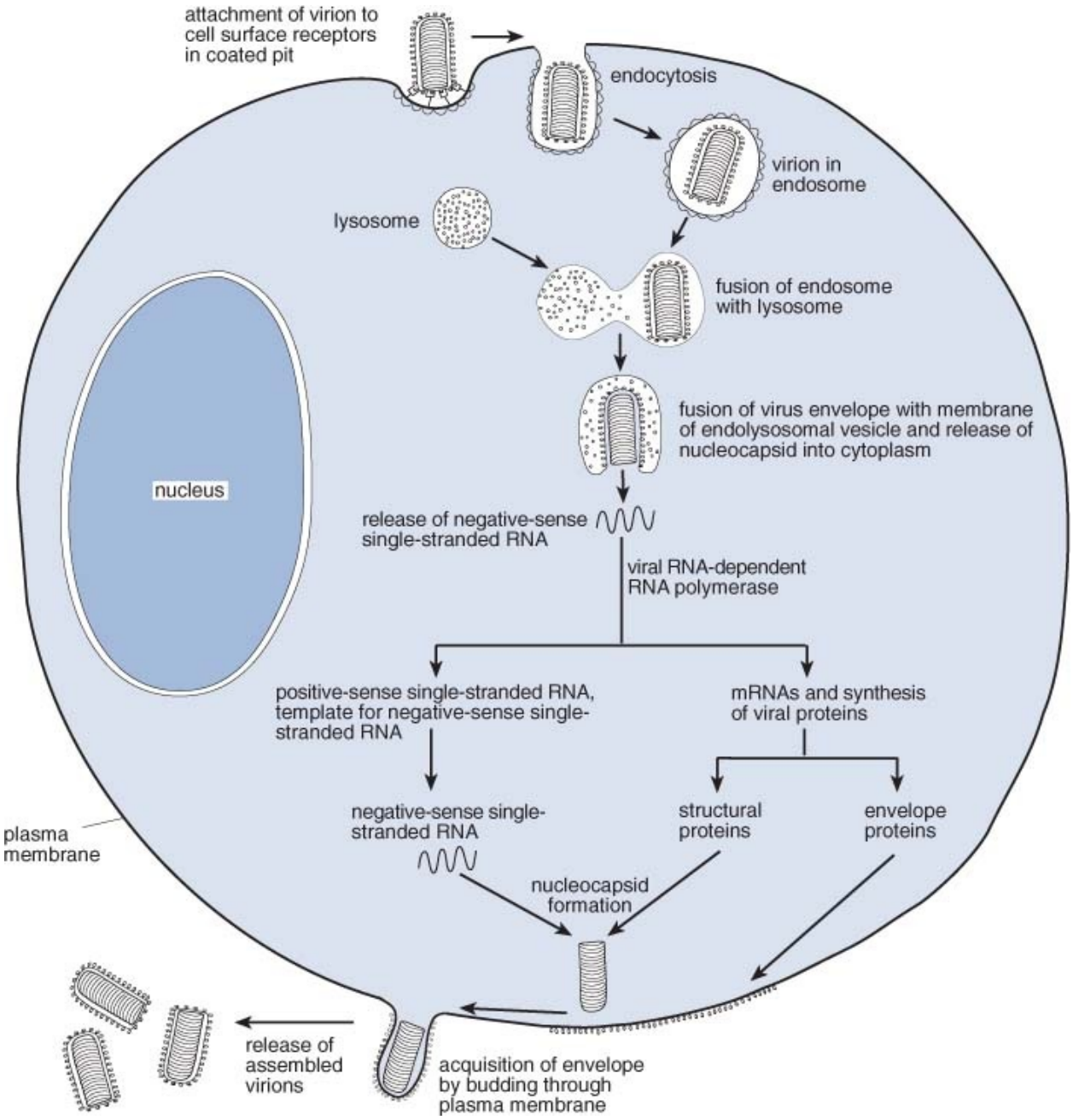
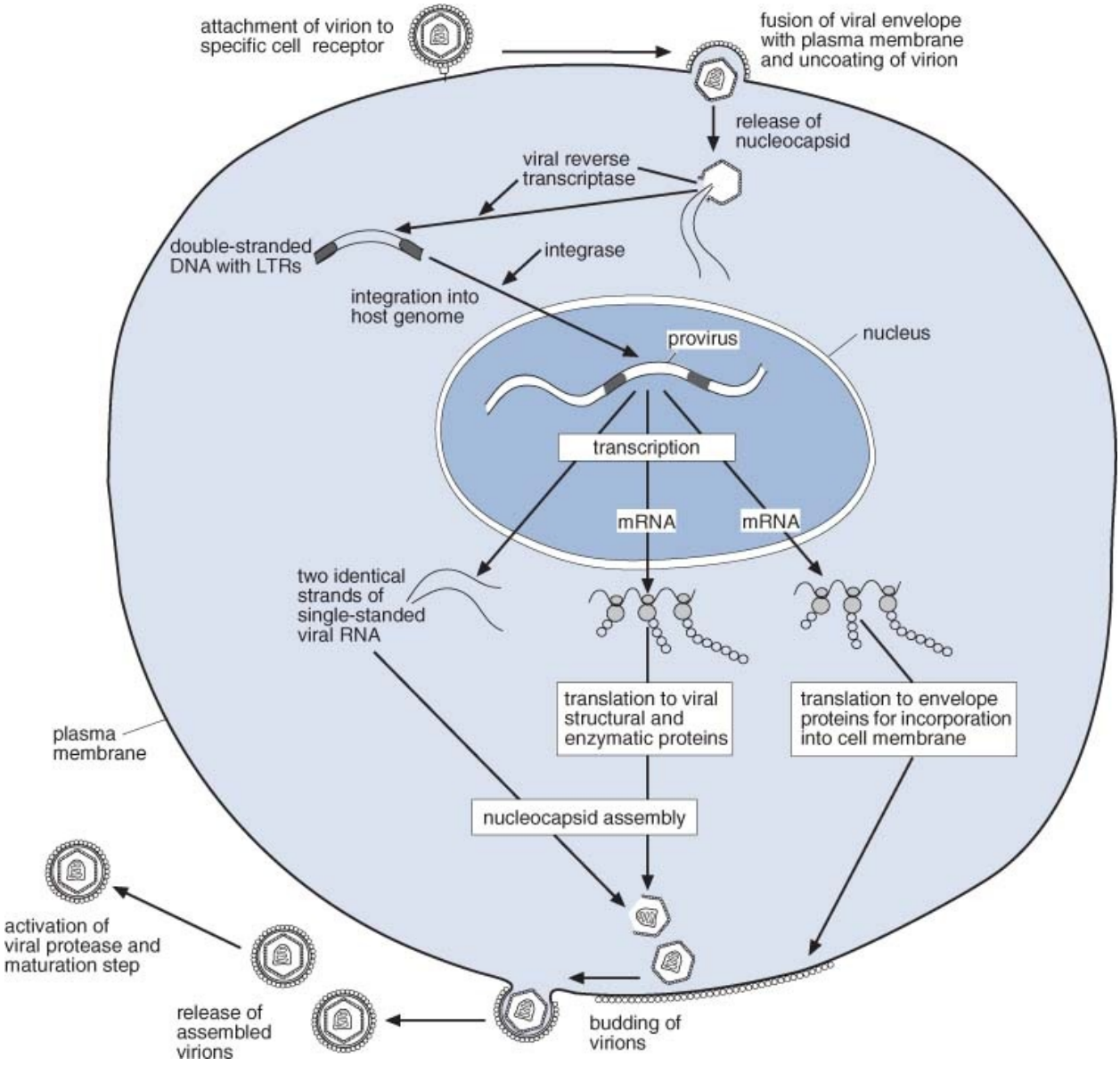


Figure 55.5 Stages in the replication of a retrovirus. LTRs: long terminal repeats.



The genome of retroviruses consists of positive-sense, single-stranded RNA which does not function as messenger RNA. Instead, a single-stranded DNA copy is produced by RNA-dependent DNA polymerase (reverse transcriptase), using the viral RNA as a template. As the second strand of DNA is formed, the parental RNA is removed from the RNA-DNA hybrid molecule. The reverse transcriptase molecule mediates two activities, DNA polymerase activity which incorporates deoxyribonucleotides on either a DNA or an RNA template, and RNase H activity which degrades RNA when it is present in a DNA/RNA duplex form. The resulting double-stranded DNA appears to enter the cell nucleus by one of two mechanisms: following breakdown of the nuclear membrane during mitosis, or through active transport of viral DNA through an intact nuclear

membrane. This may partly explain the requirement of some retroviruses for dividing cells, whereas lentiviruses can successfully infect non-dividing cells. The viral DNA is integrated into the host cell genome as a provirus through the action of DNA integrase (Fig. 55.5). Both enzymes, reverse transcriptase and integrase, are carried into the host cell inside the virion core. The integrated DNA provirus, which may be incorporated into cellular chromosomes at a number of sites, can be transcribed to new viral RNA.

Protein synthesis

Within the cell, the sites at which particular proteins are synthesized relate to the type and function of the protein. Membrane proteins and glycoproteins are synthesized on membrane-bound ribosomes, while soluble proteins including enzymes are synthesized on ribosomes free in the cytoplasm. Short specific amino acid sequences, known as sorting sequences, facilitate the incorporation of proteins at various cellular locations where they are required for metabolic activity. Most viral proteins undergo post-translational modification including proteolytic cleavage, phosphorylation and glycosylation. During glycosylation, sugar side-chains are added to viral proteins in a programmed manner as the proteins are being transferred from the rough endoplasmic reticulum to the Golgi apparatus preparatory to final assembly of intact virions and their release from the cell.

Assembly and release of virions

The mechanisms for the assembly and release of enveloped and non-enveloped viruses are distinct. Non-enveloped viruses of animals have an icosahedral structure. The structural proteins of these viruses associate spontaneously in a symmetrical and stepwise fashion to form procapsids. Subsequently, viral nucleic acid is incorporated into the procapsid. Proteolytic cleavage of specific procapsid polypeptides may be required for the final formation of infectious particles. Non-enveloped viruses are usually released following cellular disintegration (lysis). The assembly of picor-naviruses and reoviruses occurs in the cytoplasm of the cell whereas parvoviruses, adenoviruses and papillomaviruses are assembled in the nucleus.

In enveloped viruses, the final step in the process of virion assembly involves

acquisition of an envelope by budding from cell membranes. Prior to budding, cell membranes are modified by the insertion of virus-specified transmembrane glycoproteins which aggregate in patches in the plasma membrane. The presence of viral glycoproteins alters the antigenic composition of infected cells, which subsequently can become targets for cytotoxic T lymphocytes. Togavirus nucleocapsids bind to the hydrophilic domains of the virus-specified membrane proteins which project slightly into the cytoplasm and become surrounded by the altered portion of membrane. The nucleocapsids of helical viruses bind to a virus-specified matrix protein which lines the cytoplasmic side of membrane patches.

Budding of viruses through the plasma membrane does not usually breach the integrity of the membrane and, as a result, many enveloped viruses are non-cytopathic and may be associated with persistent infections. Assembly of retrovirus virions occurs by two main pathways: for retroviruses with C-type morphology, capsid assembly and envelopment occurs concurrently at the plasma membrane; in the case of capsid assembly of retroviruses with B-type and D-type morphology, it occurs at an intracytoplasmic, pericentriolar assembly site, followed by transportation to the plasma membrane and budding. Non-cytopathic viruses need to avoid rebinding to receptors on the host cell, so that efficient spread to neighbouring target cells can occur. Orthomyxoviruses and para-myxoviruses incorporate a sialidase enzyme in their envelope to promote destruction of terminal sialic acid residues on the cellular receptors used for viral attachment on the host cell surface. In the case of influenza virus, the neuraminidase is critical for virus release as it prevents nascent virions from attaching to the host cell surface receptors and to each other. Retroviruses produce an over-abundance of viral glycoproteins such that cellular receptors are bound by their ligand as they are transported to the cell surface resulting in removal or down-regulation of the receptor. Unlike most other enveloped viruses, togaviruses, paramyxoviruses and rhabdoviruses are cytolytic. Flaviviruses, coronaviruses, arteriviruses and bunyaviruses acquire their envelopes inside cells by budding through the membranes of the rough endoplasmic reticulum or the Golgi apparatus. These viruses are then transported in vesicles to the cell surface where the vesicle fuses with the plasma membrane, releasing the virion by exocytosis Herpesviruses, which replicate in the nucleus, are unique in that they bud through the inner lamella of the nuclear membrane and accumulate in the space between inner and outer lamellae, in the cisternae of the endoplasmic reticulum and in cytoplasmic vesicles (Fig. 55.2). There is still

some uncertainty over the precise mechanism of egress, and three alternative methods have been proposed: single nuclear envelopment, dual envelopment and single cytoplasmic envelopment. It is possible that different pathways are used at different times during the cycle of infection.

Release from the cell can occur either by exocytosis or by cytolysis. The assembly and release of poxviruses is a complex process taking several hours. Although replication occurs entirely in the cytoplasm of the host cell at discrete sites, termed viroplasms or ‘viral factories’, nuclear factors may be involved in transcription and assembly. Maturation proceeds to the formation of infectious intracellular mature virus, which can be detected following deliberate lysis of infected host cells *in vitro*. Following assembly, virus particles move out of the assembly area and become enveloped in a double membrane derived from the trans-Golgi network. At the periphery of the cell, fusion with the plasma membrane results in loss of the outer layer of the double membrane and release of extracellular enveloped virus.

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Further reading

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Chapter 56

Genetics and evolution of viruses

Viruses exhibit enormous genetic diversity. These minute pathogens are capable of infecting not only vertebrates but also invertebrates, plants, fungi, protozoa, algae and bacteria. Viruses utilize a variety of molecular mechanisms to compensate for their limited genetic capability. They may encode genes in different reading frames. These reading frames may overlap, may be encoded in opposite directions or may be read by frameshifting. Some viruses maximize their limited genome size by using subgenomic RNA fractions, where a portion of the RNA is translated into viral proteins. In recent years, the development of techniques in molecular and cell biology, including molecular cloning, nucleotide sequencing and the polymerase chain reaction, have greatly expanded knowledge of viral genomes. Viral genetics is concerned not only with understanding the detailed structure of the viral genome and the extent to which it determines the biological and disease-producing potential of viruses, but also with elucidation of the mechanisms of genetic change and virus evolution. Genomic change is responsible for alterations in antigenicity and pathogenicity which, in turn, may influ-ence the course of viral diseases of animals and humans. Mutation is the most frequent cause of genetic change in viruses. Less commonly, genetic interactions termed recombinations, which can occur between different viruses or between a virus and its host cell, may account for alteration in virus characteristics. This genetic variation has no specific or pre-established purpose and serves to generate repertoires of variant genomes subject to evolutionary selection forces. Virus evolution follows general Darwinian principles.

Mutation

Spontaneous and random errors in the copying of viral nucleic acid, termed mutations, can occur during the replication of viruses. The rate of mutation in the genome of DNA viruses ranges from 10^{-7} to 10^{-11} per incorporated

nucleotide, whereas the mutation rate in the genomes of RNA viruses is much higher, ranging from 10^{-3}. to 10^{-5}. When replicating in the nucleus of host cells, the genomes of DNA viruses are subjected to error correction carried out by cellular exonucleases. Some viral DNA polymerases also include a proofreading-repair activity. The lower fidelity of genome replication in RNA viruses is attributed to poor error correction by RNA replication enzymes. In an infected cell, the genomes of the progeny of an RNA virus, such as vesicular stomatitis virus with an 11 kb genome, may differ both from the parental genome and from each other by at least one nucleotide Mutation frequency has implications for genome size. DNA virus genomes are rather stable and can be very large, up to 800 kb. In contrast, the genome size of RNA viruses is typically less than 20 kb because high error rates may exceed an error threshold and result in ‘error catastrophe’. Coronaviruses are exceptional in having a genome of between 27 and 32 kb. RNA virus quasispecies have a mis-incorporation rate per nucleotide per replication of about 1/υ where υ is the length in bases of the genome. As a result, these viruses tend to be near or at their error threshold.

Exposure to X-rays, UV irradiation or chemical mutagens increases the frequency of mutation. Point mutations, resulting from single nucleotide substitutions, are the most common type of mutation. Less common types of mutation result from the deletion or insertion of one or more nucleotides. Additive effects of point mutations, occurring over several generations, may account for phenotypic variations which are influenced by selection processes. Because they frequently do not result in alteration of the amino acid composition of the coded protein, most point mutations are thus silent. In other instances, the mutation may be lethal and virus strains containing such lethal mutations are rapidly eliminated. Occasionally, mutations conferring a selective advantage allow positive selection of the mutant virus. Non-lethal mutations may accumulate rapidly in the genomes of RNA viruses giving rise to quasispecies, genetically diverse populations of viruses centred around consensus sequences. As a result, RNA viruses have enormous adaptive potential because variants are already present or can be rapidly generated. The spectrum of viruses following mutation does not behave as a collection of independent genomes, but rather as a unit of selection. Different components of the mutant spectrum can interact in both a positive (complementation effect) and a negative (suppressive or interfering effect) manner. A new area of antiviral research, termed lethal mutagenesis, exploits the inherently high mutation rates of RNA viruses and involves the administration of mutagenic agents to drive viral extinction through

violation of the error threshold and error catastrophe. Viral suppression is facilitated by a low viral load and low viral fitness. The most promising results have been obtained using a combination of mutagenic agents and antiviral compounds.

Phenotypic expression of a mutation may be reversed by a back-mutation at the nucleotide responsible for the original mutation. Alternatively, a suppressor mutation may prevent phenotypic expression of the mutant gene. The phenotypic expression of a particular mutation may give rise to clearly distinguishable mutants including conditional-lethal, antibody-escape and defective-interfering mutants.

Conditional-lethal mutants can replicate only under defined permissive conditions. Examples of such mutants include temperature-sensitive mutants, which can multiply most efficiently at temperature ranges different from parental wild-type viruses, and host-range mutants, which can infect host species different from those of parent viruses. The temperature-sensitive mutants, which replicate at temperatures slightly below core mammalian body temperature, have been particularly useful in the development of live intranasal vaccines. Such vaccines stimulate local immunity without systemic spread. When first isolated, viruses may grow poorly in cell cultures and in laboratory animals. However, adaptation can usually be achieved by serial passage, resulting in selection of rapidly growing mutants. This selection process is dependent on spontaneous mutations, particularly in genes encoding surface proteins which determine binding efficiency to host cell receptors. Host-range mutants, while often replicating more readily *in vitro*, tend to be less virulent for natural host species. This has been exploited as a method for attenuation in the production of many modified live vaccines.

Selection for antibody escape mutants can occur when viruses replicate in the presence of antibody. Because of altered antigenic surface determinants, the mutants are unaffected by neutralizing antibodies induced by the wild-type virus. Such a selection process may facilitate persistent or recurring infections.

In 1954, von Magnus noted that serial undiluted passage of influenza virus resulted in a significant decrease in infectious titre, despite the number of viral particles remaining constant. This phenomenon is due to the accumulation of defective interfering viral particles in viral stocks. Defective-interfering mutants require the presence of a complementing, helper virus, usually the wild-type virus, for replication. Most defective-interfering viral particles are deletion mutants and display a replication advantage relative to wild-type virus. Such

mutants can interfere with the replication of wild-type helper viruses, presumably by competing more effectively for host cell resources, and may, therefore, become progressively more numerous on serial passage. Defective-interfering mutants may play a role in disease, by promoting the establishment and maintenance of persistent infections.

Viral recombination

The exchange or transfer of genetic material between different but closely related viruses infecting the same cell is termed genetic recombination. This type of genetic exchange can also occur between virus and host cells. The genomes of the recombinants contain new genetic information. The alteration of genetic information may result from intramolecular recombination, copy-choice recombination, reassortment or genetic reactivation.

Intramolecular recombination usually occurs in DNA viruses and involves dissociation and reestablishment of covalent bonds within the nucleic acid in a process similar to that observed in bacteria and higher organisms. Copy-choice (template switching) recombination between positive-sense single-stranded RNA viruses occurs through a template switching mechanism. The RNA polymerase switches between template strands during synthesis of the complementary negative-sense strand. This process can occur in picornaviruses, togaviruses and corona-viruses. An exceptionally high frequency of genetic recombination has been observed in mixed infections of coronaviruses. It is suggested that western equine encephalitis virus arose through copy-choice recombination as a heterologous recombinant of the two toga-viruses, eastern equine encephalitis virus and a Sindbis-like virus. High-frequency recombination in retroviruses occurring, on average, more than once per replication event per genome, is a consequence of the retrovirus genome and replication strategy. Although the retrovirus genome is diploid, only a single provirus is generated. The two copies of the RNA genome are typically involved in the process of reverse transcription. Normally the two RNA molecules in a virion are identical but they may be distinct if derived from two viruses or viral strains resulting in a high frequency of recombination in the resulting proviral DNA. Recombination has played an important role in the variability of the envelope proteins of retroviruses, whereas the polymerase gene seems to have been protected and is highly conserved.

Reassortment can occur randomly in RNA viruses with segmented genomes,

such as orthomyxoviruses, reoviruses and bunyaviruses. In this type of recombination, genome segments of two or more related viruses infecting the same cell are exchanged. The process is an important source of genetic variability in nature, permitting rapid adaptation of viruses to new hosts, the development of viruses with new antigenic characteristics and changes in virulence. Reassortment is generally restricted to taxonomically related viruses, either viruses of the same species, such as orthomyxo-viruses, or viruses belonging to the same serogroup within a species, such as reoviruses and bunyaviruses. Genetic reassortment plays a major role in the epidemiology of human influenza A virus infections. Periodically, viruses with novel antigenic properties emerge, facilitating more rapid spread throughout the human and animal population. There is convincing evidence that dual infection of pig populations with avian and human influenza viruses accounts for the emergence of new virulent subtypes which can spread to the human population from close contact with pigs. In genetic reactivation, infectious progeny are produced from parental viruses, of which one or both are non-infectious, following mixed infection of a cell. When infectious progeny are produced from related viruses inactivated by lethal mutations at different loci in their genomes, the phenomenon is referred to as multiplicity reactivation. Cross-reactivation or genome rescue occurs when an inactivated virus becomes capable of replicating after acquiring genetic material from an infective virus.

Virus–host cell recombination

It is known that recombination between viral and cellular genetic material occurs. Such recombination may be important for virus evolution and virulence. Several retroviruses have become potentially oncogenic by incorporating cellular oncogenes into their genomes. Integration of the cellular DNA into the viral genome frequently results in a concomitant loss of viral genetic material, resulting in replication-defective progeny viruses which require helper viruses for replication. The non-cytopathic biotype of bovine virus diarrhoea virus, which causes persistent infection in cattle, may become cytopathic through a process of RNA recombination and cause mucosal disease. The specific change is cleavage of the non-structural fusion protein, NS2-3, and the subsequent separate expression of NS3. One mechanism that allows this change is insertion of one or more cellular ubiquitin gene sequences into a key region of the viral genome.

Other interactions involving viruses

Viruses may interact in a number of ways at the level of gene products. These types of interaction, which can result in phenotypic alteration of viral activity, include complementation and phenotypic mixing.

In cells with dual infections, complementation can occur if the defective gene product of one virus is substituted by the gene product of another virus. This results in survival or increased yields of the recipient virus. There is no lasting effect from this type of interaction because the genome remains unchanged. Complementation can occur between related and unrelated viruses; the defective viruses are termed satellite viruses. Adeno-associated viruses, which are members of the *Parvoviridae*, can replicate only in the nucleus of cells simultaneously infected with an ade-novirus. Hepatitis delta virus, a satellite virus of humans, requires co-infection with hepatitis B virus for replication, especially for the provision of envelope proteins.

Structural proteins may be exchanged following infection of a cell, usually by two related viruses, a process known as phenotypic mixing. One form of this type of interaction, transcapsidation, involves complete or occasionally partial exchange of capsids between non-enveloped viruses. Phenotypic mixing also occurs in enveloped viruses which acquire envelopes when budding from host cells. The progeny nucleocapsids of one virus may be released from a cell surrounded by an envelope containing glycoproteins specified by another virus. Defective oncogenic retro-viruses, which are dependent on helper viruses for replication, may derive envelopes from host cell membrane which contain glycoproteins encoded by their helper viruses. Phenotypic mixing does not involve genetic change as it is a transient event.

Viral genomic sequence analysis

Cell culture techniques are no longer required for producing sufficient quantities of virus for detailed studies. It is now possible to produce large amounts of viral nucleic acid using either molecular cloning techniques or by use of the polymerase chain reaction following extraction of viral nucleic acid from clinical samples. The nucleic acids of bovine papillomavirus and rabbit haemorrhagic disease virus have been analysed and sequenced without cultivation of these viruses *in vitro*. This has provided valuable information about both viruses, allowing alternative approaches to diagnosis and vaccination procedures. In

recent years, automated methods for sequencing viral genomes have become simpler and cheaper (see also Chapters 6 and 9). Sequences, both partial and complete, of a wide range of microorganisms are retrievable from international databanks such as GenBank (http://www.ncbi.nlm.nih.gov/GenBank).

Viral genome sequencing has been used for many years to help establish phylogenetic relationships among viruses. It has also been used to great effect in the field of molecular epidemiology. The genotyping of isolates provides information on the source and epidemiology of viral diseases of veterinary importance such as foot-and-mouth disease and Venezuelan equine encephalitis. Functional genomics is concerned with the linking of phenotypic traits to specific genes. The identification of open reading frames (ORFs) is relatively easy in viruses because translatable sequences start with the methionine codon (AUG). This facilitates the identification of genes encoding viral structural and non-structural proteins. Information of this type is of considerable value in the development of diagnostic reagents and antiviral drugs and also in the design of novel vaccines (see Chapter 5).

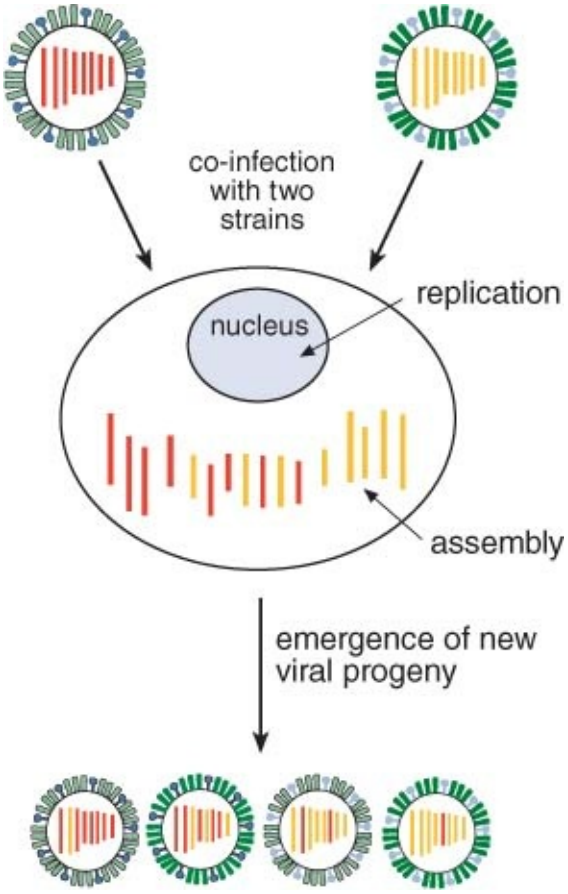
Evolution of viruses

Genetic change, arising from mutation and recombination, occurs at different rates among families of viruses. Some genetically controlled traits may confer a selective advantage which relates to prevailing conditions and selection pressures. Periodically, genomic change may contribute to the emergence of major outbreaks of new diseases. Point mutations in the genome of feline panleukopenia virus or a closely related virus can confer the ability to replicate in canine cells and have been proposed as the event responsible for the emergence of canine parvovirus which causes a serious disease in dogs, first described during the late 1970s. Genomic change may also account for infection of horses with Hendra virus and of pigs with Nipah virus. Both of these paramyxoviruses have wildlife reservoirs, probably fruit bats. Subsequent transmission of these viruses to humans through contact with infected domestic animals illustrates the important unforeseen consequences of viral genomic alteration.

Influenza A virus and myxoma virus provide important insights into the evolution of viruses. Influenza A viruses can infect a wide range of animal species. Isolates of the virus from birds and humans can infect pigs and, in this species, reassortment can occur with the emergence of new subtypes (Fig. 56.1).

Genetic changes of this type, which take place at intervals of more than 10 years, are most likely to originate in south-east Asia because of high population densities and close contact between humans and domestic pigs and ducks. Virus variants produced in this way may escape neutralization by antibodies and, by becoming the dominant circulating strain, cause pandemics. During the twentieth century, pandemics of human influenza occurred in 1900, 1918, 1957, 1968 and 2009. The pandemics of 1957 ('Asian influenza') and 1968 ('Hong Kong influenza') were caused by reassortant viruses that contained genetic segments of avian origin in a human influenza genetic background. In 2009 a new strain of influenza A virus swept through human populations around the world. Genetic studies indicated similarities to swine influenza isolates and pigs are susceptible to infection but circulation of the virus has occurred primarily in the human population (Vijaykrishna *et al*., 2010).

Figure 56.1 Reassortment of influenza A viruses following co-infection of a host cell with two different strains (8 genetic segments of RNA in each virus). Following the reassortment of these two strains, up to 2^8 or 256 different genotypes may be formed.



Myxoma virus is a poxvirus that causes a mild infection in *Sylvilagus* species, the native American rabbit. It is transmitted mechanically by biting arthropods feeding on infected animals, particularly fleas and mosquitoes. The virus causes serious, often fatal, disease in European rabbits, *Oryctolagus cuniculus*. The European rabbit, which had been introduced into Australia in the mid-nineteenth century, became a major agricultural pest. Myxoma virus was released into the Australian rabbit population in 1950 in an attempt to control numbers. Case fatality rates of more than 99% during dramatic summertime epizootics were initially recorded. The disease tended to disappear during winter months, due to the greatly reduced rabbit numbers and decreased mosquito activity. In such circumstances less virulent viral mutants, which caused a more prolonged disease course with a greater opportunity for transmission, were selected. Subsequently, attenuated strains of the virus became dominant. This resulted in a recovery rate in infected rabbits exceeding 10%, with the selection of genetically more resistant rabbits. The case fatality rate for a particular strain of virus in serial infection studies on rabbits from areas where repeated outbreaks occurred declined from 90% to 50%. However, this trend to avirulence did not continue and myxomatosis has not become a benign disease in European rabbits. It was found that a level of virulence associated with replication was required to ensure sufficiently high levels of virus in the skin to permit transmission to occur. Virulent strains of the virus became re-established in the genetically resistant populations, ensuring further efficient transmission of the virus. In 1968, the European rabbit flea was released to improve transmission of the virus in areas where mosquitoes had not been effective vectors. In rabbit populations in which this flea flourished, the seasonal incidence of myxomatosis changed from sharp summer outbreaks to protracted winter and spring outbreaks. A dynamic equilibrium currently exists between virulence of the virus and genetic resistance of the rabbit population (Fenner, 2010). However, rabbit numbers are substantially reduced compared with their flourishing population prior to the introduction of myxomatosis.

Thus, despite the restrictions imposed on viruses by their limited genome size, these agents utilize many different mechanisms in order to subvert the host to diversify, evolve rapidly, adapt and survive.

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Chapter 57

Propagation of viruses and virus-cell interactions

Viruses exhibit considerable diversity both in their ability to survive outside the host and in their cultural requirements. Some viruses, such as those causing enteric disease in animals, tolerate wide pH ranges and are relatively stable in the environment. In contrast, other viruses are labile and only survive for short periods outside the host. Enveloped viruses are readily inactivated by lipid solvents, such as chloroform and ether, and by various detergents such as sodium deoxycholate. Viruses are sensitive to ultraviolet and gamma irradiation. Because of their diploid genome, retroviruses are more resistant than other viruses to these forms of radiation. Enveloped viruses are more thermolabile than those without envelopes. As a rule, the rate of inactivation can be measured in seconds at 60°C, in minutes at 37°C, in hours at 20°C and in days at 4°C. Ice crystal formation during freezing damages viruses, especially enveloped viruses. The infectivity of viruses is retained for long periods when they are stored at −70°C or when they are freezedried Storage at −20°C in conventional freezers is an unsatisfactory method for virus preservation. Long-term storage in liquid nitrogen at −196°C can be achieved by rapid freezing of small aliquots of high-titred viral suspensions in a medium containing a high concentration of protective protein or a cryoprotective agent such as dimethyl sulphoxide. Freeze-drying involves dehydration of frozen viral suspensions under vacuum in glass ampoules which are then sealed to preserve the vacuum. Lyophilization is employed for the preservation of valuable seed stocks of viruses and for storage of modified live viral vaccines.

Propagation of viruses

As viruses replicate only in living cells, a source of viable cells is required for their growth. Tissue culture is widely used for virus propagation; inoculation of chick embryos and experimental animals is employed for the isolation and

production of particular viruses. Propagation is required for the isolation and identification of viruses involved in disease, for the titration of viruses for vaccine production and for the provision of stocks for research purposes.

Tissue culture

Techniques for tissue culture, the growth and maintenance of living tissue *in vitro*, can be grouped under two headings: explant cultures and cell cultures. Originally, methods for growing cells involved using small fragments or explants of tissue. This technique is still used for isolating viruses from animals with persistent diseases such as caprine arthritis-encephalitis In a special form of explant culture, termed organ culture, the size and type of the tissue fragment is sufficient to retain tissue architecture. Explant tracheal cultures are required for the isolation of some coronaviruses.

Digestion of tissue into individual cells is used in cell culture preparation. Dispersion of cells from tissue involves mechanical cutting or chopping of the tissue into small pieces followed by digestion, using trypsin or other proteolytic enzymes. The liquid or semi-solid culture medium in which cells are grown must supply the required environmental conditions and nutrients. It must be isotonic and be maintained at physiological pH values. There must also be a supply of inorganic ions, carbohydrate (usually glucose), amino acids, vitamins, growth factors, peptides and proteins. Some cells grow in chemically defined media but usually natural products such as foetal calf serum, yeast extract or embryo extract are incorporated in the medium. Phenol red is commonly added as a pH indicator. A bicarbonate buffer is often used to maintain the correct pH. However, exposure to air in an open system results in an increase in pH. Cells can be grown in sealed containers, or exogenous CO_2 may be provided at a rate of 5 to 10%.

Cell cultures may be primary, semi-continuous or continuous. Primary cell cultures are derived directly from tissues and contain many cell types. Tissues from foetuses or from neonatal animals are more suitable for tissue culture preparation than those from mature animals. For the isolation of a specific virus, the most sensitive systems are primary cell lines derived from the target tissue of the virus in a susceptible animal species. The cells in these cultures retain many of the characteristics of cells in the intact organ. However, the sensitivity of a given cell culture for cultivating viruses does not appear to depend solely on the organ of origin as it is frequently possible to isolate viruses that infect one body

system in cells derived from a different system. The numbers of cell divisions occurring in primary cultures are relatively low. Accordingly, primary cultures must be prepared at frequent intervals. Primary cultures can be passaged by dispersing the primary monolayer with trypsin, usually with added ethylenediamine tetra-acetic acid, and distributing the cells on to fresh surfaces to form secondary cell cultures. The number of further passages is finite and an end point, termed the Hayflick limit, is eventually reached. The Hayflick limit is related to the longevity of the animal species from which the cells derived. Cells which are nurtured may survive beyond the Hayflick limit, continue to grow and constitute a cell line. The cells of semi-continuous or diploid cell lines retain their characteristic diploid chromosomal constitution and can support the growth of a wide range of viruses. Such cell lines, most of which are predominantly fibroblastic, tend to die out between the 30th and 50th passage.

Continuous cell lines are derived from either normal or neoplastic tissue and can be passaged indefinitely. In these cell lines, referred to as heteroploid cell lines, the cells have an abnormal number of chromosomes. In general, they are not as sensitive as either primary or semi-continuous cell lines for virus isolation. However, viruses can usually be adapted to grow in continuous cell lines. Established cell lines facilitate large-scale growth of viruses for vaccine production or research purposes. Well characterized continuous cell lines can be obtained from commercial organizations which specialize in preserving and distributing stock. Samples can be purchased from the American Type Culture Collection and the European Collection of Cell Cultures. Continuous cell lines with recognized names include Madin Darby bovine kidney (MDBK) and Crandell feline kidney (CRFK). Following prolonged passaging, continuous cell lines may become contaminated with *Mycoplasma* species or viruses and may acquire altered characteristics such as increased or decreased susceptibility to viruses. Most virology laboratories freeze early stocks of cells and, periodically, revive cells from the frozen stock in order to ensure maintenance of characteristics. Cells can be stored in a viable state for long periods at temperatures below −130°C, typically at −196°C using liquid nitrogen. Cryoprotective agents such as dimethyl sulphoxide or glycerol are usually added to the medium in which cells are suspended.

Detection of viral growth in cell cultures

Viral growth in cell culture, which results in damage to infected cells, may be detectable by light microscopy. Microscopic changes in infected cells, termed

cytopathic effect (CPE), include change in shape, cell detachment, fusion leading to syncytium formation, the presence of inclusion bodies and cell death. On primary isolation, some viruses do not produce CPE. Following passage on to fresh monolayers, they may become cytopathic. The full range of the effects of viral infection on cultured cells, especially inclusion body formation, may be demonstrable only by staining. Inclusion bodies are intracellular structures which have characteristic staining features. In virus-infected cells, they may be composed of viral nucleic acid, viral protein or altered cellular material. Inclusion bodies, which may be single or multiple, may be located in the cytoplasm or the nucleus. They are described as acidophilic, when stained by eosin, and basophilic, when stained by haematoxylin. Intracytoplasmic inclusions may be found in cells infected with poxviruses, reoviruses, rabies virus and paramyxoviruses, while intranuclear inclusions occur in cells infected with adenoviruses, herpesviruses and parvoviruses. Infections with some viruses such as canine distemper virus may produce both intranuclear and intracyto-plasmic inclusion bodies.

Non-cytopathic viruses require alternative detection methods. Enveloped viruses belonging to some families insert viral glycoproteins into the plasma membrane of infected cells. These glycoproteins may induce cell fusion, producing syncytia, or promote haemadsorption, the binding of erythrocytes to the surface of infected cells. Syncytia may be formed in cell cultures infected with lentiviruses, paramyxoviruses and some herpesviruses. Cells infected with orthomyxoviruses, paramyxoviruses and togaviruses tend to exhibit haemadsorption at sites of virion budding. Haemadsorption can be detected early in the replication cycle. The glycoproteins responsible for haemadsorption, referred to as haemagglutinins, also cause clumping of erythrocytes following mixing with free virions. Many viruses, both enveloped and non-enveloped, can cause haemagglutination with eryth-rocytes from particular animal species. Feline panleukopenia virus haemagglutinates porcine eryth-rocytes while porcine parvovirus haemagglutinates chick, guinea-pig, rat, monkey, human and cat eryth-rocytes Supernatant fluid from infected cell cultures, lysed by freezing and thawing, can be examined for the presence of viral particles by electron microscopy. This is an insensitive technique and particle concentrations in excess of 10^6 per ml are required for detection.

Serological tests may be required for the specific identification of a viral species. A useful method for detection and specific identification is infection of susceptible cells grown as monolayers on coverslips in Leighton tubes or in flat-

bottomed vials. Following fixation of infected cells, virus can be identified by employing a fluorescein-labelledantibody.Monoclonal or polyclonal conjugates may be used depending on availability and the level of specificity required. This technique is independent of any cytopathic or other property of the test virus and contributes to the speed and sensitivity of the isolation procedure. However, a separate conjugate is required for the specific detection of each virus species. Specific antibodies against viruses can also be utilized in virus neutralization tests to block cytopathic effects and in haemadsorption/haemagglutination inhibition assays to inhibit the erythrocyte-binding activity of viruses. Neutralization assays are the definitive methods for distinguishing many viruses and defining serotypes. Although immunoelectron microscopy is more sensitive and specific than direct electron microscopy, it cannot be used as a general screening method for viruses in clinical samples.

Embryonated eggs

Formerly, embryonated eggs were used extensively for virus isolation. Decline in the use of embryonated eggs has resulted from improvements in tissue culture techniques. However, inoculation of embryonated eggs remains the preferred method for isolation of influenza A viruses and for many avian viruses. In order to exclude maternally-derived anti-viral antibodies, the source of the eggs requires careful selection: they should preferably be eggs from specific pathogen-free flocks. Passage in embryonated eggs has proved useful for attenuating certain viruses used in the production of modified live virus vaccines.

Embryonated eggs may be inoculated using a number of defined routes (Fig. 57.1). Viruses can be inoculated into the allantoic cavity, the amniotic cavity or the yolk sac. They can also be inoculated on to the chorioallantoic membrane (CAM). In addition, well developed chick embryos can be inoculated intravas-cularly The route selected for inoculation is largely determined by the tissue affinity of each particular virus. Evidence of the effect of viral growth includes death or dwarfing of the embryo and the formation of pocks on the CAM. Haemagglutination or immunofluorescence may be used as additional tests for the detection of virus.

Experimental animals

For ethical reasons, experimental infection of animals is now used less

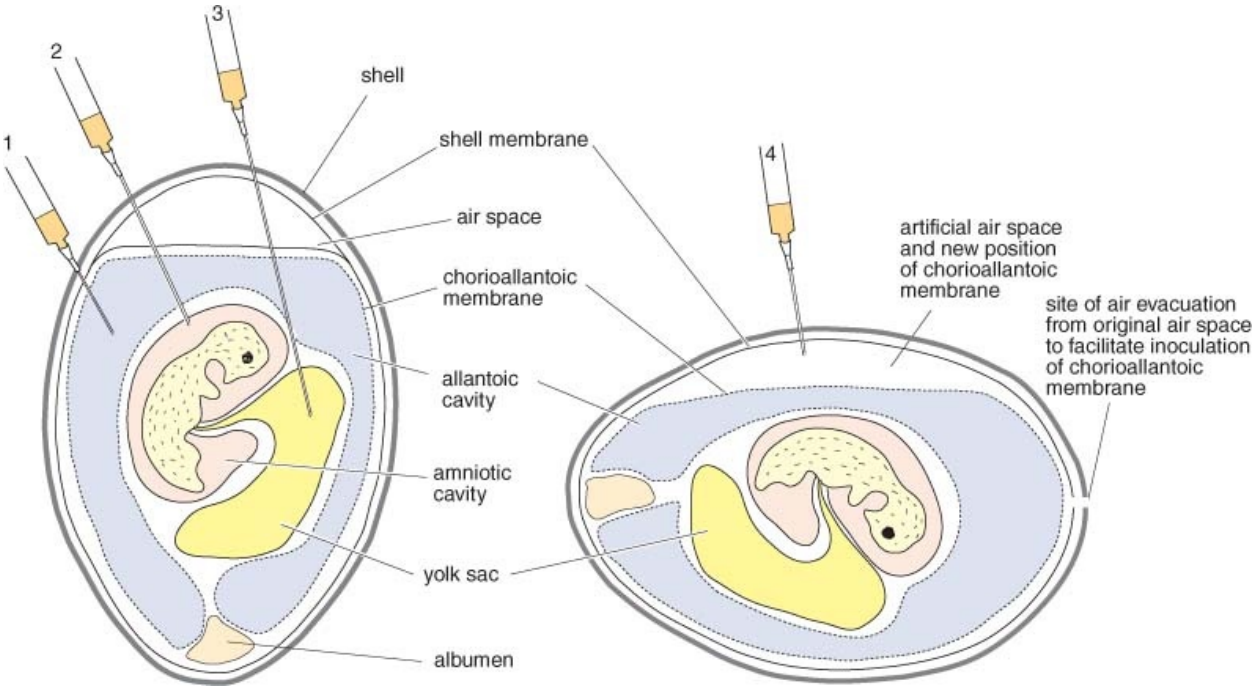
frequently than formerly. A number of alternative methods for the growth and study of viruses are available. However, for several virus families, the use of cell culture for isolation is of limited value and animal inoculation remains the preferred procedure. Suckling mice are used for the detection of arthropod-borne viruses and for rabies virus. Inoculation of the natural host species is a requirement for the isolation of some viruses. Challenge experiments in the natural host species may be necessary to evaluate vaccines. The production of antisera requires inoculation of laboratory animals with virus. Investigation of the pathogenetic mechanisms relating to viral infections and the subsequent immune response of the host frequently require inoculation of either laboratory animals or natural hosts.

Determination of virus concentration

For evaluating the efficiency of vaccines, when standardizing virus neutralization or haemagglutination-inhibition assays or when producing whole virus vaccines, accurate standardization of virus concentration is essential. It is also necessary when determining the minimum dose of virus required to produce clinical disease. Titration methods may be physical, used to measure the total number of virions present (employing electron microscopy) or biological, used to calculate the number of infectious virions by inoculating a susceptible host or tissue culture system. Alternatively, a measure of a specific viral activity such as haemagglutination may be used or the concentration of viral antigen may be determined. Some titration procedures are quantal, providing an approximation of the number of virions in a preparation. Estimation of viral concentration by quantal techniques is adequate for most experimental or diagnostic purposes. Typically, these assays are carried out using serial ten-fold dilutions of a given virus preparation to inoculate several replicate host or cell culture systems per dilution. The end point in mice or embryonated eggs is usually death and the unit is expressed as the 50% lethal dose (LD_{50}). In cell culture systems, the end point is expressed as the 50% tissue culture infective dose ($TCID_{50}$). The results are usually expressed as a $\log_{10}$ value per ml of the original undiluted specimen. The statistical methods devised by Reed-Muench and by Kärber are frequently employed in these calculations.

Figure 57.1 Routes for inoculation of viruses into embryonated eggs. 1, Into allantoic cavity; 2, into amniotic cavity; 3, into yolk sac; 4, on to chorioallantoic

membrane.



The concentration of haemagglutinating viruses is often expressed in haemagglutinating (HA) units. The HA unit is equivalent to the highest dilution of the virus suspension causing complete haemagglutination This simple titration method is insensitive because many thousands of virions are required to agglutinate sufficient numbers of erythrocytes for visual detection.

The plaque assay is a common and accurate method for quantifying viral infectivity. Serial ten-fold dilutions of the test virus preparation are inoculated on to monolayer cell cultures and allowed to adsorb for approximately 1 hour. The cell sheets are then overlaid with tissue culture medium containing agar which prevents spread of virus throughout the cell culture while permitting cell-to-cell transfer. As a result, cytopathic viruses produce foci of cell death around the replication sites, which then appear as clear areas called plaques. Based on the number of plaques and the dilution and size of the inoculum, the titration end point is calculated and expressed as plaque forming units (pfu) per ml. Although it is possible for a single virion to produce one plaque, in practice the virion : pfu ratio for most animal viruses is seldom less than 10 : 1. Even within a single virus preparation, there appears to be substantial microheterogeneity as not all virus particles in a preparation can form plaques. The multiplicity of infection (moi) is the ratio of number of infectious virus particles to a known number of cells in a culture. It might be expected that an moi of 1 pfu would result in every cell becoming infected but in practice only about two-thirds of the cell

population are found to be infected. This occurs because the distribution of virus particles among a population of cells is random with a distribution pattern that is best described mathematically by the Poisson distribution. An moi of ≥5 is required to ensure that more than 99% of cells become infected.

Table 57.1 Virus–host cell interactions.

Virus	Production of progeny virus	Outcome
Cytopathic	Productive	Necrosis
	Non-productive	Apoptosis
Non-cytopathic	Productive	Persistence Transformation
	Non-productive	Latency Transformation

Virus–host cell interactions

Virus infections may produce effects ranging from latency to cell death (Table 57.1). Infections may be productive or non-productive, consequences influenced by the ability of the virus to replicate effectively in a given cell type. When a host cell allows virus replication to proceed, it is said to be permissive. This state may not be static as occurs when latent infections are reactivated following a period of quiescence or non-productive interaction. Reactivation tends to occur when latently infected animals are subjected to stressful environmental conditions.

Mechanisms of cell injury

Cytopathic viruses kill cells which they infect. Frequently, cell necrosis is the consequence of the cumulative effects of a number of biochemical changes induced by viral replication, resulting in the production of ultrastructural lesions. Cell death due to necrosis tends to occur late in the viral replication cycle after progeny virus production is complete and cytolysis may in fact facilitate the release of virions. Reoviruses, poxviruses, picornaviruses, paramyxoviruses and rhabdoviruses can inhibit the transcription of host cell RNA to permit synthesis of viral mRNA. This alteration of host cell metabolism may relate to interference with protein synthesis due to the production of virus-encoded factors. Herpesviruses, influenza viruses and vesicular stomatitis virus inhibit processing of host cell mRNA by interfering with the splicing of the primary cellular mRNA transcripts. Rapid and pronounced shutdown of protein synthesis occurs in cells infected with picornaviruses, poxviruses and herpes-viruses. Other viruses such

as adenoviruses cause a gradual shutdown of cellular activity at a late stage in the viral replicative cycle. The viral mechanisms which block protein synthesis include competition for ribosomes from large amounts of viral mRNA, degradation of cellular mRNA by viral enzymes, interference with cellular mRNA translation and the alteration of the intracellular ionic environment in favour of viral mRNA translation. Viral proteins of lentiviruses and adenoviruses inhibit the processing of cellular proteins and their transportation from the endoplasmic reticulum. Accumulations of viral structural proteins occurring late in infection may be directly toxic for host cells. Humoral and cellular immune responses directed against viral proteins incorporated into the plasma membrane of the host cell *in vivo* may be cytotoxic.

Programmed cell death, apoptosis, can be induced by viral infection. In apoptosis, activation of cellular endonuclease leads to fragmentation of cellular DNA. The resulting DNA fragments produce a characteristic pattern of evenly spaced bands when electrophoresed in agarose gel. Apoptosis may be triggered by certain viruses in the early stages of infection, resulting in the death of individual cells prior to virus replication. This event could be an important host defence mechanism. Some viruses produce substances which block apoptosis, thus prolonging cell survival.

Non-cytopathic viral infections

Viruses such as retroviruses, which are usually non-cytopathic, do not interfere with protein synthesis in host cells. These viruses frequently produce persistent infections with progressive changes which eventually lead to cell death. Members of the sub-family *Alphaherpesvirinae* typically produce productive lytic infections, usually in epithelial cells. At the sites of productive infection, progeny viruses enter sensory nerve fibres and are transported in the axoplasm to sensory nerve ganglia. Latent infection develops within the neuronal perikarya. Viral replication is restricted because stimuli for replication are not produced in the non-dividing neurons. The intracellular viral DNA is present in a circular episomal form in association with nucleosomes, and transcription is limited to a few latency-associated transcripts (LATs). The function of LATs is not known. They do not appear to be translated into proteins and they are not essential for the maintenance of the latent state. The lack of viral protein expression allows infected neurons to remain undetected by the immune system. Multiple copies of the viral DNA may be demonstrable in latently infected host cells. Episodes of

reactivation with production of infectious progeny virus can occur periodically. The newly produced virions are transported in the sensory nerve fibres to the superficial sites of the primary infection where they may again induce lytic lesions. The mechanism of reactivation is not fully understood. Certain stimuli or stress factors, such as trauma,

Table 57.2 Some oncogenic viruses of veterinary importance.

Genome	Family	Virus
DNA	*Herpesviridae*	Marek's disease virus
	Papillomaviridae	Bovine papillomavirus Equine papillomavirus Canine oral papillomavirus
RNA	*Retroviridae*	Avian leukosis virus Feline leukaemia virus Jaagsiekte sheep retrovirus

immunosuppression, hormonal changes and intercurrent disease, can trigger reactivation. This frequently coincides with circumstances in which transmission of virus to susceptible animals may readily occur.

Retroviruses integrate a DNA copy of their RNA genome, termed provirus, into the host chromosome. This allows propagation of the provirus along with host chromosomes. Integration of provirus, while not destroying the cell, may alter the host genotype and the expression of host genes. The genomes of DNA viruses such as papillomaviruses are maintained in cells as circular episomal molecules. Viral replication, which is promoted by viral proteins, is synchronized with host cell division.

Viral oncogenesis

A number of DNA and RNA viruses cause neoplastic transformation of cells (Table 57.2). Transformation results from interference with growth signals in cells. The consequent alteration in cell growth affects saturation density, growth factor requirements and anchorage dependency. Normally, cells stop dividing once they form a confluent layer covering the available surface area of a solid substrate. Transformed cells do not exhibit contact inhibition of cell growth and continue growing, piling up into multiple layers. The normal dependence of cells for growth factors in the culture medium is often reduced or no longer present in transformed cells. Epithelial and fibroblast cells will normally grow *in vitro* only on a solid glass or plastic surface. Transformation of cells results in a loss of this dependence.

Central to the understanding of the mechanisms involved in tumour production by viruses was the discovery of oncogenes. Originally recognized in retroviruses, more than 60 oncogenes, termed v-*onc* genes, have been identified.

Cellular genes, c-*onc* genes or proto-oncogenes, corresponding to most v-*onc* genes, are present in normal cells in which they regulate cell division and differentiation. Cellular oncogenes encode proteins which function as growth factors, growth factor receptors, transcription factors and intracellular signal transducers. It is generally accepted that v-*onc* genes of retroviruses were acquired during the evolution of these viruses following virus-host cell interaction. Central to this process is the possession of a unique enzyme, reverse transcriptase, and the incorporation of a DNA copy of viral RNA into the host genome. Two important consequences of the integration of provirus into the host genome are the ability of retroviruses to transduce cellular genetic material and the insertional activation (or occasionally inactivation) of cellular genes. Retroviruses may exert an oncogenic effect by carrying v-*onc* genes (transducing retroviruses) or alternatively by causing overexpression or inappropriate expression of c-*onc* genes (cis-activating retroviruses). The v-onc genes carried by rapidly transforming retroviruses differ in certain respects from the c-*onc* genes from which they were derived. They are under the control of strong viral promoters known as long terminal repeats (LTRs), carry mutations as a result of the high error rate of reverse transcriptase and may be joined to other viral genes in a way that modifies their function. As a result, they are outside of the control of normal cellular gene regulation, and oncogene proteins may be overproduced or may function in an abnormal way leading to uncontrolled cell division. In contrast, slowly transforming retroviruses lack a v-*onc* gene and give rise to tumours in a random manner. Insertion of provirus (insertional mutagenesis) with its strong LTR promoter sequences close to a c-*onc* gene may greatly increase the production of the normal c-*onc* gene protein.

The oncogenes of DNA viruses are not usually derived from cellular genes. The DNA viruses are generally present in cells as circular episomal nucleic acid, and their oncogenes encode early viral proteins that inactivate tumour suppressor proteins such as retinoblastoma (Rb) and p53. These tumour suppressor proteins regulate mammalian cell growth by controlling the progression of the cell cycle through holding the cell at the G1 phase of the cell cycle. The strategy of the DNA viruses is to drive a resting cell to enter S phase, thereby inducing the cellular gene-encoding activities necessary for DNA replication. Normally this cell cycle deregulation is of no consequence because the infected cell eventually dies. However, if the replication cycle of the virus is curtailed (non-productive infection), overexpression of the gene products may occur, contributing to oncogenic transformation and inducing uncontrolled replication of the host cell.

In contrast to other DNA viruses associated with transformation, the genome of the herpesvirus, Marek's disease virus, appears to contain a number of v-*onc* genes similar to those of retroviruses.

Further reading

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Chapter 58

Pathogenesis of viral diseases

Viral pathogenesis, the production of disease as a result of viral infection, is concerned with the cellular events and pathological mechanisms that connect the virus to cell/tissue/organ damage and the resulting clinical signs. Although viruses are obligate cellular parasites, viral infection does not always result in apparent clinical disease. A broader definition of viral pathogenesis encompasses the process of infection following entry into a susceptible host by a virus, irrespective of outcome. The mechanisms whereby viruses produce disease relate not only to direct interference with cellular functions but also to indirect effects caused by host responses (immune/inflammatory) to infection. Virulence refers to the relative ability of viruses to produce disease in a host. It is dependent on the attributes of both the virus and its host, and is influenced by factors such as the dose and route of entry of the virus and the species, age and immune status of the host. Frequently, comparisons are made between related viruses as to their virulence, and a particular isolate may be described as more virulent or less virulent than others. Because of the complex interactions between viruses and their hosts, clinical disease does not necessarily follow exposure to a virulent virus. Viral infections may result in subacute, acute or chronic or persistent disease. Although present in the tissues, some viruses which exhibit latency produce disease only under defined environmental conditions. In slow viral infections, lesions develop gradually and clinical signs emerge only after long incubation periods. The stages in the development of a typical viral infection are illustrated in Fig. 58.1. While the stages shown illustrate the steps involved as if they were sequential and related, in reality many independent events may be taking place contemporaneously.

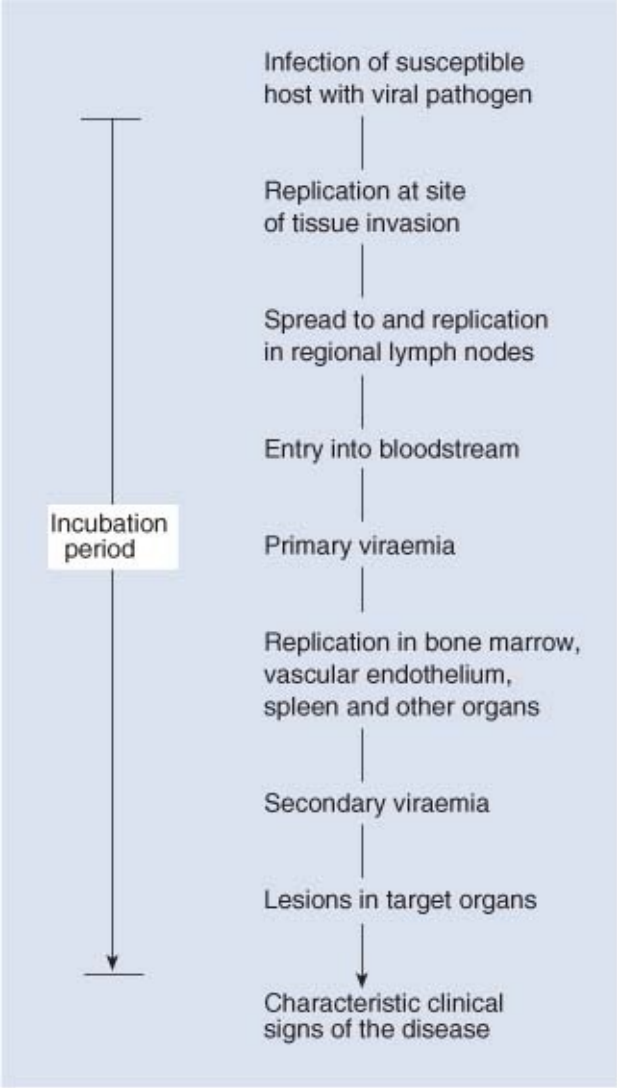
Routes of infection

Viruses can infect vertebrate hosts by a variety of routes. In many instances, viruses must first attach to and infect cells on the skin and other body surfaces.

Viruses can be introduced into the body through breaks in surface epithelia such as scratches, abrasions or wounds. They may also be introduced by parenteral inoculation either through bites or following the use of contaminated needles or surgical equipment. When intact, the stratified squamous epithelium of the epidermis usually presents an effective barrier to infection. Because they have an affinity for epidermal cells, papillomaviruses replicate in the epidermis producing local proliferative lesions. In contrast, viruses such as myxomavirus of rabbits and sheeppox virus, which also replicate in the dermis, may spread to other tissues through blood vessels and lymphatics.

Mucous membranes, which present a minimal physical barrier to infection, are coated with a layer of mucus. This viscous secretion provides some physical protection and, in addition, often contains protective immunoglobulins. In the respiratory tract, infectious agents may be moved towards the oral cavity by the coordinated activity of ciliated epithelium. Peristalsis in the intestinal tract and the flushing action of urine in the urinary tract assist in the removal of infectious agents from these anatomical locations. Some viruses, which are spread venereally, may invade through abrasions, frequently resulting from sexual activity, in the urethral, vaginal or anal mucosa.

Figure 58.1 Stages in the spread of a typical systemic viral infection. The incubation period is the interval from invasion of the host to the development of clinical signs.



In order to establish infection in the gastrointestinal tract (GIT), enteric viruses must evade local mucosal immune defences and either attach to specific receptors on epithelial cells or be engulfed by M cells, specialized epithelial cells overlying Peyer's patches in the ileum. In the GIT, prevailing environmental conditions are generally unfavourable for virus survival. Successful viral pathogens of the GIT, which damage intestinal cells, are usually unaffected by gastric acid, are tolerant to bile salts and are resistant to inactivation by proteolytic enzymes. Remarkably, the infectivity of some viruses such as coronaviruses and rotaviruses is enhanced by exposure to proteolytic enzymes. With the notable exception of coronaviruses, enteric viral pathogens are non-enveloped and therefore 'hardy'. Bile salts generally have an adverse effect on viral envelopes. The basis for the resistance of the envelopes of coronaviruses to bile salts is unknown.

Arboviruses

Arboviruses are defined as viruses maintained in nature through biological transmission between vertebrate hosts by haematophagous arthropods. The viruses multiply in the tissues of the arthropod vector. The most important arthropod vectors are mosquitoes, ticks, sandflies and midges. The vector remains infected for life. The term ‘arbovirus’ has no taxonomic status. It is applied to viruses belonging to several viral families including *Togaviridae, Flaviviridae, Reoviridae, Rhabdoviridae, Arenaviridae* and *Bunyaviridae*. Most arboviruses are maintained in complex sylvatic life cycles involving a primary vertebrate host and a primary arthropod host. Such cycles usually remain undetected unless domestic animals and humans encroach. Alternatively, due to ecological change, the virus may escape its primary cycle by means of a secondary vector or vertebrate host. As a result, the virus is brought into the peridomestic environment. Domestic animals and humans are generally ‘deadend’ hosts as they do not develop sufficient viraemia to contribute to the transmission of the virus. The majority of arbovirus infections are zoonoses, are found in tropical developing countries, and have a distinct geographical distribution. Ecological factors limiting the distribution of particular arboviruses include temperature, rainfall and distribution of both vertebrate reservoir hosts and arthropod vectors.

Dissemination in the host

Following infection, local spread from cell to cell frequently occurs. When viruses bud from cells, dissemination may be influenced by the manner and site of budding. Release of viral progeny from the apical surface of mucosal cells favours localized infection in tubular structures such as the air passages and the intestine. In contrast, release of virus from the basal surface into subepithelial tissues facilitates systemic infection. Factors which may limit the ability of some viruses to spread systemically include the absence of suitable cell receptors and lack of permissive cells. In addition, optimal viral replication may occur only in dividing cells, in cells of a certain age/differentiation state or at a temperature lower than core body temperature. These virus and host factors may play an important role in determining the tropism of a virus, and account for the selective infection of certain cells in particular organs. In subepithelial tissues, viruses frequently enter the lymphatic network and may be transported to the

regional lymph nodes either as free virions or in infected macrophages. From the nodes, virus may pass to the efferent lymphatics and the thoracic duct, eventually entering the bloodstream, the most important route of dissemination. Some viruses are transported along peripheral nerves. The preferential use of one route of spread does not necessarily exclude spread by other routes.

Haematogenous spread

Primary replication at the site of entry is frequently followed by transitory low-titred primary viraemia which results in infection of various organs including those of the reticuloendothelial system and the vascular endothelium. Further multiplication at these locations is followed by a sustained, high-titred secondary viraemia. In the bloodstream, virus may be free in the plasma or associated with cellular elements.

The body employs a number of mechanisms for clearing virus from the circulation. These include complement and antibody and the phagocytic cells of the reticuloendothedial system in the liver, spleen, lung and lymph nodes. The magnitude and duration of a viraemia are determined by the amount of virus entering the bloodstream and the effectiveness of clearance mechanisms. Large viruses are cleared more rapidly from the circulation than small viruses. There is rapid removal of viruses opsonized by antibody or by complement. Viraemias, in which virus remains free in the plasma, such as those associated with parvoviruses, flaviviruses, togaviruses and picornaviruses, are usually of short duration, with clearance generally coinciding with the appearance of neutralizing antibodies in the serum. Prolonged viraemias are features of infections with viruses such as canine distemper virus, feline leukaemia virus and Marek’s disease virus, which are associated with circulating cells. These viruses are often unaffected by the action of antibodies and complement. Some viruses like lentiviruses can replicate in monocytes or lymphocytes and, in many instances, produce persistent viraemias.

Invasion of tissues and organs from the blood can occur in a number of ways which may relate to virus interaction with macrophages or vascular endothelial cells. Viruses such as picornaviruses, retroviruses, togaviruses and parvoviruses can infect endothelial cells and, following replication, are released into the tissues of target organs. In other instances, following endocytosis by endothelial cells, viruses may be translocated to the basal surface and released into tissues by exocytosis. In some anatomical locations such as the choroid plexuses,

virions may pass from the bloodstream into surrounding tissues through fenestrated endothelium. Viruses can be transported into tissue spaces inside lymphocytes or monocytes as these cells migrate from the circulation. Engulfment and destruction of viruses by the phagocytic cells of the reticuloendothelial system are an important defence mechanism which limits viraemia. In some instances, phagocytosis of viruses by these cells may result in transfer of virions to distant sites in the body.

Neural spread

Neurotropic viruses such as rabies virus, Aujeszky's disease virus and Borna disease virus can invade the CNS through peripheral nerves. Enveloped viruses are usually transported as naked nucleocapsids by axoplasmic flow. Within the CNS, dissemination frequently involves spread across synaptic junctions. In addition, spread within peripheral nerves from the CNS to other locations may occur. Alphaherpesviruses can spread in peripheral nerves from the site of infection to ganglia, causing latent infections. Reactivation of infection may result in recrudescence of superficial lesions following transport of virus from the ganglia along nerve fibres.

Clinical signs

The signs of viral infections reflect both the site of virus replication in tissues and host responses. Some viruses kill the cells in which they replicate, producing clinical signs that relate to the anatomical location and function of the affected cells. Because of the considerable reserve and regenerative capacity of the liver, the loss of large numbers of hepatocytes may not result in significant clinical disturbance. In contrast, loss of relatively few neurons may have severe clinical consequences. In some viral infections, loss of specialized functions or reduction in functional efficiency in infected cells may induce clinical signs. Viral infections of the respiratory and intestinal tracts are frequently complicated by secondary bacterial infections, which may contribute to the development of clinical signs and associated pathology. Bacterial species present in the normal flora may contribute to these opportunistic infections. Denuded epithelial surfaces, impaired clearance mechanisms, or increased availability of bacterial nutrients may promote bacterial proliferation, leading to secondary infections.

A number of viruses, including infectious bursal disease virus of poultry and

feline immunodeficiency virus, target cells of the immune system. Progressive depletion of lymphocytes can lead to an immunodeficient state. As a consequence, affected animals may present with a variety of clinical signs, due to secondary bacterial infection. In most viral infections, the immune system has an important protective role. However, there are viral infections in which the principal lesions result from hypersensitivity reactions and subsequent immunopathological changes. Immune complexes formed in persistent viral infections such as feline infectious peritonitis and equine infectious anaemia are responsible for vasculitis and glomerulonephritis.

Abortion following viral infection is usually indicative of substantial damage to the tissues of the placenta or foetus. The effect on the foetus is often influenced by the stage of gestation and by the virulence of the virus. Infection with virulent virus early in gestation generally results in foetal death with resorption or abortion. Infection of pregnant cattle with bovine viral diarrhoea virus (BVDV) before 100 days' gestation may result in abortion, congenital defects or immunotolerance. If infection occurs at a later stage during pregnancy, calves may be born with congenital defects, whereas infection towards the end of pregnancy induces a protective immune response.

When viral epidemics occur in susceptible populations, the outcome in individual animals can range from asymptomatic infection to persistent or even fatal infection. Host factors which may influence the outcome include age, immune status, genetic factors and nutrition. Young animals are generally more susceptible to viral infections than older animals, whose increased resistance can usually be attributed to maturation of the immune system, and/or cell differentiation and to immunological memory. Reverse age resistance, although uncommon, is well documented in rabbit haemorrhagic disease. In this condition, rabbits less than 5 weeks of age do not develop disease because the target organ, the liver, does not appear to be susceptible early in life. Severe malnutrition may exacerbate certain viral diseases, a possible consequence of which may be depressed cell-mediated immunity. Success in breeding birds with enhanced resistance to avian leukosis and Marek's disease illustrates the influence of genetic factors on disease susceptibility.

Virus shedding and patterns of infection

The shedding of infectious virions from surfaces or orifices via aerosols, droplets, scabs, body fluids, excretions and secretions (saliva, blood, semen,

milk, urine, faeces) allows transfer to other susceptible hosts. Although shedding usually coincides with the onset of clinical signs, it may begin earlier in some viral infections. Respiratory viruses are usually transmitted in aerosols generated by coughing and sneezing. Enteric viruses are frequently shed in enormous quantities in faeces and can generally survive in harsh environments. Viruses which produce generalized infections may be shed by a number of routes. Body fluids such as saliva, semen, urine and milk may contain particular viruses. Arboviruses usually produce high--itred viraemias of short duration and rely on appropriate haematophagous vectors for transmission. Surgical procedures or blood sampling may facilitate spread of blood-borne viruses. Some viruses can be transmitted vertically by the transplacental route or via the birth canal. Endogenous retro-viruses are transmitted in the DNA of germ cells.

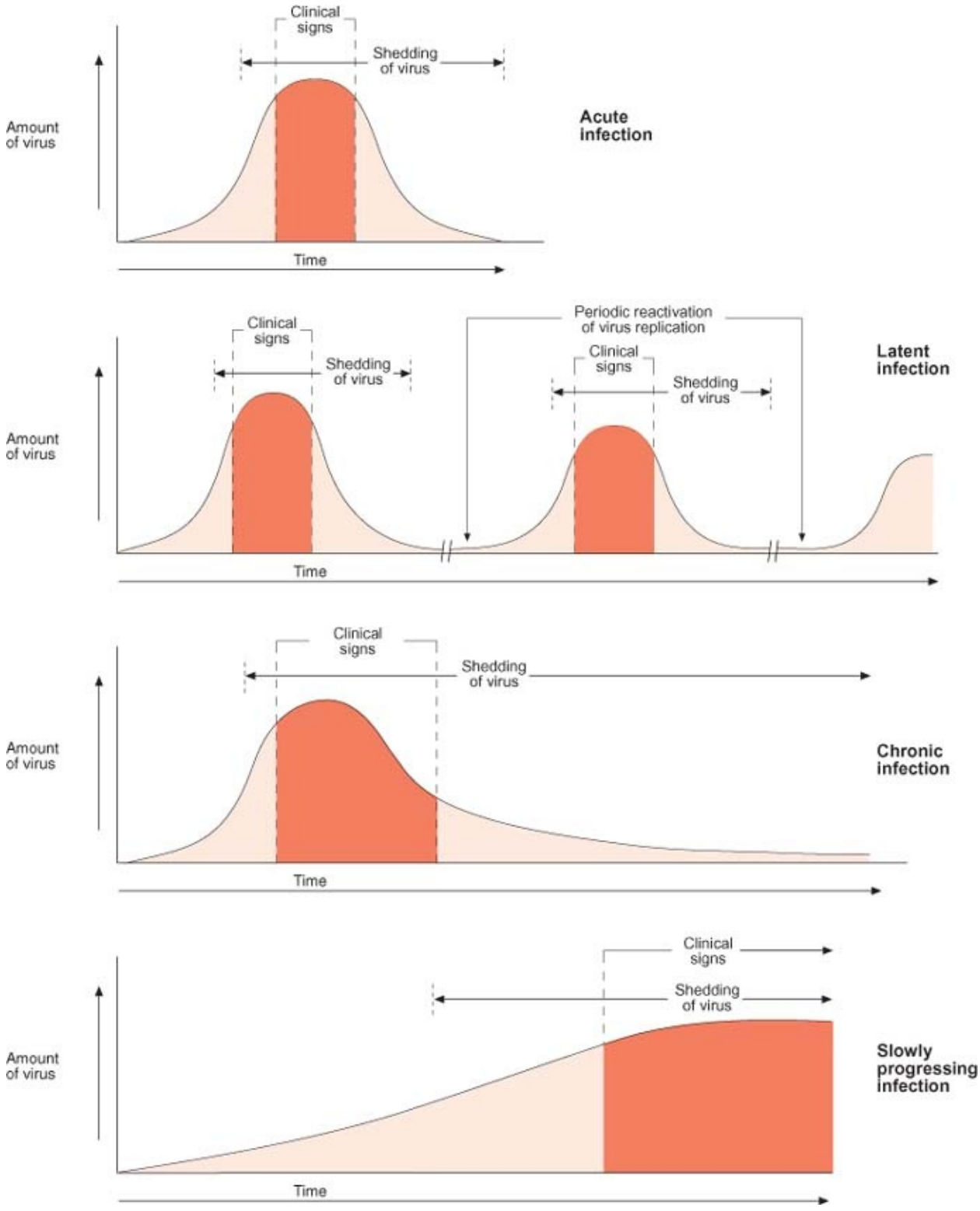
The maintenance of infection in a population requires continuous infection of susceptible animals. Two main strategies have been adopted by viruses for this purpose, namely acute infection and long-term persistence (Fig. 58.2).

Acute infection is characterized by a short clinical course, with rapid elimination of virus from the tissues. In these infections, there may be shedding of large amounts of virus over a short period. To maintain infection in a susceptible population, viruses which cause acute infections must be either highly contagious, such as influenza viruses, or capable of surviving for long periods in the environment, as is the case with parvoviruses, poxviruses and many enteric viruses.

Persistent viral infections are characterized by a prolonged course with constant or intermittent shedding. These infections, which may be acute initially, can persist in either latent or chronic forms. Latent infections are characterized by persistence of the virus in a non- productive form, either integrated into the genome of the host, as occurs with retrovirus, or as an episome, in the case of herpesviruses. Periodic reactivation of productive infection with shedding of infectious virus may occur. This type of infection is best exemplified by alphaherpesviruses, such as feline herpesvirus 1, which produce productive infections in epithelial cells and latent infections in sensory neurons. The terms persistent infections and chronic infections are sometimes used interchangeably but, in general, chronic infection implies that virus is constantly present (continuous replication) and may be shed intermittently or continuously, generally at low levels. This type of infection can occur when the immune response of the host fails to eliminate virus from the tissues within the time when immune clearance would be expected to occur.

Infections characterized by long incubation periods of months or years are referred to as slow infections. Infections of this type, produced by lentiviruses, jaagsiekte sheep retrovirus and prions, have a progressive clinical course, usually resulting in death.

Figure 58.2 Comparative features of acute, latent, chronic and slowly progressing viral infections in animals, illustrating the duration of virus persistence in the tissues. The time required for the development of clinical signs and the pattern of virus shedding in each form of disease are typical for the categories of infections illustrated. Although virus shedding typically coincides with the development of clinical signs, there is wide variation in the duration of clinical signs and virus shedding among different families of viruses. In slowly progressing viral infections, clinical signs and shedding of virus usually persist until the animal finally succumbs to the infection.



Mechanisms of persistence

Viruses that persist in the body usually employ a number of strategies to ensure

prolonged infections. For a virus to persist in host tissues, some infected cells must survive. Viruses have evolved a number of different strategies to reduce pathogenic effects on host cells. Viruses that produce non-lytic infections are likely to cause chronic infections. A number of viruses that are normally lytic have been shown to be capable of establishing persistent infections. Alphaherpesviruses, by exploiting the fact that sensory neurons are only partly permissive, have the ability to set up latent infections in such cells. In these non-dividing cells, the viruses persist as circular episomal DNA until adverse environmental factors induce immunosuppression, which permits virus replication. Foot-and-mouth disease virus can persist for long periods in the pharyngeal region of animals that have recovered from the clinical disease. These animals may act as a source of infection for susceptible animals. Adenoviruses may persist in the body by producing low-grade, cycling infection with small numbers of cells infected at any given time. The evolution of defective-interfering or less cytolytic variants may permit this type of infection to occur.

Viruses have evolved several strategies to evade the immune response and so avoid elimination by an immunocompetent host. Certain tissues in the body, referred to as immunologically privileged sites, are exempt from immune surveillance. The blood-brain barrier restricts contact between lymphocytes and CNS tissues. In addition, neurons do not express the major histocompatibility complex (MHC) class I or class II molecules which are required for T cell recognition of virus-infected cells, while other cells in the body, expressing low levels of MHC class I, are targets for virus infection. Certain viruses cause adjacent cells to fuse together, permitting the viral genome to spread contiguously from cell to cell, thus avoiding neutralizing antibody. This phenomenon is thought to be important in a number of late-onset, progressive, fatal, nervous diseases where the virus spreads gradually from neuron to neuron. Subacute sclerosing panencephalitis, which is a rare complication of measles virus infection, and old dog encephalitis, which is a complication of canine distemper, are conditions in which this form of viral spread may occur. During latent infection of host cells by herpesviruses, viral gene expression is diminished and viral proteins are not expressed on the cell surface. Such infected cells are not easily recognized by the immune system. Viruses, particularly RNA viruses, undergo frequent mutation. Variants with altered epitopes at sites important for antibody neutralization or T cell recognition can emerge. These variants may evade immune detection and become the predominant infecting

strain. This type of antigenic variation has been described in influenza virus infections as a consequence of alterations in the surface glycoproteins, haemagglutinin and neuraminidase. Although these viruses do not persist in individual infected animals, they may persist in animal populations. Lentiviruses, such as equine infectious anaemia (EIA) virus, exhibit significant antigenic variation during replication in infected animals. Recurrent episodes of clinical disease can occur in individual horses with EIA, in association with the emergence of antibody-escape variants and subsequent viraemia. Infection with feline immunodeficiency virus (FIV), a lentivirus, results in the destruction of CD4+ lymphocytes, but the bone marrow responds by increasing the rate of production of naïve CD4+ cells. The host's immune response against FIV limits virus replication and the bone marrow continues to produce adequate levels of these lymphocytes for months or years. Antigenic variation, which allows the virus to circumvent the immune system, ensures a high rate of virus production. A dynamic equilibrium becomes established between virus production and clearance by the body. Eventually, the bone marrow is unable to compensate, with a resultant drop in $CD4^+$ cell levels and immunodeficiency. It has been shown that some viruses can down-regulate or block expression of host cell surface markers such as MHC class I and class II molecules. Some viral proteins appear to act defensively by interfering with the function of anti-viral cytokines. Immune tolerance of the type associated with congenital BVDV infection permits persistent infection in calves. Affected calves have a lifelong viraemia and are the principal source of infection for other cattle.

An important epidemiological parameter used in the mathematical modeling of the spread of infectious disease through a population is reproduction number or reproduction rate (R). The basic reproduction number (R_0) is defined as the mean number of secondary cases arising from a typical single infected case in a population, with no immunity to the disease and without any control interventions. This value is affected by the ability of the virus to infect susceptible hosts, the duration of infectivity of an infected host and the population density of susceptible hosts. Underlying assumptions include equal susceptibility and exposure of host animals and uniform mixing of infected and susceptible animals. An important threshold is defined when $R_0 = 1$; values greater than 1 indicate that each primary case will give rise to more than one secondary case and the infection will spread. Large values of R_0 indicate the possibility of a major epidemic. Values of R_0 less than 1 indicate that the

outbreak will eventually die out, even if a number of secondary cases occur. The proportion of a given population that must be vaccinated to provide sufficient herd immunity to prevent sustained spread of an infection is given by $1-1/R_0$. The rate at which an epidemic develops is expressed by R_0 in association with the generation time (T_g), that is, the time from shedding of virus in primary infections to shedding of virus in the resulting secondary cases.

Further reading

Mims, C.A., Nash, A. and Stephen, J. (2000). *Mims' Pathogenesis of Infectious Disease*. Fifth Edition. Academic Press, Amsterdam.

Virgin, S. (2007). Pathogenesis of viral infection. In *Fields' Virology. Volume* 1. Fifth Edition. Eds D.M. Knipe and P. Howley. Lippincott, Williams and Wilkins, Philadelphia. pp. 327–388.

Chapter 59

Laboratory diagnosis of viral infections

Many viral diseases of animals can be diagnosed on the basis of clinical signs, together with post-mortem findings and histopathological changes. However, confirmation of the involvement of specific viral pathogens frequently requires specific laboratory procedures. Surveillance for particular viruses is an important aspect of the management of valuable animals, such as bulls and stallions, used for artificial insemination, which have the potential to spread infection to many other animals. As part of international trade regulations, certification of freedom from certain viral diseases must accompany animals exported to countries in which the diseases are exotic. Moreover, rapid and accurate laboratory confirmation of exotic viral diseases, including those with zoonotic potential, is essential for the successful implementation of eradication policies and for the protection of human health. Surveillance of animal populations for new, emerging or re-emerging viral diseases is an important responsibility of national veterinary services.

More than 200 major viral diseases of veterinary importance affect animal species. Because of the considerable resources required for the provision of comprehensive diagnostic services in virology, national diagnostic services are usually concerned with those diseases prevalent in a country. Moreover, laboratories frequently provide a diagnostic service for particular animal species. Special laboratory containment facilities are mandatory for some viruses, such as foot-and-mouth disease virus, which cause highly contagious diseases. The Office International des épizooties (OIE) in Paris, also known as the World Organization for Animal Health, monitors and publishes details of significant animal disease outbreaks worldwide. Compilation and publication of this information relies on international cooperation and a network of laboratories dealing with viral diseases of international importance.

Collection, preservation and transportation of

samples

Failure to provide a suitably selected specimen for the diagnostic laboratory is the single most important cause of unreliable laboratory results. Important factors relating to specimen collection and submission include: the timing of specimen collection from a sick animal; the type, quality and quantity of the specimen; the time taken for the specimen to reach the laboratory, the suitability of the medium in which the sample is stored during transportation and the conditions under which transportation took place. Ideally, specimens for laboratory examination should be collected as early as possible from affected animals when viral titres are highest and before secondary bacterial or fungal infections become established. It is advisable to collect samples from apparently normal in-contact animals as some of these animals may be actively shedding virus. The specimens selected for laboratory examination should relate to the clinical signs or to lesion distribution post mortem. Swabs from the oropharynx or nasopharyngeal aspirates are suitable specimens for investigation of respiratory diseases. In enteric viral diseases, large numbers of virus particles are shed in faeces. In those diseases characterized by viraemia, virus may be demonstrable in cells of the buffy coat layer.

Preservation of the infectivity or antigenicity of viruses may be required for particular tests. As many viruses are labile, specimens for virus isolation should be collected into transport medium, refrigerated and transmitted to the laboratory without delay. Samples should be frozen at −70°C if delay in delivery is anticipated. Freezing in a domestic freezer at −20°C decreases the infectivity of most viruses. Transport medium consists of buffered isotonic saline containing a high concentration of protein, such as bovine albumin or foetal calf serum, which prolongs virus survival. Antibiotics and antifungal drugs are added in order to inhibit the growth of contaminants. Samples for electron microscopy in which demonstration of virion morphology is the primary objective require less exacting conditions for storage and transportation. When collecting blood samples for molecular diagnostic tests, it should be noted that the anticoagulant heparin is inhibitory for some polymerase chain reaction procedures. Air-dried smears for fluorescent antibody (FA) staining should be fixed in either acetone or methanol for up to 10 minutes in order to preserve viral antigens. This fixation process allows penetration of FA conjugates into cells. A similar fixation procedure is required for cryostat sections of frozen tissues prior to FA staining. Formalin-fixed tissue samples embedded in paraffin wax can be stored for many

years and used to demonstrate the presence of viral antigen by immunohistochemical techniques.

Guidance from clinicians regarding the possible aetiology of the disease under investigation is essential for deriving maximum benefit from laboratory tests. This requires an accurate assessment of the history and clinical signs together with a tentative clinical diagnosis. In some instances, post-mortem and histopathological examination of tissues may be sufficient for diagnostic purposes, particularly if specific inclusion bodies are found in infected tissues.

Detection of virus, viral antigens or nucleic acid

The presence of virus in tissues can be confirmed by isolation of live virus, by demonstration of virus particles or viral antigen and by detecting viral nucleic acid. Laboratory diagnostic tests based on molecular biology and antigen detection methods have become the methods of choice for the rapid diagnosis of viral diseases.

Isolation of live virus

Virus isolation using cell culture, fertile eggs or experimental animals is the standard against which other diagnostic methods are usually compared. A monolayer composed of a particular cell type cannot be expected to support the growth of the diverse range of viruses which cause animal diseases in a host of different cell types. Laboratories usually have a limited range of cell lines most often used for virus isolation and appropriate for the range of samples received. Embryonated eggs are widely used for the isolation of influenza A virus and avian viruses. Because of ethical considerations and cost, virus isolation in experimental animals is now seldom employed.

Virus isolation is a sensitive procedure when cultural conditions are optimal for a particular virus and also generates a supply of virus for further studies. This method is suitable for the detection of many different viruses, including viruses not suspected from the clinical assessment and not previously recognized in clinical disease conditions in animals. However, it is labour-intensive, slow and expensive. A number of blind passages may be required before a virus becomes adapted to a particular cell line and, as a consequence, a test result may not be

available for some weeks. Because some viruses do not produce a cytopathic effect, additional detection procedures such as haemadsorption and FA staining may be needed to demonstrate their presence in cell cultures. Even when a virus produces a pronounced cytopathic effect, additional tests are frequently required for definitive identification. Shell vial culture refers to a modified cell culture technique whereby cells are grown on a coverslip placed at the base of a vial, the inoculum is added and the vial is centrifuged. Following a suitable incubation period, the coverslip is removed and subjected to immunofluorescent staining. This method frequently provides enhanced sensitivity compared with conventional viral culture. Mixed cell culture systems, in which the simultaneous culture of a number of different cell types takes place in a monolayer, have been used for virus isolation. This system permits the isolation of a broader range of viruses than a conventional monolayer and their subsequent detection using monoclonal antibodies.

Electron microscopy

Viruses can be demonstrated in diagnostic specimens by electron microscopy. This method can be used not only to recognize mixed viral infections, but also to detect viruses that cannot be grown *in vitro* such as noroviruses. Although this technique is particularly useful for identifying enteric viruses, it has serious limitations, including limited sensitivity, high capital costs and the need for a skilled operator. Large numbers of viral particles (usually greater than 10^7 per ml) must be present in clinical samples to ensure detection. Sufficient concentrations of virus may be found in samples of faeces, scabs, vesicular fluids and wart tissues to allow detection. Moreover, viruses with similar morphology may be difficult to distinguish using electron microscopy. Members of a viral family usually have identical morphology and cannot be distinguished on this basis alone and the use of additional techniques, such as antibody labelling, may be necessary to definitively identify the viral species. In a few viral families, such as *Poxviridae* and *Reoviridae*, morphological differences do exist at the generic level.

A number of methods are used for the preparation of samples for examination. When preparing fluid samples, low-speed centrifugation to remove large particulate debris is followed by ultracentrifugation to sediment virus particles. Negative staining with heavy metal compounds such as phosphotungstic acid or uranyl acetate increases contrast so that the brighter virions stand out against a

dark background. The addition of antiserum to the specimen in immunoelectron microscopy enhances the sensitivity of the procedure by clumping virus particles and facilitating their recovery by centrifugation. Alternatively, antiserum, when applied to the copper grid used for examining the specimen, can aggregate virions in the specimen.

Immunofluorescence and immunohistochemistry

Antiviral antibodies labelled with fluorochromes can be used to detect viral antigens in clinical specimens, particularly respiratory specimens. Enzyme-labelled antiviral antibodies can also be used for this purpose. When using procedures involving immunofluorescence, a microscope with a powerful light source (xenon arc lamp or mercury vapour lamp) is required in order to visualize fluorescing material (Fig. 59.1). Specific antibodies labelled with fluorochromes, usually fluorescein isothiocyanate (FITC) or rhodamine isothiocyanate (RITC), are employed to detect virus-infected cells in specimens. The fluorochrome, exposed to light of a particular wavelength, emits light at a slightly longer wavelength, allowing demonstration of the labelled antibody bound to virus particles. The technique is used to detect virus-infected cells in fixed smears, cryostat sections or monolayers.

Direct and indirect immunofluorescent techniques can be used to demonstrate virus or viral antigen in specimens (Fig. 59.2). In direct immunofluorescence, conjugated antibody, specific for the particular virus, is applied to the fixed specimen. Following incubation, the specimen is washed to remove unbound antibody prior to microscopic examination. The indirect method employs unlabelled antiviral antiserum and a labelled antiglobulin, specific for the species of animal from which the unlabelled antiserum was derived. Immunofluorescence techniques are rapid and sensitive, but require careful interpretation as non-specific fluorescence can occur in certain specimens.

Figure 59.1 Schematic diagram of a fluorescence microscope.

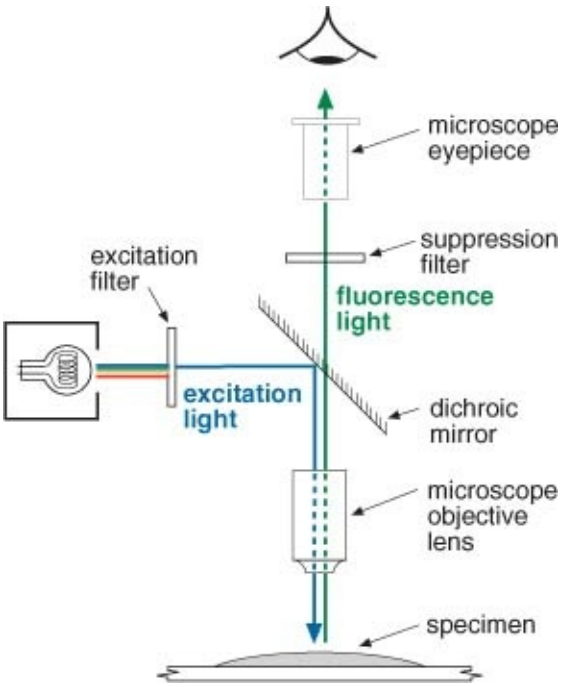
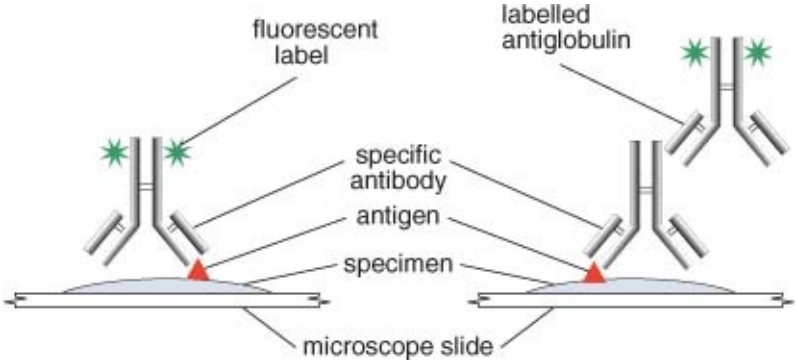


Figure 59.2 Direct and indirect immunofluorescent techniques for demonstrating viral antigen in cryostat (frozen tissue) sections, cell smears or monolayers. In the direct method, specific antibody is labelled with the fluorophore, whereas, in the indirect method, specific antibody bound to the viral antigen is demonstrated by the addition of labelled antiglobulin for the species of origin of the antibody.



Enzyme-labelled antibodies can also be used to identify viruses or viral antigen in clinical specimens. Horseradish peroxidase is the most common enzyme employed for conjugation with specific antiserum. After binding to viral antigen, antibody is identified by the addition of hydrogen peroxide and a benzidine derivative to the preparation. During the ensuing reaction, the colourless soluble benzidine derivative is converted to a coloured insoluble precipitate. For this technique, paraffin-embedded and resin-embedded tissues can be used and stained preparations do not fade when stored for long periods. Moreover, the stained preparations can be examined by conventional light

microscopy. Endogenous tissue peroxidases, however, may produce misleading reactions unless suitable controls are included.

An avidin–biotin system can be used for enhancing immunohistochemical reactions. Biotin can be covalently linked to antibody without interfering with its antigen-binding capacity. Avidin, bound to either a fluorochrome or an enzyme, has a high affinity for the biotin molecules linked to the antibody.

Solid-phase immunoassays

In these assays, either antigen or antibody is immobilized on to a surface. Suitable surfaces include polystyrene or synthetic membranes for enzyme immunoassays and radioimmunoassays; latex beads are often employed for agglutination tests. These assays are sensitive and relatively uncomplicated. Commercial tests based on these methods have been developed for particular viruses.

Radioimmunoassays employ antibodies labelled with radioisotopes, and the bound antibody is measured using a gamma counter. These types of assays have largely been superseded by safer immunoassay procedures. Enzyme immunoassays, usually termed enzyme-linked immunosorbent assays (ELISA), are currently widely used for the immunodiagnosis of viral infections. In these assays, antibodies are labelled with enzymes which produce a colour change when they react with appropriate substrates. This colour change can be assessed visually or spectrophotometrically. For the detection of virus, wells in polystyrene plates are coated with specific antiviral antibody and test material is added (Fig. 59.3). If present in the test material, viral antigen binds to the antibody during incubation and is not removed by washing. Enzyme-labelled antibody specific for the viral antigen is then added. Following incubation and washing, substrate for the enzyme is added; a colour change indicates a positive reaction.

Large numbers of samples can be processed quickly using ELISA. Rapid one-step assays suitable for use in veterinary practices are commercially available. Many of these kits utilize a membrane as the solid phase in order to increase the surface area on which antigen–antibody reactions can occur, thereby reducing incubation and washing times.

Latex particles, coated with antiviral antibodies, agglutinate in the presence of viral antigen. Special equipment is not required for these tests, which are simple and inexpensive. However, factors that decrease the reliability of these tests

include nonspecific reactions and prozone effects, due to high antigen concentrations.

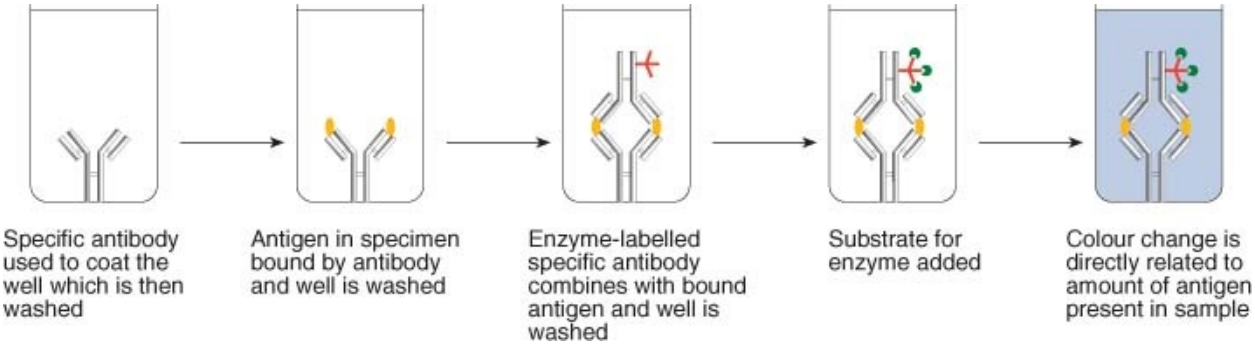
Immunodiffusion

This procedure is carried out in agar. The technique involves placing a fluid sample containing the virus under test in a well in the agar opposite a well containing antiserum. As the fluids diffuse out of the wells, a line of precipitate forms if the sample under test contains viral antigen. Although this test is easy to perform and inexpensive, it is relatively insensitive.

Complement fixation test for antigen detection

When antigen binds to antibody to form immune complexes, complement becomes activated and fixes to the complexes. In the complement fixation test, the test sample is added to a known antiserum which has been heat-inactivated to destroy complement. Following incubation, a precise amount of guinea-pig complement is added. If viral antigen is present in the test sample, immune complexes formed during incubation fix the guinea-pig complement. Sheep red cells treated with specific rabbit antibody are added as an indicator to detect residual complement activity. If the guinea-pig complement is not bound, indicating the absence of viral antigen from the test sample, red cell lysis occurs. If viral antigen is present in the test sample, red cell lysis does not occur. The CFT requires rigorous standardization of reagents and careful interpretation.

Figure 59.3 Steps in ELISA for detecting antigen in a test sample (antigen capture method).



Haemagglutination and haemadsorption

Viruses belonging to several families including *Orthomyxoviridae*,

Paramyxoviridae, *Adenoviridae*, *Parvoviridae* and *Togaviridae* can interact with erythrocytes of many animal species causing haemagglutination. This unique ability derives from viral glycoproteins (haemagglutinins) which attach to receptors on erythrocytes, resulting in the formation of aggregates which settle out of suspension. The haemagglutinins of orthomyxoviruses and paramyxoviruses allow these viruses to bind to neuraminic acid-containing receptors on erythrocytes. These viruses also possess neuraminidases which can destroy the erythrocyte receptors, causing dissociation. The surface structures (spikes) of influenza viruses which contain haemagglutinin and neuraminidase activity can be used to type influenza virus A isolates using specific antibody. As large numbers of virus particles are required to produce visible haemagglutination, this test is relatively insensitive.

The term haemadsorption is used to describe binding of erythrocytes to cells infected with haemagglutinating viruses. The haemagglutinating glycoproteins of these viruses are incorporated into cell membranes during viral replication.

Nucleic acid detection

The sensitivity and versatility of methods for the detection of viral nucleic acids have greatly improved in recent years and they are now becoming the method of choice for viral identification. These methods are particularly valuable when dealing with viruses which are either difficult to grow or cannot be grown *in vitro*. They are useful for latent infections in which infectious virus is absent and also for specimens containing inactivated virus. Cloned viral DNA is available for probing of samples and tissues by nucleic acid hybridization. An advantage of applying hybridization procedures to intact cells or tissue sections is that cellular integrity is retained allowing identification of the exact site of virus location. Hybridization techniques, however, have been largely superseded in recent years by the more sensitive polymerase chain reaction (PCR), which has the advantage of amplifying the target gene sequences. An important modification of PCR is reverse transcriptase polymerase chain reaction (RT-PCR), which has extended the application of the technique to RNA viruses. Because of their exquisite sensitivity, PCR techniques require rigorous standardization to exclude cross-contamination and to ensure reproducibility and reliability. When collecting clinical specimens, contamination from the environment or from other sources must be avoided. The possible presence of viral ‘passengers’ and degraded viral genomes becomes an important issue as the

sensitivity of detection tests increases. As manipulation of the amplified product is not necessary in real time PCR, this method is less prone to contamination problems than conventional PCR.

Diagnostic serology

Serological procedures can be used for the retrospective diagnosis of viral diseases and for epidemiological surveys. These procedures can be automated and diagnostic reagents for many viral pathogens are commercially available. Single blood samples from animals in susceptible populations suffice for establishing the prevalence of a disease. When using serological procedures for the diagnosis of endemic disease in flocks or herds, paired serum samples taken at an interval of at least 3 weeks are required to demonstrate rising antibody titres. Initial samples should be collected during the acute phase, when clinical signs are first evident, and the second samples during convalescence. A single blood sample may be adequate for diagnosis if reagents are available for demonstrating IgM antibodies, which are indicative of a primary immune response. Difficulties with the interpretation of serological tests may arise due to cross-reactions with antigenically-related viruses or if the acute-phase serum is collected too late to detect a primary immune response. In young animals, passively acquired maternal antibodies may persist for several months, which can lead to misinterpretation of results.

Enzyme-linked immunosorbent assay

The procedure used for carrying out ELISA to detect antibodies is different from that previously described for the demonstration of virus in specimens. The essential difference is that viral antigen is bound to the solid phase (polystyrene wells or membranes). Dilutions of test serum are added and allowed to react with the antigen. After washing, enzyme-conjugated antiglobulin is added and, following further incubation and washing, appropriate substrate is added. The intensity of the colour change is proportional to the amount of antibody in the test serum (Fig. 59.4). Either polyclonal or monoclonal labelled conjugates may be used; the choice depends on whether or not the immunoglobulin isotype is required for estimating the duration of the infection.

Figure 59.4 Steps in ELISA for detecting antibody in a test sample (indirect method).

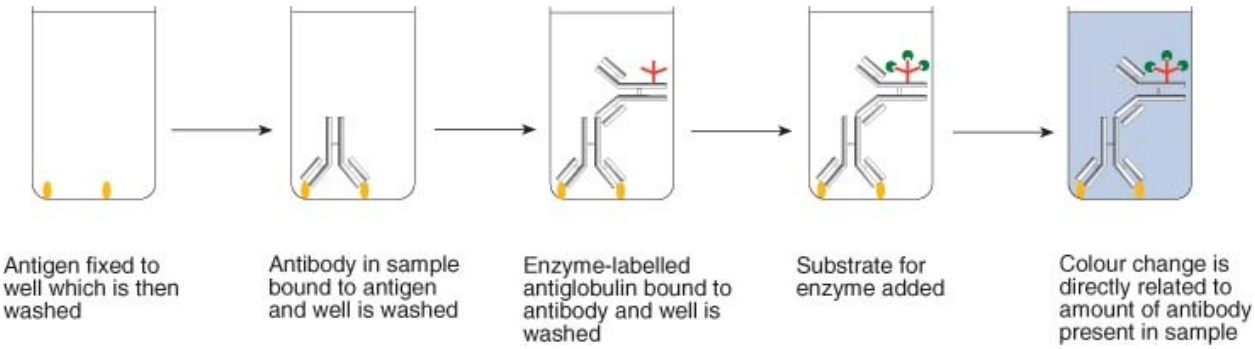
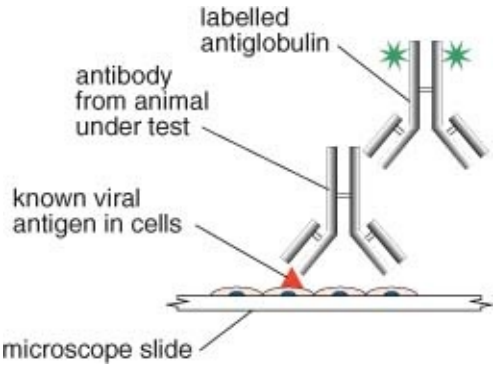


Figure 59.5 Indirect immunofluorescence technique for demonstrating antibodies in serum.



Immunofluorescence for antibody detection

Using indirect immunofluorescence, test serum is added to known viral antigen fixed on a microscope slide (Fig. 59.5). Following incubation, the slide is washed and, after the addition of fluorescein isothiocyanate-conjugated antiglobulin, incubated for a further period. The slide is again washed before examination using a fluorescence microscope. The test is both sensitive and rapid, but requires careful interpretation.

Serum neutralization test

This test is highly specific and sensitive for viruses which produce cytopathic effects (CPE). It is considered to be the definitive standard against which other serological tests are compared. Neutralizing antibodies usually correlate closely with immune protection. In this test, which is typically carried out in microtitre plates, a constant amount of stock virus is added to doubling dilutions of a test serum. Cells susceptible to the virus are added to the wells. The presence of neutralizing antibodies in the serum prevents infection of the cells and CPE. The titre of the serum is the highest dilution at which the virus is neutralized. The

neutralizing effect of test serum can also be evaluated in susceptible experimental animals and in chick embryos. Neutralizing antibodies tend to persist in recovered animals for long periods, often for many years.

Haemagglutination inhibition test

Because viruses in certain families have the ability to haemagglutinate, the inhibitory effect of antibodies on haemagglutination can be used for the diagnosis of infection with these viruses. The haemagglutination inhibition (HAI) test is specific, reliable and easily performed. The test, usually carried out in microtitre plates, involves serial twofold dilutions of serum to which a known concentration (four haemagglutinating units) of virus is added. The highest dilution of the serum which inhibits erythrocyte agglutination is the HAI titre of the test serum. Non-specific inhibitors of haemagglutination, sometimes present in sera, can be inactivated by heating or by treatment with kaolin, trypsin, periodate or bacterial neuraminidase.

Complement fixation tests

Due to difficulties with standardization, complement fixation tests have been superseded by more convenient diagnostic tests such as ELISA. Complement-fixing antibodies tend to appear before neutralizing antibodies but do not persist. Sera from some species are difficult to titrate by this method, owing to the presence of anti-complementary activity.

Western blotting (immunoblotting) technique

This test, which was developed primarily as a research procedure for the identification of antigenic proteins, can also be used for the diagnosis of viral disease. Purified virus, solubilized with an anionic detergent such as sodium dodecyl sulphate, is electrophoresed in a polyacrylamide gel. Separated proteins are transferred electrophoretically on to a nitrocellulose membrane, which is then washed, dried and cut into longitudinal strips. Following incubation in test sera, the strips are washed and incubated with enzyme-labelled antiglobulin. Addition of substrate produces an insoluble coloured product where test antibody is bound to the separated viral proteins.

Interpretation of test results

Because false-positive and false-negative results can occur in many test procedures, inclusion of positive and negative controls is essential. The sensitivity and specificity of a particular diagnostic test should be established. In the case of a diagnostic test applied to identify infected animals in a population, the sensitivity of the test, expressed as a percentage, is the number of animals identified as positive out of the total number of animals infected. The specificity of the test is the percentage of uninfected animals in which the result is negative. In order to detect all animals with an important viral infection, a test with high sensitivity is required. For laboratory confirmation of a viral infection in an individual animal, a test with high specificity is essential.

The detection of virus or the demonstration of antibody to a specific virus does not necessarily confirm an aetiological link with a disease state. With the increasing use of very sensitive, non-culture-based amplification methods capable of detecting latent or very low level persistent infections, care should be taken when interpreting results obtained by such sensitive laboratory methods. For the conclusive confirmation of test results, it may be necessary to demonstrate a correlation between the site of virus recovery and the nature and extent of lesions. Circumstantial evidence for the aetiological involvement of a virus in a clinically affected animal is supported by the recovery of the same virus from susceptible in-contact animals. Moreover, a rising antibody titre to the putative causal virus, demonstrated by the use of paired serum samples, is of diagnostic importance. Published reports on the potential importance of a similar disease syndrome and its aetiology may point to the requirement for particular laboratory investigations.

Further reading

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Chapter 60

Antiviral chemotherapy

Introduction

In common with bacterial infections, some viral infections may be confined to particular tissues or organs while other viral infections may become generalized, affecting many tissues and systems. When feasible, immunization is the preferred method for controlling viral infections in humans and animals. However, the absence of vaccines for many major viral diseases and the emergence of new virulent virus subtypes in animals, leading to zoonotic infections, has confirmed the necessity for effective antiviral chemotherapy not only for treatment of the human population but also for prophylactic medication of those exposed to sources of infection. Although antiviral chemotherapy is now a well established part of human treatment regimes, its place in veterinary medicine is less well defined. Cost, the necessity for rapid and reliable diagnosis before commencing treatment and the circumstances in which antiviral drugs can be used therapeutically have curtailed this form of therapy in animals. Despite present trends in the use of antiviral drugs in animals, it is probable that this form of medication will follow the pattern of antibacterial and anthelmintic use in animals as cost factors and other circumstances change in future years.

Unlike bacteria or fungi, viruses cannot replicate independently and, because of this restriction, they are obligate intracellular parasites. Host cells provide the requirements for viral replication including energy, protein synthesis and RNA or DNA replication. Because viruses utilize the host cell’s biosynthetic systems for replication, development and delivery of antiviral therapy present many challenges relating to efficacy and toxicity for the host. Most antiviral drugs have narrow therapeutic margins and the efficacy of antiviral chemotherapy is further complicated by viral latency, a feature of many herpesviruses.

Virus replication occurs in sequential steps. Attachment and penetration follow the binding of viral attachment protein to a cell surface receptor. Once within the cell, uncoating with release of the viral genome follows. Expression of the viral genome, replication of the genome and translation of viral proteins is followed

by post-translational modifications of viral proteins, assembly of virion components and release by budding or cell lysis.

Effective antiviral drugs inhibit virus-specific events related to virus replication rather than host cell synthetic activities, such as nucleic acid or protein synthesis. Most antiviral drugs interfere with viral-encoded enzymes or viral structures essential for replication. Many antiviral drugs are nucleic acid analogues which interfere with DNA and RNA synthesis. Other mechanisms of action include interference with viral cell binding, interruption of virus uncoating and inhibition of virus progeny release from infected host cells. In addition to their antiviral activity, some antiviral substances such as interferons possess immunomodulatory activity. A major obstacle in the development of antiviral drugs is the inherent toxicity of inhibitory compounds for host cells. The other limiting factor in antiviral chemotherapy is the development of resistance.

Development of antiviral drugs

Developments in antiviral chemotherapy lagged behind the sustained progress achieved in antibiotic discoveries from the 1940s to the 1960s. From the 1970s to the present, antiviral chemotherapy has progressed steadily in response to the risks posed by the threat of influenza pandemics, the rapid spread of human immunodeficiency virus across the world and the inadequacy of conventional chemotherapy for the treatment of viral infections in patients with immunological incompetence. Some of the major developments relating to the synthesis and subsequent use of antiviral drugs are presented in Fig. 60.1.

Viral infections and strategies for interrupting virus replication

All viruses which cause disease in humans and animals replicate in host cells and, accordingly, they follow a similar sequence during their replicative cycle. Although they may share similar features during replication, diagnosis of viral infections often presents clinicians and laboratory staff with many challenges. Acute viral infections have relatively short incubation periods, whereas slowly progressing viral infections have long incubation periods. A consequence of chronic viral infections is the prolonged shedding of virus by the infected

animal. Latent viral infections are characterized by periodic reactivation of virus replication, often related to stressful environmental conditions or immunosuppression associated with intercurrent infection, administration of immunosuppressive drugs or other factors. Latency, a feature of some families of viruses such as herpesviruses, is not affected by treatment with antiviral drugs. Because currently available antiviral drugs are virustatic, successful antiviral chemotherapy relies on host immunological competence. For viral infections such as those caused by retroviruses, the host's immune response is unable to clear the viral pathogens from the tissues and life-long antiviral therapy is required.

Antiviral therapy is aimed at preventing virus entry into host cells, interfering with uncoating, genome replication or assembly and release of virus from host cells. Stages of viral replication and possible points at which antiviral drugs or components of the immune system can interrupt replicative events are presented in Table 60.1. The major classes of antiviral drugs, grouped according to their modes of action, are reviewed briefly in Table 60.2.

Immunomodulators

A number of immunomodulatory drugs which promote protective immune response to viral pathogens have been described. Some of these compounds enhance innate immune responses and, although they lack direct antiviral effects, they may induce cytokines and chemokines with antiviral activity. Among the important cytokines with antiviral activity, interferons feature prominently. Interferons have important immunomodulatory activity also. They bind to specific cell surface receptors which initiate intracellular events including induction of particular enzymes, inhibition of cell proliferation and enhancement of immune responses.

There are two types of functionally overlapping interferons, type 1 and type 2. Type 1 interferons, which mediate the early immune responses to viral infections, include variants of interferon-α (IFN-α) and a single interferon-β (IFN-β). Mononuclear phagocytes are the principal source of IFN-α, while fibroblasts and other cells produce IFN-β. Type 2 interferon or interferon-γ (IFN-γ) is produced following antigenic stimulation of T cells and NK cells.

The primary role of type 1 interferons is defence against viruses. They induce an antiviral state in most nucleated cells and also activate NK cells. Interferon-γ

has indirect antiviral activity but also functions as an immunomodulatory cytokine through its ability to activate macrophages and thereby contribute to the initiation of specific antiviral resistance mediated by antibodies and T lymphocytes.

Type 1 interferons are produced by infected cells when they detect virus replication and also by cells associated with innate immune responses which detect the presence of virus by toll-like receptors. Whether produced by infected cells or sentinel immune cells, type 1 interferons act on nearby cells inhibiting virus replication by a number of mechanisms at different stages of the replicative cycle. Among the antiviral mechanisms associated with IFN-α and IFN-β are production of a double-stranded RNA-dependent protein kinase R, PKR, a latent endonuclease, RNaseL, and Mx proteins. Release of PKR interferes with translation of viral mRNA. The RNaseL degrades viral mRNA and also ribosomal RNA, thereby inhibiting protein synthesis. The Mx proteins may block transcription of a range of RNA viruses and also interfere with virus particle assembly. These proteins, however, have little effect on DNA viruses. Interferon-γ induces only PKR but, in addition, has a major role in macrophage activation which results in phagocytosis of viruses and virus-infected cells, destruction of virus-infected cells and production of tumour necrosis factor-α (TNF-α) and IFN -α.

Figure 60.1 Major developments relating to the discovery, synthesis and therapeutic use of antiviral drugs from the 1950s onwards. In some instances, many years elapsed between the discovery of a drug and the demonstration of its antiviral activity.

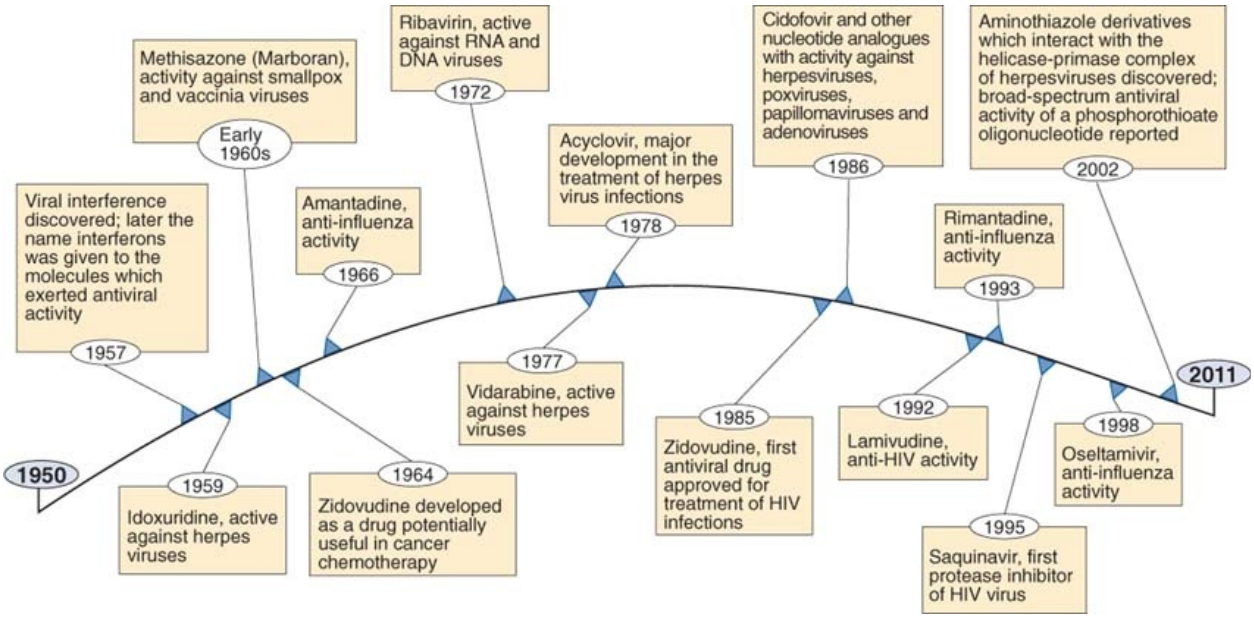


Table 60.1 Categories of antiviral drugs and immune components indicating the stages at which they act during virus replication.

Categories of drugs or immune components with antiviral activity	Stage of replication where antiviral drugs or immune components act
Peptide analogues of attachment proteins; fusion protein inhibitors; neutralizing antibodies	Attachment to host cell
Ion channel blockers	Uncoating
Inhibitors of viral DNA polymerase, RNA polymerase, reverse transcriptase	Transcription of viral genome
Nucleoside analogues	Replication of viral genome
Interferons, antisense oligonucleotides	Translation of viral proteins
Protease inhibitors	Post-translational changes in proteins
Interferons	Assembly of virion components
Neuraminidase inhibitors; specific antibodies plus complement; destruction of infected cells by cytotoxic T cells or NK cells	Release of virions by budding or cell lysis

Interferons produced by recombinant technology and also by chemical synthesis are available for treating a number of viral infections in humans and also for use in animals. Two subtypes of recombinant IFN-α (rIFN-α), rIFN-α-2a and rIFN-α-2b, have been used for treating viral hepatitis and other viral diseases. Recently, rIFN-α-2a modified by covalent attachment of a branched-chain polyethylene glycol moiety has become available. This modified interferon, referred to as pegylated interferon, is absorbed slowly from the injection site and has a much longer half-life than conventional interferon.

Imiquimod and inosine pranobex are among the immunostimulating drugs which have been used to enhance immune responses to viral infections. Inosine pranobex is reported to have immunopotentiating activity and has been used to treat superficial infections with herpesviruses. Imiquimod, a novel

immunomodulating agent, binds to toll-like receptors TLR-7 and TLR-8 and stimulates innate immune responses including production of interferons.

Ion channel blocking compounds

The anti-influenza drugs amantadine and rimantadine inhibit virus replication at an early stage in the replicative cycle of influenza A viruses. The mechanism of action of these antiviral drugs relates to virus uncoating shortly after endocytosis of virus by the host cell.

Amantadine

The antiviral drug amantadine, which has long been known as a specific inhibitor of influenza A virus, is a tricyclic amine. Amantadine inhibits an early step in the replication of influenza A virus and its antiviral activity is confined to influenza A virus.

Following attachment to host cell sialic acid moieties on cell surface glycoproteins by means of influenza envelope glycoprotein spikes or haemagglutinins, the virus is endocytosed. At this early stage of its replication cycle, the virus is contained in a membrane-bound compartment, the endosome. As part of its normal function, the endosome becomes acidified. The low pH causes a conformational change in the virion haemagglutinin protein, and fusion of the virion envelope and the endosomal membrane occurs, releasing the nucleocapsid into the cytoplasm of the host cell. However, in the presence of amantadine, the matrix protein, M1, does not dissociate from the ribonucleoprotein which remains in the cytoplasm instead of entering the nucleus. The M2 protein in the nucleocapsid seems to form a polymeric tube-like structure through which hydrogen ions from the acidified endosome enter the virion and dissociate M1 from the ribonucleoprotein (Fig. 60.2). By interfering with the ion channel function of the M2 protein, amantadine inhibits acid-mediated dissociation of the ribonucleoprotein complex early in replication, a process essential for uncoating of the single-stranded RNA genome.

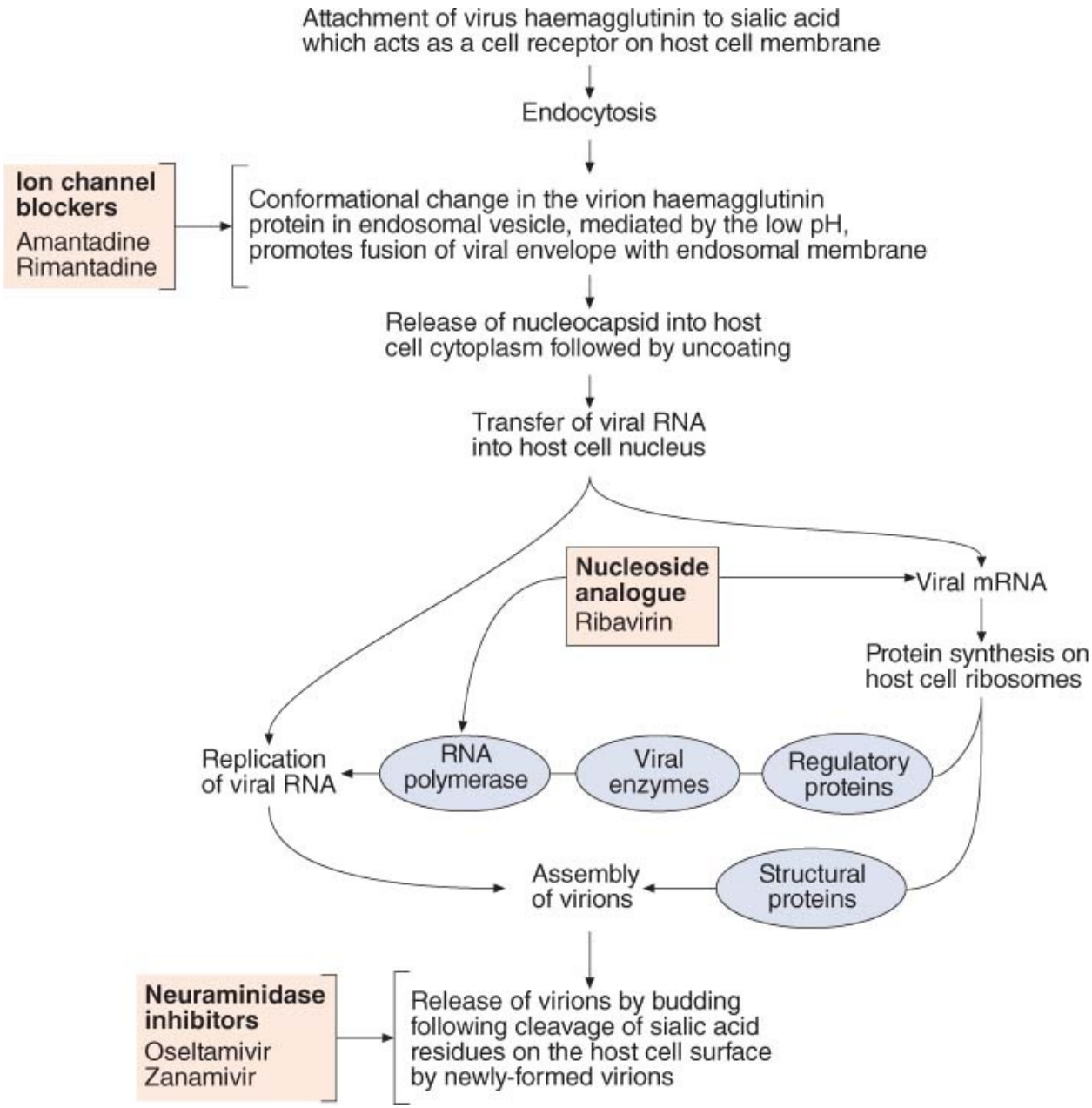
Primary drug resistance to amantadine is uncommon in human isolates of influenza A virus. Resistance in some avian and swine isolates has been reported. Single nucleotide changes leading to amino acid substitutions in the transmembrane region of M2 protein have been associated with resistance to amantadine.

Table 60.2 The chemical nature, mode of action and antiviral spectrum of selected antiviral drugs.

Antiviral drug	Chemical nature / Mode of action	Antiviral spectrum	Comments
Acyclovir	Nucleoside analogue / Inhibits viral DNA polymerase	Herpesviruses, particularly herpes simplex virus	Not effective against latent viral infections
Amantadine	Tricyclic amine / Ion channel blocker which interferes with virus uncoating	Influenza A virus; other viruses which encode proteins that form ion channels may be susceptible	Published reports on the antiviral activity of amantadine in animals are limited
Amprenavir	Amino sulphonamide non-peptide protease inhibitor / Active site inhibitor of HIV protease	Human immunodeficiency virus	The therapeutic role of protease inhibitors in viral diseases of animals is not well defined
Cidofovir	Cytidine nucleotide analogue / Inhibits viral DNA synthesis	Herpesviruses, poxviruses, papillomaviruses, adenoviruses	Long tissue half-life allows infrequent dosing
Delaviridine	Bis-heteroarylpiperazine compound / Disrupts the catalytic activity of HIV-1 reverse transcriptase	Human immunodeficiency type 1 virus	Cross-resistance to other drugs in this class usually applies
Enfuvirtide	Synthetic peptide / Prevents fusion of HIV-1 with host cell membrane	Human immunodeficiency type 1 virus	Retains activity against viruses which have become resistant to other classes of antiretroviral drugs
Famciclovir and penciclovir	Nucleoside analogues / Inhibit viral DNA polymerase	Herpesviruses	Development of resistance during clinical use is reported to be low
Foscarnet	Inorganic pyrophosphate analogue / Inhibits viral DNA polymerase	Herpesviruses; some activity against retroviruses	Its clinical use is limited to herpesviruses
Ganciclovir	Acyclic guanine nucleoside analogue / Inhibits viral DNA synthesis and interferes with viral rather than cellular DNA polymerase	Herpesviruses	It is especially active against cytomegalovirus
Idoxuridine	Iodinated thymidine analogue / Incorporated into DNA with interference in nucleic acid synthesis and viral gene expression	Herpesviruses and poxviruses	Because of its toxic effects if given systemically, it is used for topical treatment only
Immunomodulators, including interferons	Proteins, other novel compounds / Inhibit virus replication or promote protective antiviral immune responses	Although most RNA viruses are inhibited by interferons, many DNA viruses are relatively insensitive to their antiviral effects	Because of the complex interactions between interferons and other components of the immune system, these molecules may have both direct and indirect antiviral activity

Lamivudine	Nucleoside analogue / Inhibits reverse transcriptase activity of retroviruses and also inhibits the DNA polymerase of hepatitis B virus	Retroviruses and hepatitis B virus	When combined with zidovudine, a marked synergistic antiviral effect results
Nevirapine	Dipyridodiazepinone compound / Disrupts the catalytic activity of HIV-1 reverse transcriptase	Human immunodeficiency type 1 virus	Cross-resistance with other drugs in this class usually occurs
Oseltamivir	Analogue of sialic acid / Interacts with neuraminidase, inhibiting its activity	Influenza A virus and influenza B virus	Can be used prophylactically and therapeutically
Ribavirin	Purine nucleoside analogue / Inhibits RNA polymerase of influenza viruses; appears to have multiple sites of action against a number of RNA and DNA viruses	Although potentially active against a number of RNA and DNA viruses, it is effective against a limited number of viral infections	Causes suppression of bone marrow; inhibits lymphocyte responses and alters cytokine profiles *in vitro*
Rimantadine	Tricyclic amine / Ion channel blocker which interferes with virus uncoating	Influenza A virus; may be effective against some viruses which utilize ion channels	Published reports on the antiviral activity of rimantadine in animals are limited
Ritonavir	Peptidomimetic HIV protease inhibitor / Binds to the active site of HIV protease	Human immunodeficiency virus	The therapeutic role of protease inhibitors in viral diseases of animals is not well defined
Saquinavir	Peptidomimetic hydroxyethylamine HIV protease inhibitor / Binds to the active site of HIV protease	Human immunodeficiency virus	Can be used in combination with nucleoside reverse transcriptase inhibitors
Stavudine	Nucleoside analogue / Reverse transcriptase inhibitor	Human immunodeficiency virus	Cross-resistance to multiple nucleoside analogues has been reported
Trifluridine	Fluorinated analogue of thymidine / Competitive inhibitor of the incorporation of thymidine into DNA	Wide antiviral activity *in vitro*; used topically for herpesvirus keratoconjunctivitis in humans and ocular herpesvirus infections in animals	Unsuitable for systemic use because of its toxicity
Valacyclovir	L-valyl ester of acyclovir, a nucleoside analogue/Inhibits viral DNA synthesis	Herpesviruses	Enhanced oral bioavailability offers many advantages over acyclovir
Zanamivir	Analogue of sialic acid / Interacts with neuraminidase, inhibiting its activity	Influenza A virus and influenza B virus	Can be used for the prevention and treatment of influenza viruses
Zidovudine	Thymidine analogue / Terminates elongation of proviral DNA chain	Wide range of mammalian retroviruses	Cross-resistance to other thymidine analogues occurs

Figure 60.2 Points in the replicative cycle of an influenza virus, an RNA virus, at which antiviral drugs interfere with replication or with release of newly-formed viruses from host cell surface.



Rimantadine

Like amantadine, rimantadine is a specific inhibitor of influenza A virus. Both drugs can be used for the treatment and prevention of infection. Rimantadine, an α-methyl derivative of amantadine, is a tricyclic amine with much greater antiviral activity than amantadine. In addition to its antiviral activity, rimantadine has inhibitory activity in vitro for *Trypanosoma brucei*.

As described for amantadine, the mechanism of action of rimantadine relates to interference with virus uncoating shortly after endocytosis. The functioning of

the M2 protein in the nucleocapsid of the virus, which is associated with uncoating, is disrupted by rimantadine. When this occurs, acid-mediated dissociation of the ribonucleoprotein complex, a process essential for uncoating of the single-stranded RNA genome, does not take place.

Primary drug resistance to rimantadine in human isolates of influenza A virus is uncommon. Resistance in some avian and swine isolates has been reported. Alteration of M2 protein or haemagglutinin protein may contribute to the development of resistance. Rimantadine and amantadine share cross-susceptibility and resistance patterns. Resistance may occur more readily in immunocompromised patients treated with these two antiviral drugs than in individuals with a normal immune system.

Neuraminidase inhibitors

Inhibitors of influenza neuraminidase activity interfere with release of influenza A virus and influenza B virus from host cells. When influenza viruses complete their replicative cycle, they bud from the cell membrane. Release of newly formed virions from infected cells requires neuraminidase for cleavage of sialic acid residues from the cell membrane envelope present on the budding virions. If this does not take place, the binding of haemagglutinin protruding from the virion surface with persisting sialic acid residues on newly released adjacent virions causes aggregation of the virions on the cell surface.

The neuraminidase inhibitors oseltamivir and zanamivir are sialic acid analogues which specifically inhibit influenza A virus and influenza B virus neuraminidase activity (Fig. 60.2).

Oseltamivir

Studies in laboratory animals have demonstrated that neuraminidase activity at the time of virion release is essential for disease production. Such studies have also shown that oseltamivir and zanamivir, both neuraminidase inhibitors, were effective antiinfluenza drugs when used prophylactically and also therapeutically.

Oseltamivir is a transition-state analogue of sialic acid which is a potent selective inhibitor of influenza A and B neuraminidase activity. The interaction of oseltamivir with neuraminidase causes a conformational change in the enzyme’s active site which inhibits its activity. As a consequence of

neuraminidase inhibition, aggregation and clumping of virions occur at the infected cell surface, reducing the spread of virus within the respiratory tract.

Although influenza variants containing haemagglutinin and neuraminidase mutations which are less inhibited by oseltamivir than wild-type viruses have been reported, such isolates have reduced infectivity and virulence in animal models.

Zanamivir

The neuraminidase inhibitor, zanamivir, like oseltamivir, is a sialic acid analogue which specifically inhibits influenza A virus and influenza B virus neuraminidase activity. Unlike oseltamivir, zanamivir is not bio-available if administered orally and it is given as a dry powder by intranasal inhalation or by oral inhalation in humans. Neuraminidase inhibition by zanamivir results in aggregation and clumping of viruses at the infected cell surface, reducing the spread of virus within the respiratory tract.

Resistance to zanamivir is associated with mutations in viral haemagglutinin or neuraminidase. Zanamivir-resistant variants usually have decreased infectivity for laboratory animals.

Antiviral drugs which inhibit viral genome replication

Many antiviral drugs inhibit viral genome replication. Most of these drugs are nucleoside analogues which inhibit viral polymerases, especially DNA polymerases. Before these compounds can exert their antiviral effect, they must undergo intracellular phosphorylation to the active triphosphate form. Phosphorylated nucleoside analogues inhibit polymerases by competing with natural substrates and they are usually incorporated into the growing DNA chain where they often terminate elongation.

Acyclovir and related drugs in this category, which include famciclovir, penciclovir, ganciclovir and valacyclovir, are all acyclic nucleoside analogues. These antiviral drugs, which are especially effective against herpesviruses, inhibit viral DNA polymerase or inhibit viral DNA synthesis by slowing and eventually terminating chain elongation (Fig. 60.3).

Acyclovir

The nucleoside analogue acyclovir is structurally similar to the natural nucleoside deoxyguanosine (Fig. 60.4). This acyclic guanosine nucleoside was among the first antiviral drugs approved for clinical use in humans and its antiviral activity is restricted to herpesviruses. Acyclovir has selective activity against a number of herpesviruses including herpes simplex and varicella-zoster viruses. There is limited published information on the clinical efficacy of acyclovir for the treatment of herpesvirus infections in animals.

Acyclovir inhibits DNA polymerase in a number of herpesviruses. Before this antiviral drug can exert its effect, however, it must be phosphorylated. Herpes simplex virus encodes a thymidine kinase which activates the drug by phosphorylation to acyclovir monophosphate, and host cell enzymes complete the conversion to the diphosphate form and finally to the triphosphate form. Because initial phosphorylation does not take place in uninfected cells, the production of acyclovir triphosphate is confined to herpesvirus-infected cells. Acyclovir triphosphate competitively inhibits viral DNA polymerases and, to a lesser extent, cellular DNA polymerases. The acyclovir triphosphate also becomes incorporated into viral DNA where it acts as a chain terminator. A feature of herpesvirus infections in humans and animals is latency, which is associated with periodic symptomatic and asymptomatic recurrence. Antiviral drugs do not eliminate latent viral infections.

Figure 60.3 Replicative cycle of a herpesvirus, a DNA virus, and point at which antiviral drugs interfere with replication.

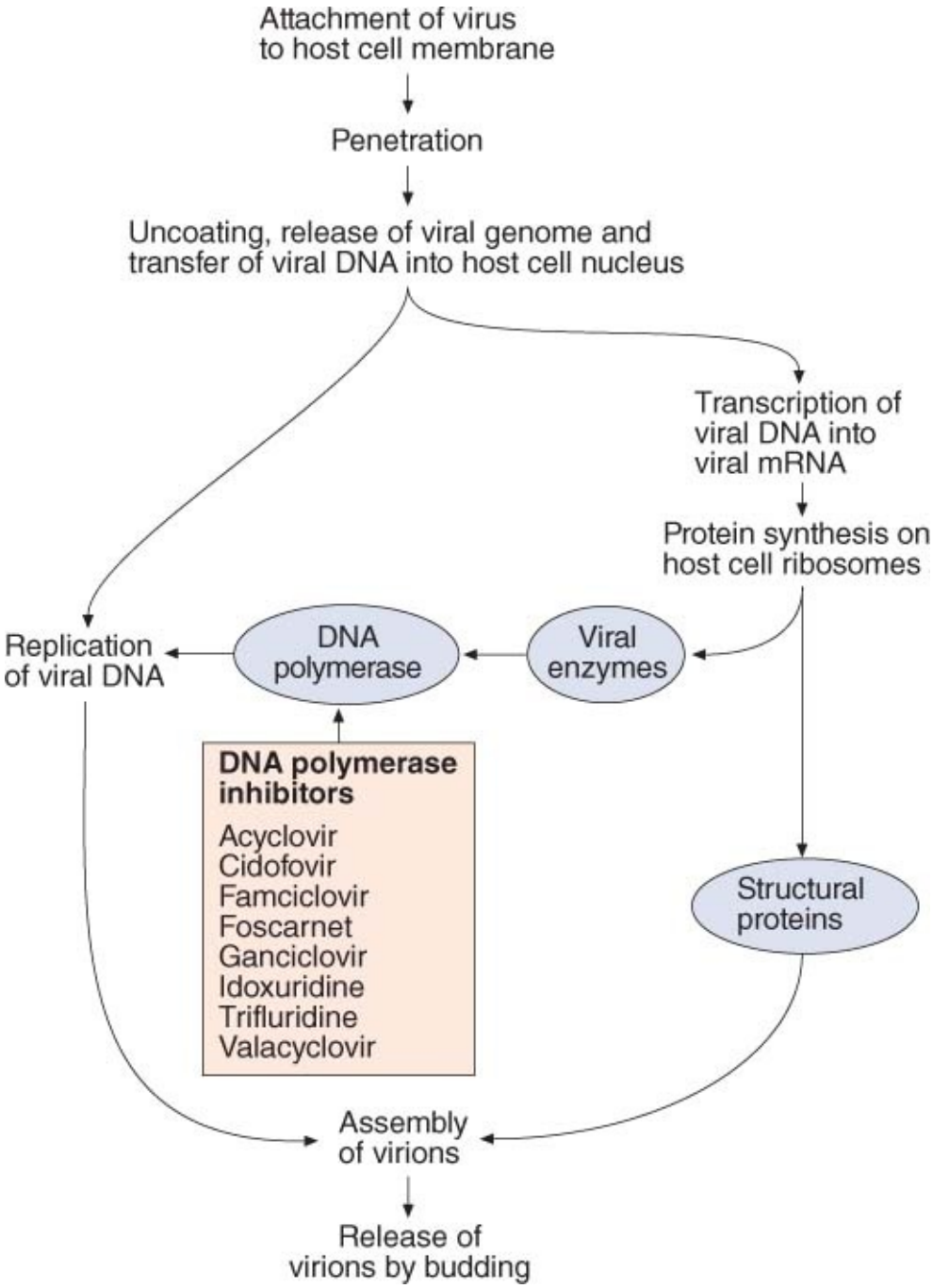
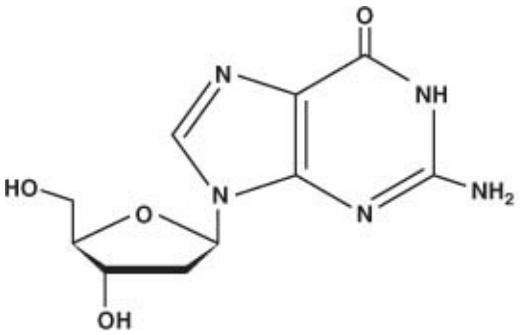
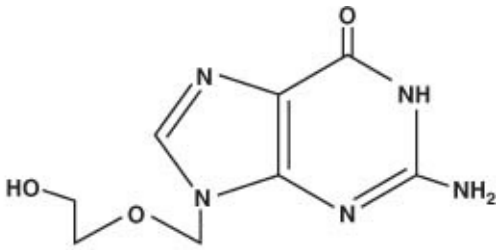


Figure 60.4 Structural similarity of the nucleoside analogue, acyclovir, to the natural nucleoside deoxyguanosine.



Deoxyguanosine



Acyclovir

Treatment with acyclovir has been reported to decrease mortality in parakeets and psittacine birds with herpesvirus infections. This antiviral drug is also used for treating feline viral rhinotracheitis.

Resistance to acyclovir is linked to absence or partial production of thymidine kinase, altered thymidine substrate specificity or alteration of viral DNA polymerase in a manner which prevents acyclovir binding.

Cidofovir

The cytidine nucleotide analogue cidofovir has inhibitory activity against herpesviruses, poxviruses, papillomaviruses and adenoviruses. Cidofovir has a long tissue half-life and in many animal models long intervals between doses do not diminish its antiviral activity. Single doses may be effective against poxvirus infections.

Cidofovir inhibits viral DNA synthesis by slowing and gradually terminating chain elongation. Resistance to cidofovir is attributed to mutations in viral DNA polymerase.

Famciclovir and penciclovir

A prodrug derivative of penciclovir, famciclovir is metabolized to the active compound, penciclovir *in vivo*. Famciclovir is a diacetyl ester of 6-deoxypenciclovir. Penciclovir is an acyclic guanine nucleoside analogue and its mode of action and spectrum of activity are similar to those of acyclovir. This antiviral drug is an inhibitor of viral DNA synthesis, and penciclovir triphosphate acts as a competitive inhibitor of viral DNA polymerase. Penciclovir, which inhibits herpesviruses in a manner similar to acyclovir, becomes concentrated in and persists in infected cells to a greater extent than

acyclovir.

Resistance to penciclovir and famciclovir among herpesviruses develops in the same manner as observed with acyclovir. However, resistance during clinical use is reported to be low.

Foscarnet

The antiviral drug foscarnet is an inorganic pyrophosphate analogue which inhibits viral DNA polymerase by binding directly to the pyrophosphate binding site. Foscarnet, trisodium phosphonoformate, inhibits replication of herpesvirus DNA polymerases and HIV reverse transcriptase. Its clinical use, however, is limited to herpesvirus infections.

Foscarnet reversibly blocks the pyrophosphate binding site of the viral polymerase in a non-competitive manner and inhibits cleavage of pyrophosphate from deoxynucleoside triphosphates. Although foscarnet inhibits DNA polymerase by a mechanism that differs from nucleoside analogues, many mutant viruses which are resistant to nucleoside analogues are also resistant to foscarnet. Herpesvirus resistance to foscarnet is associated with point mutations in the gene encoding viral DNA polymerase.

Ganciclovir

The acyclic guanine nucleoside analogue, ganciclovir, is similar in structure to acyclovir. Ganciclovir has inhibitory activity against many herpesviruses but it is especially active against cytomegalovirus. Phosphorylation of ganciclovir within herpes-infected cells is initiated by viral kinases. Ganciclovir diphosphate and ganciclovir triphosphate are formed by cellular enzymes. The triphosphate form is a competitive inhibitor of deoxyguanosine triphosphate incorporation into DNA and it preferentially interferes with viral DNA polymerase rather than the DNA polymerase of host cells. Incorporation of ganciclovir triphosphate into viral DNA does not result in chain termination as is the case with acyclovir triphosphate incorporation. When ganciclovir triphosphate is incorporated into host cells, it exerts a radiomimetic effect, especially on bone marrow cells.

Resistance to ganciclovir results from reduced intracellular phosphorylation and mutations in viral DNA polymerase.

Idoxuridine

The iodinated thymidine analogue, idoxuridine, was among the first specific

antiviral compounds used therapeutically. Its antiviral activity does not compare with the high selectivity of modern antiviral compounds. Idoxuridine inhibits the replication of a number of DNA viruses including herpesviruses and poxviruses. It resembles acyclovir in its mode of action as it is first converted to a monophosphate nucleotide by viral thymidine kinase with subsequent synthesis of diphosphate and triphosphate nucleosides by cellular kinases. Idoxuridine triphosphate inhibits viral and cellular DNA synthesis. Much of its antiviral activity is attributed to its incorporation into viral nucleic acid with subsequent interference with viral gene expression. Because of its toxicity following systemic administration, it is used only as a topical agent. Ophthalmic solutions containing idoxuridine are used for treating herpesvirus keratitis in animals.

Resistance to idoxuridine, which develops quickly, is due to mechanisms similar to those that mediate resistance to acyclovir. These include altered thymidine substrate specificity and alteration of viral DNA polymerase resulting in interference with idoxuridine binding.

Ribavirin

This antiviral drug inhibits the replication of a wide range of DNA and RNA viruses. Ribavirin, a purine nucleoside analogue, is phosphorylated successively by cellular kinases to ribavirin-monophosphate, ribavirin-diphosphate and ribavirin-triphosphate nucleotides. The triphosphate form inhibits the RNA polymerase of the influenza viruses, and other mechanisms of antiviral activity probably operate in the inhibition of a range of RNA and DNA viruses. The mode of action of ribavirin is probably relatively non-specific (Fig. 60.2).

Ribavirin, administered as an aerosol, is used to treat severe respiratory syncytial virus bronchopneumonia in children. Oral ribavirin, combined with pegylated interferon-a-2a, is active against hepatitis C virus infection. Prompt administration of ribavirin to patients with Lassa fever caused by an arenavirus results in a dramatic reduction in mortality. Ribavirin is active against feline infectious peritonitis virus *in vitro* but, when used to treat cats experimentally infected with calicivirus, it induced bone marrow suppression and other adverse reactions.

The relatively broad antiviral spectrum of ribavirin is often accompanied by an associated lack of potency at non-toxic levels. Lack of specificity of an antiviral drug often correlates with limited clinical activity and this applies to ribavirin also. Undesirable effects of ribavirin therapy include bone marrow suppression

and oxidative damage to membranes.

Emergence of resistance to ribavirin has been recorded infrequently. Because of the uncertainty associated with its mode of action, the underlying mechanisms leading to the development of resistance are not well defined.

Trifluridine

This fluorinated analogue of thymidine has *in vitro* inhibitory activity against a wide range of viruses including herpesviruses, vaccinia virus and some adenoviruses. Trifluridine is highly toxic and unsuitable for systemic use. The triphosphate form of trifluridine is a competitive inhibitor of the incorporation of thymidine triphosphate into DNA. This antiviral compound, which was synthesized more than 40 years ago, is still the treatment of choice for herpesvirus keratoconjunctivitis in humans. It is used as a 1% ophthalmic solution. If given systemically, trifluridine is reported to be mutagenic and teratogenic in animals. It is used topically for the treatment of herpetic keratitis in animals.

Resistance to trifluridine has been described in clinical isolates of herpesviruses. The basis of resistance is attributed to altered thymidine kinase substrate specificity.

Valacyclovir

The L-valyl ester of acyclovir, valacyclovir, is efficiently absorbed after oral administration and rapidly converted to acyclovir. The enhanced oral bioavailability of acyclovir produced from valacyclovir has allowed the development of better treatment regimes than with acyclovir.

Valacyclovir inhibits viral DNA synthesis by the same mechanisms outlined for acyclovir. In common with acyclovir, valacyclovir has inhibitory activity against herpesviruses and is reported to be especially effective against herpes zoster infections in humans.

Antiretroviral drugs

Antiretroviral fusion inhibitors

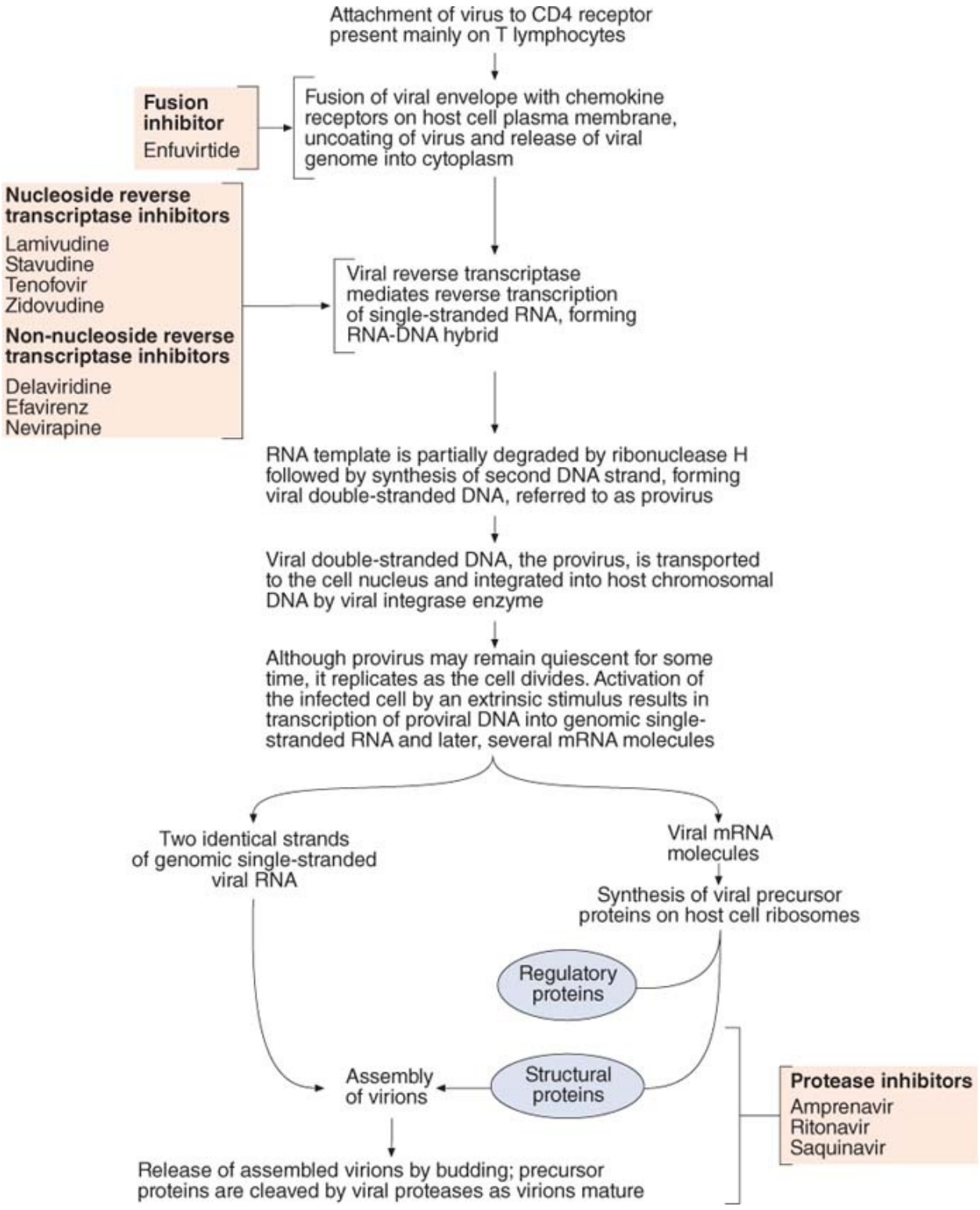
Antiviral drugs which interfere with virus attachment and entry into host cells

prevent subsequent stages of virus infection. Such drugs also provide an opportunity for components of the immune system to clear viruses from body fluids and host tissues.

Enfuvirtide

The synthetic peptide enfuvirtide prevents the fusion of HIV-1 with the host cell outer membrane, thereby preventing infection of $CD4^+$ T cells. The sequence of the synthetic peptide is derived from a portion of the transmembrane gp41 region of HIV-1 which mediates fusion of the virus membrane lipid bilayer with that of the host cell. Enfuvirtide inhibits infection of $CD4^+$ T cells by free virus particles and also prevents cell-to-cell transmission of HIV-1 *in vitro* (Fig. 60.5). Because of its unique mechanism of action, enfuvirtide retains activity against viruses which have become resistant to other classes of antiretroviral drugs. Human immunodeficiency type 1 strains develop resistance to enfuvirtide through specific mutations in the enfuvirtide-binding domain of gp41.

Figure 60.5 Replicative cycle of a retrovirus such as human immunodeficiency virus type 1, an RNA virus, and points at which antiviral drugs interrupt virus replication or interfere with virus maturation.



Non-nucleoside reverse transcriptase inhibitors

These antiviral drugs selectively inhibit HIV-1 by binding to a site on the reverse

transcriptase that differs from that bound by natural nucleoside analogues. These compounds induce conformational change in reverse transcriptase which disrupts its catalytic activity. Delaviridine and nevirapine, two examples of these antiviral drugs, are non-competitive inhibitors of HIV-1 reverse transcriptase. These drugs do not require intracellular phosphorylation to acquire antiviral activity.

Delaviridine

The non-nucleoside reverse transcriptase inhibitor delaviridine is a bis-heteroarylpiperazine compound which selectively inhibits HIV-1. Delaviridine induces a conformational change in reverse transcriptase which disrupts its catalytic activity. Because the target site of this antiviral drug is HIV-1-specific and is non-essential for the enzyme, resistance can develop quickly. If resistance to delaviridine develops, cross-resistance to other antiviral drugs in this class usually follows.

Nevirapine

In common with other non-nucleoside reverse transcriptase inhibitors, nevirapine binds to sites on the enzyme different from the substrate. Nevirapine is a dipyridodiazepinone compound which selectively inhibits HIV-1 but not HIV-2. This antiviral drug is a non-competitive inhibitor which binds to a site on HIV- 1 reverse transcriptase that is distant from the active site, inducing a conformational change in the enzyme which disrupts its catalytic activity. Because the target site is HIV-specific and is not essential for the functioning of the enzyme, resistance to nevirapine develops rapidly. If resistance to nevirapine occurs, cross- resistance to other antiviral drugs in this class usually follows.

Nucleoside reverse transcriptase inhibitors

A number of nucleoside analogues, including lamivudine, stavudine and zidovudine, are inhibitors of HIV reverse transcriptase. Stavudine is a synthetic nucleoside analogue and lamivudine is a cytosine analogue.

These nucleoside reverse transcriptase inhibitors are activated intracellularly by phosphorylation with cellular kinases, and their triphosphate forms competitively inhibit reverse transcriptase. The triphosphate form of these antiviral agents terminates elongation of the proviral DNA chain (Fig. 60.5).

Lamivudine

The cytosine analogue lamivudine is a reverse transcriptase inhibitor of HIV-1 and HIV-2 and an inhibitor of the DNA polymerase of hepatitis B virus. Cellular enzymes convert lamivudine to the triphosphate form which competitively inhibits hepatitis B DNA polymerase.

Resistance to lamivudine, which occurs with single amino acid substitutions, develops rapidly in patients with HIV infections treated with this drug alone. Such resistance confers cross-resistance to related antiviral drugs. In combination with zidovudine, lamivudine has a marked synergistic antiviral effect.

Stavudine

Nucleoside inhibitors of HIV reverse transcriptase act as DNA chain terminators after they are converted to the triphosphate form intracellularly. Stavudine, a synthetic nucleoside analogue, is a reverse transcriptase inhibitor which is active against HIV-1 and HIV-2. The triphosphate form of stavudine terminates the elongation of proviral DNA and, accordingly, HIV replication.

Resistance to stavudine shares common features with resistance to another thymidine analogue, zidovudine. Mutations that lead to resistance to these two antiviral drugs are referred to as thymidine-associated mutations. Resistance mechanisms relate to mutations in reverse transcriptase.

Zidovudine

Nucleoside reverse transcriptase inhibitors include zidovudine, an analogue of thymidine. Zidovudine was the first antiviral drug approved for the treatment of HIV infection. It has inhibitory activity against a range of retroviruses including HIV-1, HIV-2, human T cell lymphotropic viruses and other mammalian viruses. Because it becomes incorporated by the enzymatic activity of reverse transcriptase into nascent DNA, zidovudine triphosphate terminates the elongation of the proviral DNA chain (Fig. 60.5). Selectivity does not exist at the activation step and, accordingly, phosphorylated zidovudine accumulates in most dividing cells. This leads to toxicity which is characterized by bone marrow suppression resulting in neutropenia and anaemia.

Treatment with zidovudine was reported to reduce clinical signs of disease in feline immunodeficiency virus-infected cats. Although infection persists despite

treatment, therapy with this antiviral drug may prolong the lives of infected cats.

Resistance to zidovudine is associated with mutations in viral reverse transcriptase resulting in decreased affinity of the enzyme for this antiviral drug. Cross-resistance to other thymidine analogues also occurs.

Antiviral drugs which act as protease inhibitors

Together with genome replication, production of virus proteins is an essential part of the replicative cycle of all viruses. For many viruses, including HIV-1, assembly of proteins and nucleic acid into viral particles does not produce an infectious virion. An additional step, referred to as maturation, is required. New virus proteins require cleavage by virus- specific proteases to become fully functional. The HIV-1 protease cleaves gag and pol polyproteins yielding shorter mature HIV proteins. The gag proteins constitute essential structural proteins including nucleocapsid proteins, whereas the pol proteins possess enzymatic activity including reverse transcriptase, protease and integrase functions. Thus, HIV protease has a central role in converting HIV virus particles into mature infectious viruses. Cleavage events are initiated when newly formed virions begin to bud from an infected cell. Inhibition of protease results in the production of non-infectious viruses. HIV protease inhibitors are peptide-like chemicals which competitively inhibit the activity of the viral protease. When combined with antiretroviral drugs, HIV protease inhibitors produce long-term suppression of viraemia, increased $CD4^+$ T lymphocyte numbers and reduced disease progression. Antiviral drugs which target HIV protease include amprenavir, ritonavir and saquinavir. When introduced into an infected cell before virion budding commences, protease inhibitors prevent polyprotein cleavage and result in the production of non-infectious virus.

Amprenavir

Amprenavir is an amino sulphonamide non-peptide HIV protease inhibitor. This antiviral drug acts by binding to the active site of HIV protease, preventing the processing of gag and pol polyprotein precursors and, as a consequence of this interference, immature non-infectious virus particles are produced (Fig. 60.5).

As amprenavir is an active-site inhibitor of HIV protease, resistance to this drug is conferred by mutations in the viral protease cleavage site and amino acid substitutions. It has been reported that cleavage site mutations can improve the fitness of protease mutants.

Ritonavir

Protease inhibitors share a number of characteristics: they bind competitively to proteases of HIV-1 and HIV-2, preventing the post-translational breakdown of viral polyprotein into shorter, mature proteins required for assembly of the virion and for budding, without the need for intracellular activation of proteases.

Ritonavir is a peptidomimetic HIV protease inhibitor which reversibly binds to the active site of HIV protease, thereby preventing polypeptide processing. Infected cells exposed to ritonavir continue to produce viral proteins but these proteins are unsuitable for virion assembly and for maturation. Although virus particles are produced in the presence of ritonavir, they are not infectious.

Resistance to ritonavir develops because of accumulation of mutations in the corresponding gene leading to amino acid substitutions at active enzymatic sites or at other sites known to interact with inhibitors. Viruses with protease mutations, however, appear to be less fit than wild-type viruses.

Saquinavir

As the first licensed virus protease inhibitor, saquinavir was an active inhibitor of HIV replication *in vitro*. In clinical trials, however, it had poor oral bioavailablity. Saquinavir is a peptidomimetic hydroxyethylamine HIV protease inhibitor which inhibits replication of HIV-1 and HIV-2. It reversibly binds to the active site of HIV protease, preventing polypeptide processing and virion maturation.

Resistance to saquinavir develops because of accumulation of mutations with amino acid substitutions at the active site.

Resistance to antiviral drugs

All forms of microorganisms, including viruses, can readily become resistant to inhibitory drugs. As most antiviral compounds are highly selective and usually target a specific viral protein, often enzymes involved in viral nucleic acid synthesis or viral protein processing, a natural consequence is that point mutations in the virus genome can result in drug-resistant variants. The development of resistance, which often limits the usefulness of antiviral drugs, has been reported for the majority of antiviral compounds currently in use. As current antiviral drugs inhibit active replication, viral replication is likely to

resume when treatment concludes. Effective antiviral host immune responses are therefore essential for clinical recovery from infection. Failure of antiviral therapy may relate to the host's immunological incompetence or the emergence of drug-resistant variants. Development of resistance, a multifactorial process, may involve the selection and use of the antiviral compounds, drug concentration at the site of infection, initial virus susceptibility to the treatment, inherent characteristics of the invading virus and the immune status of the host.

Factors which affect the development of drug resistance

The frequency of drug-resistant mutants in a population of viruses and the rate at which mutations arise depend on many factors. A factor of major importance is the mutation rate of the virus: the higher the rate of mutation, the more rapidly resistance develops. Viral mutation rates are mostly determined by the fidelity of the polymerases which replicate the viral genome. Among DNA viruses, there is a relatively low error rate because DNA polymerases include exonuclease proofreading functions which can eliminate errors. In contrast, RNA viruses have very high intrinsic mutation rates with no proofreading or repair mechanisms involved. This high rate of mutation combined with a large virion population can give rise quickly to considerable diversity within a single host. Based on virus mutation rates alone, viruses could evolve to resist any antiviral drug unless the drug in question succeeded in reducing virus replication to a negligible level. This can be achieved in practice by drug combinations aimed at reducing virus replication to a low level.

Although some antiviral drugs are potential chemical mutagens, most mutants appear to arise naturally and these virions have a selective advantage in the presence of the inhibitor. Drug therapy, therefore, does not induce the mutation; rather it provides the necessary selective pressure for the promotion of drug-resistant viruses which arise naturally.

Another aspect of antiviral drug resistance is the target size for mutation: the greater the number of sites where mutations confer drug resistance, the more rapidly antiviral resistance is likely to occur. Mutations which give rise to amino acid substitutions in or close to the site which interacts with the inhibitory compound, leading to resistance, are referred to as primary mutations. Such mutations usually arise early after exposure to the antiviral compound. Additional mutations which arise during the course of treatment and which

contribute to the overall level of resistance are termed secondary mutations. Even in the absence of antiviral chemotherapy, a small number of virions in a given population become resistant to inhibitory drugs. Such pre-existing drug-resistant mutants continue to replicate when selective pressure is applied through administration of antiviral drugs.

Some drug-resistant mutants have altered pathogenicity, a consequence of alteration of virus fitness. For a virus which is drug-resistant to be capable of causing disease, it must mutate not only to evade drug inhibition but also to retain those characteristics required for disease production.

Apart from immunomodulatory compounds which promote either non-specific or specific protective antiviral immune responses, development of resistance to antiviral drugs is an inevitable consequence of antiviral chemotherapy. Of the many strategies that can be applied to the control of viral diseases in humans and animals, vaccination is the preferred option. In the absence of effective vaccination, antiviral chemotherapy offers the possibility of prophylactic and therapeutic treatment for a defined number of viral pathogens.

Future developments

For the past two decades, the choice of antiviral chemotherapy was limited to a narrow range of compounds with activity against herpesviruses, some influenza viruses and retroviruses. In recent years, molecular developments have ushered in a new era which heralds fundamental changes in the choice, safety and cost of antiviral compounds. The ability of pharmaceutical companies to synthesize antiviral drugs with greater specificity than those currently in clinical use offers the possibility of less toxic, more effective compounds for a wider range of viral pathogens than has been available in recent years.

Because symptoms of acute infection often occur after viral replication has been taking place for several days, the benefit of chemotherapy for viral infections is often less evident than for bacterial infections. Successful antiviral therapy depends to a considerable extent on the host’s immunological competence. As the antiviral drugs currently available are virustatic, the outcome of viral infection is ultimately determined by components of innate immune responses, especially interferons and NK cells, and by specific cellmediated immune responses together with the production of neutralizing antibodies or antibodies that contribute to the development of antibody-dependent cell-

mediated cytotoxicity.

Future antiviral therapeutic developments will depend on the identification of new molecular targets during viral replication and the synthesis of appropriate antiviral agents. A new target for the inhibition of herpes simplex virus, the helicase–primase complex, has been reported. The helicase–primase complex comprises a group of three proteins concerned with unwinding the double-stranded DNA and priming the daughter strand during DNA replication. An aminothiazolylphenyl-containing compound, BILS 179 BS, was reported to inhibit the enzymatic activities of herpes simplex virus helicase-primase (Crute *et al*., 2002). The mechanism of action of these types of inhibitors is through the stabilization of the enzyme–nucleic acid interactions which prevent progression through helicase–primase catalytic cycles. Both *in vitro* and *in vivo* anti-herpes simplex virus activity was demonstrated with this antiviral compound. Another helicase–primase inhibitor belonging to the thiazolyl-amides, BAY 57-1293, with marked anti-herpes simplex activity, has been reported to bind to the helicase– primase subunits simultaneously (Kleymann *et al*, 2002). This antiviral compound had potent therapeutic antiviral activity in mice experimentally infected with herpes simplex virus type 1 (Biswas *et al*., 2007). This compound was also effective in athymic nude mice, animals which have defective cell-mediated immune responses.

Helicase inhibitors, which are aminothiazole derivatives, appear to be highly potent in tissue culture and in animal models.

Broad-spectrum antiviral drugs are not currently available for clinical use. A phosphorothioate oligonucleotide, referred to as REP-9, is reported to have antiviral activity against a range of enveloped viruses (Field and Vere Hodge, 2008). This oligonucleotide, which has both hydrophobic and hydrophilic surfaces, is reported to be active against herpesviruses, orthomyxoviruses, paramyxoviruses and retroviruses. The antiviral effects of small interfering RNAs (siRNAs) have been demonstrated *in vitro* for a range of viruses which affect humans and animals but the application of this approach using *in vivo* test systems has been limited owing to the technical difficulties of delivering sufficient amounts of siRNAs intracellularly.

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Section VI
Viruses and Prions

Chapter 61

Herpesviridae

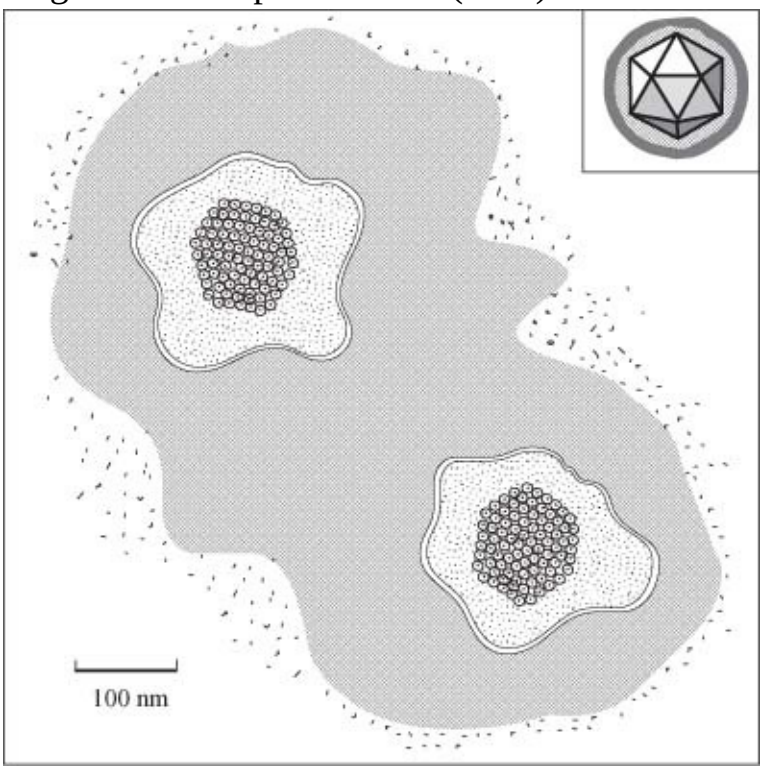
The family *Herpesviridae* contains more than 100 viruses. Fish, amphibians, reptiles, birds and mammals including humans are susceptible to herpesvirus infection. These viruses are of special importance because of their widespread occurrence, their evolutionary diversity and their involvement in many important diseases of domestic animals and humans. The name, herpesvirus (Greek *herpein*, to creep), refers to the sequential appearance and local extension of lesions in human infection. Herpesvirus virions are enveloped and range from 200 nm to 250 nm in diameter. They contain double-stranded DNA within an icosahedral capsid which has a diameter of 125 nm (Fig. 61.1). A layer of amorphous material, the tegument, lies between the envelope and the capsid. Herpesviruses enter cells by fusing with the plasma membrane. Replication occurs in the cell nucleus. The method by which mature capsids leave the nucleus and gain their envelope is currently a matter of debate. Nucleocapsids appear to bud through the inner nuclear membrane. However, it is unclear whether this envelope is retained or if fusion with the outer nuclear membrane causes the envelope to be lost with subsequent re-envelopment occurring in a Golgi or post-Golgi compartment. At least ten viral encoded glycoproteins are incorporated into the envelope. Enveloped virions are released from the cell by exocytosis. Infection involving virus replication results in cell death. However, herpesviruses are best known for their ability to produce latent infections. The mechanisms controlling latency are not well understood but are thought to involve a ‘default’ mechanism whereby immediate early gene expression fails to occur, resulting in the maintenance of the viral genome as a circular episomal element. A possible trigger for alphaherpesvirus latency may be infection of neurons, which are only partly permissive. Resumption of the productive cycle of infection is dependent on changes in the infected cell due to external stimuli or cell differentiation. Intranuclear inclusions are characteristic of herpesvirus infections. Extension of viral infection occurs through points of cell contact without exposure of virus to neutralizing antibodies in blood or interstitial fluids. Protective antibody responses are usually directed against the envelope

glycoproteins. Herpes-virus virions, which are fragile and sensitive to detergents and lipid solvents, are unstable in the environment.

Key points

- Enveloped DNA viruses with icosahedral symmetry
- Replicate in nucleus
- Labile in the environment
- Three subfamilies of veterinary importance: *Alphaher-pesvirinae*, *Betaherpesvirinae*, *Gammaherpesvirinae*
- Cause diseases of the respiratory, reproductive and nervous systems; may cause cell transformation in some species
- Latency is a common outcome of infection with these viruses

Figure 61.1 Herpesvirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



The family is divided into three subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaher-pesvirinae* (Fig. 61.2). The subfamilies comprise 12 genera. Recent, newly created genera include *Proboscivirus*, containing a virus of elephants, and the genera *Macavirus* and *Percavirus*, which are made up of viruses previously assigned to the genus *Rha-dinovirus*. A former genus, *Ictalurivirus*, containing herpesviruses of fish, has now been assigned to a

separate family *Alloherpesviridae* within the order *Herpes-virales*. Alphaherpesviruses replicate and spread rapidly, destroying host cells and often establishing latent infections in neurons located in sensory ganglia. Betaherpesviruses, which replicate and spread slowly, cause infected cells to enlarge, hence their common name cytomegaloviruses. They may become latent in cells of the monocyte series. Gammaherpesviruses, which infect lymphocytes, can produce latent infections in these cells. When lymphocytes become infected, there is minimal expression of viral antigen. A number of gammaherpesvirus species also replicate in epithelial and fibroblastic cells causing cytolysis. Some gammaherpesviruses are implicated in neoplastic transformation of lymphocytes.

Clinical infections

Herpesviruses establish life-long infections with periodic reactivation resulting in bouts of clinical disease. Shedding of virus may be periodic or continuous. Following a cell-associated viraemia, systemic infection is usual. Members of the genus *Simplexvirus*, however, tend to produce a more limited infection involving only the epithelium at the site of inoculation and the sensory nerves that innervate the site. During latency, the episomal viral genome remains circular and gene expression is limited. Reactivation of infection is associated with various stress factors including transportation, adverse weather conditions, overcrowding and intercurrent infection. Natural infections with particular herpesviruses are usually restricted to defined host species. Because these viruses are highly adapted to their natural hosts, infections may be inapparent or mild. However, in very young or immunosuppressed animals, or as a result of infection of an alternative host, infection can be life-threatening. Some herpesviruses, such as Marek's disease virus and human herpesvirus 4 (Epstein-Barr virus), are implicated in neoplastic transformation of cells.

Herpesviruses can cause respiratory, genital, mammary and CNS diseases in cattle (Tables 61.1, 61.2). Aujeszky's disease, which affects pigs and other domestic species, is the major porcine herpesvirus infection (Table 61.3). In horses, herpesviruses can cause respiratory, neurological and venereal diseases, and abortion along with neonatal infection (Table 61.4). The herpesviruses of domestic carnivores are presented in Table 61.5 and those of birds in Table 61.6.

Table 61.1 Herpesvirus infections of ruminants.

Virus	Genus	Comments
Bovine		Causes respiratory (infectious bovine rhinotracheitis) and genital (infectious pustular vulvovaginitis,

herpesvirus 1	*Varicellovirus*	balanoposthitis) infections. Occurs worldwide
Bovine herpesvirus 2	*Simplexvirus*	Causes ulcerative mammillitis in temperate regions and pseudo-lumpy-skin-disease in tropical and subtropical regions
Bovine herpesvirus 5	*Varicellovirus*	Causes encephalitis in calves; described in several countries
Ovine herpesvirus 2	*Macavirus*	Causes subclinical infection in sheep and goats worldwide. Causes malignant catarrhal fever in cattle and in some wild ruminants
Alcelaphine herpesvirus 1	*Macavirus*	Causes subclinical infection in wildebeest in Africa and also in captivity. Causes malignant catarrhal fever in cattle, deer and in other susceptible ruminants

Figure 61.2 Classification of herpesviruses of domestic animals. Viruses in red cause OIE-listed diseases.

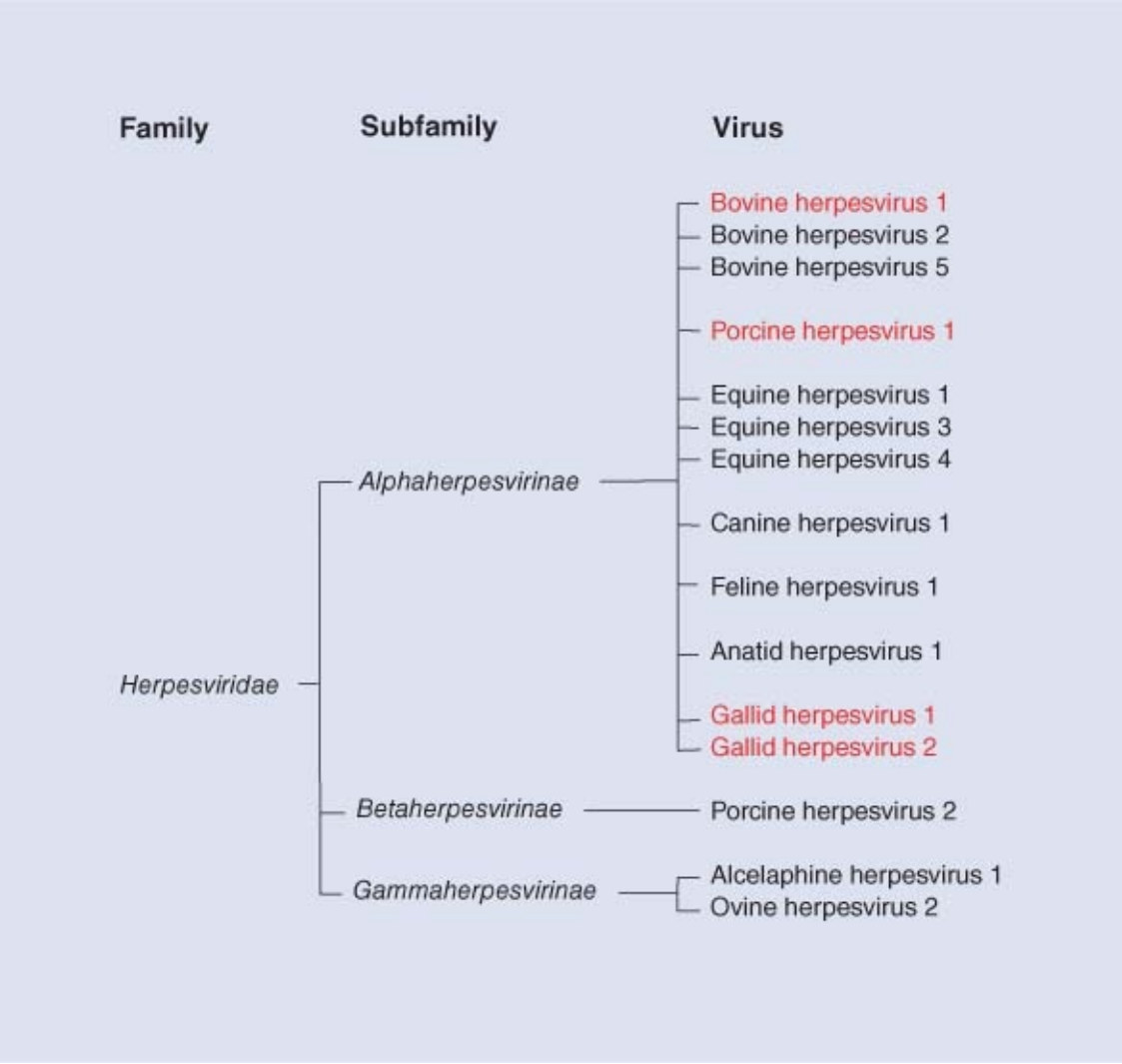


Table 61.2 Subtypes of bovine herpesvirus 1 and their clinical significance.

Subtype	Comments
Subtype 1.1	Implicated in respiratory disease; may cause abortion. Usually included in vaccines
Subtype 1.2a	Implicated in infectious balanoposthitis/infectious pustular vulvovaginitis syndrome and may cause mild respiratory disease; may also cause abortion

Subtype 1.2b	This subtype, which is implicated in infectious balanoposthitis/infectious pustular vulvovaginitis syndrome, has not been associated with abortion

Table 61.3 Herpesvirus infections of pigs.

Virus	Genus	Comments
Porcine herpesvirus 1 (Aujeszky's disease virus)	*Varicellovirus*	Causes Aujeszky's disease in pigs. Encephalitis, pneumonia and abortion are features of the disease. In many species other than pigs, pseudorabies manifests as a neurological disease with marked pruritus. Occurs worldwide
Porcine herpesvirus 2	Unassigned	Causes disease of the upper respiratory tract in young pigs (inclusion body rhinitis)

Table 61.4 Herpesvirus infections of horses.

Virus	Genus	Comments
Equine herpesvirus 1	*Varicellovirus*	Causes abortion, respiratory disease, neonatal infection and neurological disease. Occurs worldwide
Equine herpesvirus 3	*Varicellovirus*	Causes mild venereal infection (coital exanthema) in both mares and stallions
Equine herpesvirus 4	*Varicellovirus*	Causes rhinopneumonitis in young horses and sporadic abortion. Occurs worldwide

Table 61.5 Herpesvirus infections of domestic carnivores.

Virus	Genus	Comments
Canine herpesvirus 1	*Varicellovirus*	Causes a fatal generalized infection in neonatal pups
Feline herpesvirus 1	*Varicellovirus*	Causes feline viral rhinotracheitis in young cats

Table 61.6 Herpesvirus infections of birds.

Virus	Genus	Comments
Gallid herpesvirus 1	*Iltovirus*	Causes infectious laryngotracheitis. Present in many countries
Gallid herpesvirus 2 (Marek's disease virus)	*Mardivirus*	Causes Marek's disease, a lymphoproliferative condition in 12-to 24-week-old chickens. Occurs worldwide
Anatid herpesvirus 1	Unassigned	Causes acute disease in ducks (duck plague), geese and swans characterized by oculonasal discharge, diarrhoea and high mortality. Occurs worldwide

Infectious bovine rhinotracheitis and pustular vulvovaginitis

Bovine herpesvirus 1 (BHV-1), which infects domestic and range cattle, is associated with several clinical conditions including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, conjunctivitis and respiratory disease in calves. Infection with BHV-1 is an important cause of losses in livestock worldwide. A single antigenic type of BHV-1, which contains subtypes 1.1, 1.2a and 1.2b, has been recognized using restriction enzyme analysis (Table 61.2).

Epidemiology

Transmission occurs via respiratory or genital secretions. Aerosol transmission is most efficient over short distances and is facilitated by the close grouping of

animals. The reactivation of latent infections may occur following stressful events such as transportation or parturition. Virus is transported intra-axonally to the original portal of entry. These animals frequently show no clinical signs when the infection is reactivated but shed virus into the environment and are an important means of perpetuating infection in herds. The semen of infected bulls may contain virus and infection may follow natural service or artificial insemination. In addition, aborted foetuses can be an important source of infection.

Pathogenesis and pathology

The virus that causes infectious bovine rhinotracheitis is usually acquired through aerosols. Replication occurs in the mucous membranes of the upper respiratory tract and large amounts of virus are shed in nasal secretions. Virus also enters local nerve cell endings and is transported intra-axonally to the trigeminal ganglion where it remains latent. It is also thought that latent infection can become established in the germinal centres of the pharyngeal tonsils. In most instances, infection is contained within 2 weeks by a strong immune response. However, tissue necrosis may facilitate secondary bacterial infection with severe systemic effects and, possibly, death. Rarely, viraemia in pregnant cows may produce foetal infection and abortion. Necrotic foci may be present in various organs of aborted foetuses, particularly in the liver.

Following genital infection with subtypes 1.2a and 1.2b, virus replicates in the mucosa of the vagina or prepuce, and latent infection may become established in the sacral ganglia. Focal necrotic lesions on genital mucosae may eventually coalesce to form large ulcers. An intense inflammatory reaction can develop in the reproductive tract with secondary bacterial infection leading to endometritis. Viraemia is not a feature of genital infection with BHV-1 subtypes and infected pregnant cows rarely abort.

Clinical signs

In outbreaks of disease, either the respiratory or the genital form usually predominates. The incubation period is up to 6 days. The severity of the clinical signs in the respiratory form of the disease are largely determined by the extent of secondary bacterial infection. Affected animals develop a high temperature and nasal discharge accompanied by anorexia. The nares are inflamed (‘red nose’) and conjunctivitis, lacrimal discharge and corneal opacity are often

present. In uncomplicated infections, animals recover after about a week. If bacterial infection becomes established, animals develop dyspnoea, coughing and open-mouth breathing. Death may ensue. In severe outbreaks in feedlot cattle, morbidity may approach 100% and mortality may be as high as 10%.

Cows with infectious pustular vulvovaginitis exhibit vaginal discharge and frequent urination. Animals usually recover within 2 weeks. However, secondary bacterial infection may result in metritis, temporary infertility and purulent vaginal discharge persisting for several weeks. Infected bulls have lesions on penile and preputial mucosae.

A fatal generalized disease with fever, oculonasal discharge, respiratory distress, diarrhoea, incoordination and convulsions has been described in young calves.

Diagnosis

- Swabs collected from the eyes, nares and genitalia of several affected animals during the early acute phase of the disease are suitable for virus isolation (Nettleton *et al*, 1983). Because the virus is fragile, specimens for transportation to the laboratory should be placed in viral transport medium and kept refrigerated. The virus produces a rapid cytopathic effect in bovine cell lines.
- Smears from ocular, nasal or genital swabs and frozen sections of tissues from aborted foetuses can be used for the rapid demonstration of viral antigen by immunofluorescence. Viral antigen can also be detected using ELISA.
- The presence of characteristic gross and microscopic lesions in aborted foetuses is suggestive of infection with BHV-1.
- The polymerase chain reaction has been adapted for detection of BHV-1 DNA in appropriate samples (Moore *et al*., 2000).
- Real-time PCR assays are now widely used instead of virus isolation for detection of virus (Kramps, 2008).
- Evidence of a rising antibody titre in paired serum samples using virus neutralization or ELISA is indicative of active infection. Commercial ELISA kits are available including gE-ELISA formats designed for use in conjunction with marker vaccines.
- As part of a surveillance programme, bulk milk samples can be tested for antibodies using ELISA.

Control

Inactivated, subunit and modified live vaccines are available for the control of BHV-1 (van Oirschot *et al*., 1996). Vaccination reduces the severity of clinical signs but may not prevent infection and the establishment of latent infection with possible future re-excretion. Regular re-vaccination at 6-monthly intervals can help to decrease the risk of re-excretion. Modified-live vaccines, which may cause abortion, should not be administered to pregnant animals. Vaccine strains, which are temperature-sensitive, have a cut-off temperature in the same range as body temperature and should be administered by the intranasal route. Replication of these mutants in nasal mucosa is not impaired as the temperature at this site is lower than that of the body. Marker or DIVA (differentiate infected from vaccinated animals) vaccines, based either on genetically engineered viruses lacking one or more genes encoding surface glycoproteins such as gE, or on subunit preparations containing one or more glycoproteins, are available. A serological test, usually a blocking ELISA, is used to detect antibodies against a glycoprotein not present in the marker vaccine. The availability of marker vaccines enables either eradication or reduction of infection in national herds to proceed economically. Successful eradication programmes based on test and slaughter policies have been carried out in Denmark and Switzerland.

Bovine herpes mammillitis and pseudo-lumpy-skin disease

Infection with bovine herpesvirus 2 (BHV-2) is associated with outbreaks of a severe ulcerative condition of the teats of dairy cows. The condition has been reported in many countries around the world. Bovine herpesvirus 2 infection resulting in a generalized mild skin infection, termed pseudo-lumpy-skin disease to distinguish it from the more serious lumpy skin disease caused by a poxvirus, has been reported in tropical and subtropical regions. Isolates of the virus appear to be serologically and genetically similar regardless of whether they have come from cases of mammillitis or pseudo-lumpy-skin disease.

Epidemiology

In temperate regions, outbreaks of herpes mammillitis are sporadic and usually occur in autumn or early winter. Latent infection and subsequent reactivation may be important factors in the spread and perpetuation of infection within

herds. In cows calving for the first time, lesions appear a few days after parturition. Serous exudate from lesions contains large quantities of virus, and transmission to other cows in the herd occurs through direct and indirect contact during milking. Infection occurs through small abrasions in the skin. Insects may transmit the virus from animal to animal mechanically. Calves suckling affected cows can become infected and may transmit the virus. In Africa, a wide range of wild animal species appear to act as subclinical reservoirs of infection. Insect transmission is considered to be important in warm climates and may account for the occurrence of the generalized skin form of the disease in those regions.

Pathogenesis

The virus replicates optimally at a temperature lower than normal body temperature. Following intradermal or subcutaneous inoculation, BHV-2 replicates without dissemination to other sites. In contrast, generalized infection with widespread skin nodules develops in experimental animals following intravenous inoculation of BHV-2.

Clinical signs

The number of animals displaying clinical signs during outbreaks is variable, and subclinical infection is common. First-lactation cows which have recently calved, particularly animals with udder oedema, are most severely affected. The incubation period is up to 8 days. Lesions appear as thickened plaques on one or more teats. Ulceration of the skin leads to scar formation. The lesions are painful and there is a reduction in milk yield due to difficulty with milking. In severe cases, lesions may also appear on the skin of the udder. Circular ulcers may be present on the lips, nostrils or muzzle of calves suckling affected cows. In pseudo-lumpy-skin disease, a variable number of nodules appear on the skin over the neck, shoulders, back and perineum. The nodules, which are circular and hard, have depressed centres. They heal without scar formation within a couple of weeks.

Diagnosis

Diagnosis is based on virus isolation in tissue culture. The optimal incubation temperature for inoculated cell cultures is 32°C. A PCR assay has been developed for the detection of BHV-2 in skin lesions (d'Offay *et al.*, 2003). Serological tests have been used in attempts to demonstrate a significant rise in

titre but frequently the first serum sample already has a high titre.

Control

Commercial vaccines are not available. Affected animals should be isolated and milked separately. Teat dipping and disinfection of milking machine clusters between cows is advisable. The most susceptible animals, first-lactation cows, should be milked first. Insect control measures may help to limit the spread of disease within a herd.

Malignant catarrhal fever

This severe, sporadic disease of cattle, deer and other ruminants is frequently fatal. The disease has also been described in pigs in a number of European countries. Malignant catarrhal fever (MCF) is caused by two related but distinct viruses, alcelaphine herpesvirus 1 (A1HV-1) and ovine herpesvirus 2 (OvHV-2). As the wildebeest is the natural host of AlHV-1, infection with this virus is confined to Africa. Sheep are the natural hosts of OvHV-2 and infection occurs worldwide in sheep and goats. In these species, infection is common and subclinical.

Epidemiology

Alcelaphine herpesvirus 1 is transmitted vertically and horizontally in wildebeest populations. There is evidence that latency occurs in lymphoid cells. Some wildebeest calves are infected transplacentally but most acquire infection shortly after birth through nasal secretions from their mothers or from other calves. Viraemia persists in young wildebeest for the first few months of life. This facilitates shedding of large quantities of virus in nasal and ocular secretions. In-contact cattle may become infected. The pattern of virus shedding is thought to be similar in sheep especially at lambing, when transmission to cattle and farmed deer is most likely to occur. Infection in cattle and deer is thought to be acquired through contact with young lambs. However, recent studies suggest that sheep from 6 to 9 months of age are the group most likely to transmit infection (Li *et al.*, 2004). Cattle and deer are considered to be ‘end-hosts’ because they do not appear to transmit virus.

Pathogenesis

The pathogenesis of MCF is poorly understood. It is presumed that virus enters the body through the upper respiratory tract. A cell-associated viraemia occurs. However, virus is difficult to detect at the site of lesions and it has been suggested that tissue changes in MCF may have an immunopathological basis. Cellmediated reactions have been implicated in lesion development. Lymphocytes, predominantly $CD8^+$ T lymphocytes, accumulate in a variety of organs, frequently associated with tissue necrosis. It has been proposed that tissue damage results from the unregulated activity of virus-infected cytotoxic T lymphocytes or virus-infected NK cells (Russell *et al*., 2009).

Clinical signs

The incubation period, although variable, generally lasts 3 to 4 weeks. The most common clinical presentation is characterized by sudden onset of fever, oculonasal discharge, enlarged lymph nodes, conjunctivitis, corneal opacity and erosive mucosal lesions in the upper respiratory tract. Profuse mucopurulent nasal discharge leads to encrustation of the muzzle. Some animals display neurological signs including muscle tremors, incoordination and head pressing. An intestinal form of the disease presents with diarrhoea or dysentery.

The course of this usually fatal disease is up to 7 days. Some animals may linger for weeks or months and may recover (O' Toole *et al*., 1997). In peracute disease, particularly in deer, death may occur without premonitory signs. There is evidence that mild or inapparent infections may occur in cattle at risk (Powers *et al*., 2005).

Diagnosis

Diagnosis is based on clinical presentation and post-mortem findings of extensive vasculitis characterized histologically by fibrinoid degeneration and marked lymphoid infiltration. Ulceration of surface epithelia is a prominent feature of MCF. Viral DNA can be detected in circulating leukocytes, fresh tissues and paraffin-embedded tissues using PCR (Muller-Doblies *et al*., 1998; Crawford *et al*, 1999). Although a competitive inhibition ELISA has been developed for the detection of serum antibodies to A1HV-1 and OvHV-2 (Li *et al*., 1994), it is not as reliable as PCR or histopathological examination. This competitive ELISA can be used to determine the prevalence of antibodies in sheep populations. Although A1HV-1 can be isolated in bovine thyroid cells from the buffy coat of animals with wildebeest-associated MCF, ovine

herpesvirus 2 has not yet been grown in tissue culture.

Control

As a vaccine is not available, control depends on the separation of susceptible species from reservoir hosts. Using the PCR assay, identification and elimination of sheep harbouring OvHV-2 in order to establish a virus-free flock may be worthwhile in defined circumstances.

Aujeszky's disease

This disease is caused by porcine herpesvirus 1, also referred to as Aujeszky's disease virus (ADV). A single serotype of the virus is recognized. The pig, in which subclinical and latent infections can occur, is the natural host of the virus. Other domestic animals are susceptible and infection in these incidental hosts, often referred to as pseudorabies, is usually fatal.

Epidemiology

Infection is endemic in the pig populations of most countries. The disease has been eradicated from many countries in recent years. Outbreaks of disease in naive herds can be devastating, with rapid spread to pigs of all ages. The virus is shed in oronasal secretions, milk and semen. Transmission usually occurs by nose-to-nose contact or by aerosols. Transplacental transmission occurs and aborted foetuses are a source of virus. Although the virus is not stable in the environment, it may remain infectious for a few days under suitable conditions. Wind-borne transmission over distances of a few kilometres has been recorded. Sheep, which are highly susceptible, may acquire infection following direct contact with pigs or when sharing the same airspace. Scavenging carnivores may become infected after eating pig meat; cats are particularly susceptible to infection as are hunting dogs fed infected pig carcasses. The opportunity for transmission of virus from these animals is limited because the incubation period is short and the brief clinical course is invariably fatal in these incidental hosts.

Pathogenesis

Following infection, the virus replicates in the epithelium of the nasopharynx and tonsils. Virus spreads from these primary sites to regional lymph nodes and subsequently to the CNS along axons of the cranial nerves. Virulent strains of

ADV produce a brief viraemia and become widely distributed throughout the body, particularly in the respiratory tract. Virus replication in alveolar macrophages interferes with their phagocytic function. Transplacental transfer results in generalized infection of foetuses. Infected animals excrete virus for up to 3 weeks following infection. Latency occurs in a high percentage of infected animals, with virus localizing in the trigeminal ganglia and tonsils.

Clinical signs

The age and susceptibility of infected pigs and the virulence of the infecting strain influence the severity of the clinical signs. Young pigs are most severely affected; mortality may approach 100% in suckling piglets. In neonatal piglets, the incubation period may be as short as 36 hours compared with 5 days in older pigs. Neurological signs, including incoordination, tremors, paddling and convulsions, predominate in young pigs. Affected animals usually die within 2 days. Mortality is much lower in weaned pigs although neurological and respiratory signs are often present. Fever, weight loss and signs of respiratory disease including sneezing, coughing, nasal discharge and dyspnoea may be evident in fatteners. Neurological signs are uncommon in these older animals and they usually recover within a week. Infection in sows early in pregnancy usually results in resorption of foetuses and return to oestrus. Later in pregnancy, infection frequently produces abortion; full-term piglets may be stillborn or weak. In herds with endemic ADV infection, neonatal animals are protected by maternally derived antibody.

Disease in other domestic animals occurs sporadically and is characterized by neurological signs resembling those of rabies. Intense pruritus (‘mad itch’) leading to self-mutilation is a feature of the disease, particularly in ruminants. The clinical course is short with most affected animals dying within a few days.

Diagnosis

The history, clinical signs and lesions may suggest ADV infection. Laboratory confirmation is based on virus isolation, detection of viral antigen, serology and histopathological findings.

- Specimens of brain, spleen and lung from acutely affected animals are suitable for virus isolation. In the event of a delay between collection and tissue culture inoculation, samples should be refrigerated.
- Cryostat sections of tonsil or brain are suitable for detection of viral antigen

by immunofluorescence.

- Both PCR and real time PCR assays have been developed for the detection of the viral genome in secretions and tissues (McKillen *et al*., 2007).
- Serological tests, including virus neutralization and ELISA, are available for detecting ADV antibodies. In young pigs, maternally derived antibodies may be present up to the age of 4 months. Differential ELISA methods have been introduced for the detection of antibodies to the surface glycoproteins gC, gE and gG. These assays are designed to differentiate animals infected with field virus from those vaccinated with a gene-deleted mutant lacking a particular surface glycoprotein, gE being the usual deletion used.

Control

If used strategically, vaccination can prevent the development of clinical disease. Modified live, inactivated and gene-deleted vaccines are available. Vaccines have been produced in which the thymidine kinase (TK) gene has been deleted together with a gene encoding one of the non-essential surface glycoproteins. Because endogenous levels of TK are low in neurons, this gene-deleted virus cannot replicate in neurons and as a consequence is significantly reduced in virulence. However, such vaccinal strains may fail to prevent latent infection caused by field virus in the trigeminal ganglia. In addition, the possibility of recombination of field virus with a gene-deleted virus and the subsequent generation of a virulent ADV has been suggested (Maes *et al*., 1997). Eradication of Aujeszky's disease can be achieved by depopulation, by test and removal, or by segregation of litters. The development and use of effective DIVA or marker vaccines in conjunction with the appropriate serological assays has been pioneered in the field of Aujeszky's disease, making eradication feasible and economically worthwhile. The disease has been eradicated from domestic pigs in several European countries, Canada and New Zealand. Eradication programmes are underway in several other countries including Ireland. In the USA, domesticated pigs are free of the disease but infection is still present in feral pigs.

Equine rhinopneumonitis and equine herpesvirus abortion

Equine herpesvirus 1 (EHV-1) and equine herpesvirus 4 (EHV-4), which are

endemic in horse populations worldwide, are responsible for outbreaks of respiratory disease in young horses and for abortion. Before 1981, it was considered that the pathogen was a single virus, EHV-1, composed of two subtypes. However, restriction endonuclease analysis showed that the subtypes were two distinct viruses. Infection with EHV-1 is associated with respiratory disease, abortion, fatal generalized disease in neonatal foals and encephalomyelitis. Although infection with EHV-4 is primarily associated with respiratory disease, sporadic abortions have also been attributed to this virus.

Epidemiology

Close contact facilitates transmission of these fragile viruses. Transmission usually occurs by the respiratory route following contact with infected nasal secretions, aborted foetuses, placentae or uterine fluids. Both EHV-1 and EHV-4 can occur as latent infections. Serological surveys using a type-specific ELISA have shown that there is a high prevalence of antibody to EHV-4, approaching 100% in some populations (Gilkerson *et al*, 1999b). It is thought that episodes of reactivation of latent EHV-4 infection without coincident clinical disease occur in adult horses, resulting in transmission to foals. The prevalence of antibody to EHV-1 is about 30% in adult horses; it is lower in foals (Gilkerson *et al*, 1999b). It appears that foals become infected with EHV-1 from their dams or from other lactating mares in the group. Foal-to-foal spread may occur both before and after weaning (Gilkerson *et al*., 1999a). Even without aborting, mares infected with EHV-1 are potential sources of infection. When reactivation of infection in a latent carrier mare occurs in a stud farm, exposure of non-immune, in-contact pregnant mares may lead to an abortion storm.

Pathogenesis

These viruses replicate initially in the upper respiratory tract and regional lymph nodes extending in some cases to the lower respiratory tract and lungs. Latent infection of the trigeminal ganglia with both EHV-1 and EHV-4 may occur. Infections with EHV-4 appear to be restricted to the respiratory tract and viraemia is uncommon. In contrast, local replication of EHV-1 may be followed by a lymphocyte-associated viraemia which disseminates the virus around the body and can result in abortion or neurological disease. Latent infection of lymphocytes with EHV-1 has been demonstrated and can be followed by reactivation. The virus can spread directly from infected leukocytes to

contiguous cells, thus avoiding neutralization by circulating antibody. Equine herpesvirus 1 has a predilection for vascular endothelium. Vasculitis and thrombosis in the placenta along with transplacental infection of the foetus result in abortion. Vasculitis and thrombosis, associated with EHV-1 infection, may also occur in the CNS especially in the spinal cord, with resultant ischaemia and myelomalacia. Neurological changes appear to be related to infection with particular neuropathogenic strains of EHV-1, as a result of a single amino acid substitution in the highly conserved DNA polymerase (Nugent *et al*., 2006). It has been reported that horses regularly vaccinated with killed vaccines are at an increased risk of developing equine herpesvirus-associated neurological disease and an anamnestic immune response is considered an important prerequisite for the immune-mediated vascular damage associated with the condition (Borchers *et al*., 2006).

Clinical signs

Respiratory disease caused by EHV-4 occurs in foals over 2 months of age, in weanlings and in yearlings. Following an incubation period of 2 to 10 days, there are signs of fever, pharyngitis and serous nasal discharge. Secondary bacterial infection is common, giving rise to mucopurulent nasal discharge, coughing and, in some cases, bronchopneumonia. In the absence of serious secondary infection, recovery usually occurs within 2 weeks. Respiratory disease associated with EHV-1 is clinically indistinguishable from that caused by EHV-4; outbreaks of disease caused by EHV-1, however, are less common. Immunity, following primary respiratory tract infection, lasts only a few months and is restricted to antigenically similar viruses. Multiple infections result in significant cross-protection against heterologous herpesvirus. Mares which abort following infection with EHV-1 rarely show premonitory signs. Abortion occurs several weeks or months after exposure, usually during the last 4 months of gestation. Such infected mares rarely abort during subsequent pregnancies and their fertility is unaffected. Infection close to term may result in the birth of an infected foal which usually dies due to interstitial pneumonia and viral damage in other tissues, sometimes complicated by secondary bacterial infection. Although neurological signs associated with EHV-1 infection are relatively uncommon, they may present in several horses during an outbreak of abortion or respiratory disease on a farm. The signs range from slight incoordination to paralysis, recumbency and death.

Diagnosis

- Virus isolation and identification are used routinely for the laboratory confirmation of herpesvirus infection in horses. Nasopharyngeal swabs should be collected during the early stages of respiratory infections and dispatched in suitable transport medium to the laboratory.
- Viral antigen may be demonstrated in cryostat sections of lung, liver and spleen from aborted foetuses using immunofluorescence.
- The characteristic gross and microscopic lesions, particularly intranuclear inclusions in areas of hepatic necrosis, may be sufficient for confirmation of herpesvirus abortion. Vasculitis is commonly found in the myeloencephalopathy caused by herpesvirus.
- The polymerase chain reaction is widely used for the detection of viral DNA in clinical specimens. It is capable of differentiating EHV-1 from EHV-4 (Varrasso *et al*., 2001).
- Demonstration of a fourfold rise in antibody titre in paired serum samples is useful for confirmation of a recent outbreak. Most serological tests do not distinguish infection with EHV-1 from infection with EHV-4 because of antigenic cross-reactivity. ELISA-type assays employing monoclonal antibody or recombinant glycoprotein G antigens can distinguish the two viruses (Crabb *et al*., 1995). Complement-fixing antibodies decline to undetectable levels within a few months and, accordingly, are useful for identifying recent infection.

Control

Effective management practices and vaccination are essential for control. Animals returning from sales, races or other events should be segregated for up to 4 weeks. On large stud farms, horses should be kept in small, physically separated groups. It is essential that pregnant mares be segregated in a stress-free environment. Following an outbreak of disease, affected animals should be isolated. Premises should be disinfected and movement should be restricted until animals on the premises have been clear of the disease for at least a month.

Modified live and inactivated virus vaccines are commercially available. Vaccination with live vaccines is not permitted in some countries. Many vaccine preparations contain both EHV-1 and EHV-4. As vaccination is not considered to be fully protective, frequent boosters are recommended. Vaccination appears to reduce the severity of clinical signs and to decrease the likelihood of abortion. It

is a valuable adjunct to sound management practices aimed at controlling the disease.

Equine coital exanthema

This benign venereal disease of horses, caused by equine herpesvirus 3 (EHV-3), is thought to occur worldwide.

Epidemiology

Serological surveys indicate that the prevalence of infection in breeding animals is about 50%. The reported incidence of the disease is much lower, presumably because many infections are subclinical. The principal mode of transmission is venereal but transfer of EHV-3 may also occur through contaminated instruments. Immunity to reinfection is short lived and clinical signs may develop in the same animal in consecutive breeding seasons.

Pathogenesis

Although latent infection with EHV-3 has not been conclusively demonstrated, it is thought that it probably occurs in sacral ganglia and that outbreaks of disease are initiated by reactivation of latent infection. The virus, which has a tropism for keratinized epithelium, is temperature sensitive and replication is restricted at core body temperature. Viraemia and abortion are not associated with EHV-3 infection.

Clinical signs

The incubation period is up to 10 days. Lesions on external genitalia appear initially as red papules which develop into vesicles and pustules. The pustules rupture leaving ulcers that may coalesce. Lesions are occasionally encountered on the teats, lips and nares. Secondary bacterial infection is common. In uncomplicated cases, lesions heal within 2 weeks. On pigmented skin, sites of healed lesions appear as white spots. Infection can affect the fertility of stallions as they may refuse to serve mares when penile lesions are severe.

Diagnosis

Clinical diagnosis is based on the distribution and appearance of the lesions. Electron microscopy of lesion scrapings or virus isolation in tissue culture at

34°C can be used to confirm infection. Viral DNA can be detected in skin lesions by PCR (Kleiboeker and Chapman, 2004). Virus neutralization and ELISA are suitable assays for demonstrating a rising antibody titre in paired serum samples. However, in some cases the antibody response may be minimal and failure to demonstrate a significant rise is not necessarily evidence of absence of infection.

Control

Affected horses should be isolated and should not be used for breeding until lesions have completely healed. Disposable gloves should be used for genital examination and equipment should be thoroughly disinfected after use. An effective vaccine is not available.

Canine herpesvirus infection

Infection in domestic and wild *Canidae* caused by canine herpesvirus 1 (CHV-1) is common worldwide. Clinical disease caused by the virus is uncommon, but when infection occurs in neonatal pups it results in generalized disease with accompanying high mortality.

Epidemiology

Prevalence rates, based on serological surveys of dogs, are 88% in England and 42% in The Netherlands (Reading and Field, 1998; Rijsewijk *et al*., 1999). Infection usually occurs by the oronasal route following direct contact between infected and susceptible animals. During periods of stress, latent infections may be reactivated with shedding of virus. The sites of latency include sensory ganglia (Burr *et al*., 1996; Miyoshi *et al*., 1999). Virus is shed in oronasal and vaginal secretions. Newborn pups, which can acquire infection either *in utero* or during parturition, may be an additional source of infection for littermates.

Pathogenesis

Following infection, CHV-1 replicates in the nasal mucosa, pharynx and tonsils. The virus replicates most effectively at temperatures below normal adult body temperature with the result that infection in adults is usually confined to the upper respiratory tract or to the external genitalia. Because the hypothalamic regulatory centre is not fully operational in pups under 4 weeks of age, they are particularly dependent on ambient temperature and maternal contact for

maintenance of normal body temperature. A cell-associated viraemia and widespread viral replication in visceral organs can occur in infected neonatal animals with decreased body temperatures.

Clinical signs

In adult dogs and pups over 4 weeks of age, infection is usually asymptomatic. Occasionally, vesicular lesions on external genitalia and mild vaginitis or balanoposthitis may be observed. Primary infection of pregnant bitches may result in abortion, stillbirths and infertility. Pups infected during parturition or shortly after birth develop clinical signs within days. Affected pups stop sucking, show signs of abdominal pain, whine incessantly and die within days. Morbidity and mortality rates tend to be high in affected litters. Bitches whose pups are affected tend to produce healthy litters subsequently. Pups which receive colostral antibodies may become infected without developing clinical signs.

Diagnosis

Diagnostically significant post-mortem findings include focal areas of necrosis and haemorrhage, particularly in the kidneys. Intranuclear inclusions are usually present. Virus isolation can be carried out in canine cell lines from fresh specimens of liver, kidney, lung and spleen. *In situ* hybridization and PCR protocols are available.

Control

A commercial subunit vaccine is available and has been shown to decrease pup mortality following administration to pregnant bitches (Poulet *et al*., 2001). Affected bitches and their litters should be isolated to prevent infection of other whelping bitches. Heating lamps and pads, which raise the body temperature of pups to 39°C, may help to reduce the severity of infection if initiated prior to or at the time of exposure to the virus.

Feline viral rhinotracheitis

This acute upper respiratory tract infection of young cats is caused by feline herpesvirus 1 (FHV-1). The virus, which is distributed worldwide, accounts for about 40% of respiratory infections in cats. Both domestic and wild species of *Felidae* are susceptible.

Epidemiology

Close contact is required for transmission. The prevalence of infection is higher in cats in colonies than in cats reared individually. Virus is shed in oral and oculonasal secretions. Due to its relative lability, survival in the environment is short. Most recovered cats are latently infected. Reactivation with virus replication and shedding is particularly associated with periods of stress such as parturition, lactation or change of housing. Several days elapse between exposure to stress and shedding of virus. The kittens of carrier queens may become infected subclinically while protected by maternally-derived antibody. These kittens may become carriers and, as adults, perpetuate the infection. In common with many herpesvirus infections in other species, the trigeminal ganglia are important sites of latency.

Pathogenesis

Initially, FHV-1 replicates in oronasal or conjunctival tissues before infecting the epithelium of the upper respiratory tract. Viraemia and generalized infection does not appear to occur except in animals at the extremes of age and in immunocompromised cats. Secondary bacterial infections, which commonly occur, exacerbate the clinical signs.

Clinical signs

The incubation period is usually short, about 2 days, but may be up to 6 days. Young cats display signs of acute upper respiratory tract infection including fever, sneezing, inappetence, hypersalivation, conjunctivitis and oculonasal discharge. Crusts form around the eyes, sometimes causing the eyelids to stick together. In more severe disease, pneumonia or ulcerative keratitis may be evident. The mortality rate is low except in young or immunosuppressed animals. Rarely, cats may present with facial and nasal dermatitis, which has been linked to reactivation of latent infection (Hargis and Ginn, 1999).

Diagnosis

Clinical differentiation of feline viral rhinotracheitis from feline calicivirus infection is difficult.

- Virus can be isolated in feline cell lines from oropharyngeal or conjunctival swabs.

- Specific viral antigen can be demonstrated in acetone-fixed nasal and conjunctival smears using immunofluorescence.
- Several PCR protocols have been published and compared for sensitivity (Maggs and Clarke, 2005). In terms of clinical relevance, particularly in the absence of characteristic clinical signs, a positive result may simply be an incidental finding as all latently infected cats may shed virus intermittently. Quantitative assessment using real-time PCR may be more informative (Vogtlin *et al*., 2002).
- The virus neutralization test or ELISA can be used on paired serum samples to demonstrate a rising titre for confirmation of diagnosis.

Treatment and control

Treatment is generally non-specific and supportive. Nucleoside analogues such as trifluridine, idoxuridine, vidarabine and acyclovir have been used topically for FHV-1 ocular disease with some success, but large-scale clinical trials have not been carried out. Other treatments, including feline interferon and L-lysine, have also been investigated. Antibiotics are frequently used to control secondary bacterial infections. The protection provided by vaccination is incomplete as vaccinated cats can become infected but clinical signs and shedding tend to be much reduced. Inactivated vaccines are suitable for use in pregnant queens and help to boost levels of maternal antibody available for kittens. Intranasal vaccines may give rise to mild upper respiratory signs. When cats are at low risk of exposure to infection, booster vaccinations at 3-year intervals may be sufficient (Elston *et al*., 1998; Scott and Geissinger, 1999). Commercial vaccine preparations also contain feline calicivirus. Developments in vaccine production include the use of avirulent deletion mutant virus strains and, in addition, the insertion of the feline calicivirus capsid gene into the modified FHV-1 genome (Gaskell and Willoughby, 1999). Good husbandry practices and disease control procedures should be implemented in catteries in conjunction with regular vaccination schedules to minimize the impact of clinical disease. In breeding colonies where the disease is endemic, additional measures should be implemented including the isolation of queens and their kittens, early weaning of kittens into isolation and vaccination at an early age.

Infectious laryngotracheitis

This highly contagious respiratory disease of chickens and sometimes pheasants

is caused by gallid herpes-virus 1 (GaHV-1). Infectious laryngotracheitis (ILT) occurs in many countries. Although strains of GaHV-1 vary in virulence, they are antigenically homogeneous. In areas of intensive poultry production, the disease is usually well controlled by a combination of vaccination and biosecurity. However, the virus tends to persist in small enterprises and specialized chicken flocks as an endemic infection.

Epidemiology

Typically, infection is transmitted through aerosols, especially among intensively reared birds. Latency occurs in the trigeminal ganglia, and carrier birds may shed virus intermittently after periods of stress such as the onset of laying or when groups of birds are mixed. Indirect transmission from one production unit to another may occur through contaminated fomites.

Pathogenesis

Following inhalation, virus replicates locally in the upper respiratory tract. Spread along sensory nerves results in localization in the trigeminal ganglia.

Clinical signs

The incubation period is up to 12 days. The epidemic form of the disease, caused by virulent strains of GaHV-1, is characterized by coughing, gasping, moist rales, oculonasal discharge, expectoration of bloodstained mucus and head shaking. Mortality may reach 70%. Death is often due to severe obstructive haemorrhagic laryngotracheitis. Mild respiratory signs, conjunctivitis and decreased egg production are features of infection with strains of low virulence.

Diagnosis

In severe outbreaks of ILT, the clinical signs and postmortem findings may be sufficiently characteristic for diagnosis. In outbreaks of the mild form of the disease, laboratory confirmation is necessary. Virus isolation can be carried out on the chorioallantoic membrane of embryonated eggs or in avian cell cultures. Rapid methods of diagnosis include demonstration of herpesvirus particles in tracheal samples by electron microscopy and detection of viral antigen in smears or frozen sections by immunofluorescence. Viral antigen can be detected in tracheal samples by ELISA or agar gel immunodiffusion (AGID). The PCR

assay is considered to be more sensitive than virus isolation for the detection of GaHV-1 and permits the differentiation of field and vaccinal strains using restriction fragment length polymorphism (RFLP) analysis of amplified fragments. Real time PCR assays have been described. Antibodies to GaHV-1 can be demonstrated by virus neutralization, ELISA or AGID.

Control

Flock management systems and vaccination protocols form the basis of control methods. In broiler flocks, short production cycles and all-in all-out management systems ensure that they remain free of the disease. In layer flocks, vaccination is usually carried out using live vaccines administered by aerosols or in drinking water. Vaccination protects against clinical disease but is ineffective against infection by field virus and the establishment of latency. Genetically engineered vaccines are currently being evaluated.

Marek's disease

This contagious lymphoproliferative disease of chickens is caused by gallid herpesvirus 2 (Marek's disease virus), which is a cell-associated and oncogenic virus. The disease, which is of major economic significance in the poultry industry, occurs worldwide. Herpesviruses of chickens and turkeys in the genus *Mardivirus* can be divided into three serotypes or species. Serotype 1 (gallid herpesvirus 2) includes all pathogenic strains and the attenuated variants derived from these strains; serotype 2 (gallid herpesvirus 3) contains avirulent and non-oncogenic strains; the third serotype, meleagrid herpesvirus 1, is an avirulent herpesvirus of turkeys. Serotype 1 strains can be categorized as mildly virulent, virulent and highly virulent.

Epidemiology

Productive replication with release of infective virus occurs only in the epithelium of the feather follicle. Cell-free virus is released from the follicles along with desquamated cells. This dander can remain infective for several months in poultry-house dust and litter. Infected birds remain carriers for life, and their chicks, which are protected initially by maternally-derived antibody, acquire infection within a few weeks, usually by the respiratory route. In addition to the virulence of the infecting strain of herpesvirus, host factors that contribute to the severity of the disease include genotype, sex and age at the time

of infection. The best characterized genetic locus associated with resistance to Marek's disease is the chicken major histocompatibility complex (MHC). Female birds are more susceptible to the disease than male birds; the reason for this difference is unknown. Resistance to the development of disease increases with age. Transportation, vaccination, handling and beak trimming are stress factors that increase susceptibility to disease.

Pathogenesis and pathology

Following inhalation, virus replicates locally before transfer, probably within macrophages, to the major lymphoid organs where it causes cytolysis, primarily of B cells. Latent infection occurs in T cells activated by the cytolytic process involving B cells. A persistent cell-associated viraemia results in dissemination of the virus throughout the body. Infection of epithelial cells in feather follicles occurs about 2 weeks after infection. Cytolysis of these epithelial cells results in shedding of virus particles into the environment. Genetically susceptible chickens are prone to tumour development and immunosuppression, associated with apoptosis of T cells and thymocytes along with down-regulation of CD8 molecule expression. Lymphomatous lesions may become evident in these birds from 2 weeks to several months after infection. Transformation of T cells probably relates to oncogenes in certain serotype 1 strains. Multiple copies of the virus genome are found in transformed cells, both as episomal DNA and integrated into host cell DNA. Several genomic regions with potential roles in transformation have been identified (Venugopal, 2001) including *meq* (which resembles the Jun/Fos family of oncogenic transcription factor proteins), a basic-leucine zipper gene (Calnek, 1998). Defective immune surveillance mechanisms can allow transformed cells to form lymphoid tumours. The peripheral nerves are often affected, exhibiting proliferative (type A), inflammatory (type B) or minor infiltrative (type C) changes. Demyelination, which is found in type A and type B lesions, causes paralysis. The acute form of Marek's disease is characterized by diffuse infiltration of many internal organs with neoplastic lymphoid cells. A surface antigen expressed on transformed lymphocytes, formerly called Marek's disease tumour-associated antigen (MATSA), is now considered to be merely a marker for activated T cells.

Clinical signs

Birds between 12 and 24 weeks of age are most commonly affected. Clinically,

Marek’s disease presents as partial or complete paralysis of the legs and wings. The mortality rate rarely exceeds 15% with deaths occurring over a number of weeks or months. In the acute form of the disease, birds are severely depressed before death or may die without clinical evidence of disease. The mortality rate in the acute form of the disease is usually between 10% and 30%; outbreaks with mortality as high as 70% have been reported.

Diagnosis

The diagnosis of Marek’s disease is based on clinical signs and pathological findings.

- Paralysis of legs and wings in conjunction with thickening of the peripheral nerves is typical of Marek’s disease.
- In adult birds, nerve involvement is not always evident. In these birds, differentiation from lymphoid leukosis is particularly important.
- Differentiation from lymphoid leukosis is based on the age of affected birds, the incidence of clinical cases and the histopathological findings.
- Virus can be isolated from the buffy coat of blood samples or suspensions of spleen or lymphoma cells from infected birds. Chicken kidney cells or duck embryo fibroblasts can be used for isolation of the virus.
- Viral antigen can be detected in preparations of skin or feather tips, using a radial precipitin test.
- Serum antibodies to GaHV-2 may be demonstrated using AGID, ELISA, immunofluorescence or virus neutralization.
- Primers, which can distinguish attenuated and wild-type strains, have been developed for PCR assays. Suitable sources of viral DNA include blood and feather tips.
- In the absence of characteristic clinical signs, infection with gallid herpesvirus 2 is not indicative of Marek’s disease in a flock.

Control

The use of appropriate management strategies, genetically resistant stock and vaccination have reduced losses from Marek’s disease. Disinfection, all-in all-out policies, and rearing young chicks away from older birds for the first 2 or 3 months of life reduce exposure to infection, decreasing the likelihood of high mortality. A range of modified live vaccines containing the three avian herpesvirus serotypes are commercially available. Although a single dose of

virus injected into day-old chicks provides good lifelong protection, it does not prevent superinfection with virulent field viruses. A synergistic protective effect is obtained by using two or three strains in a vaccine. Consequently meleagrid herpesvirus 1 is commonly incorporated into bivalent or trivalent vaccines. Novel vaccines based on recombinant DNA technology are being developed because of a reduction in the efficacy of conventional vaccines due to emergence of virulent mutants (Witter, 1998). Automated *in ovo* vaccination at the eighteenth day of incubation has replaced conventional vaccination methods in large commercial units (Ricks *et al*., 1999).

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Chapter 62

Papillomaviridae

Viruses in the family *Papillomaviridae* (Latin *papilla*, nipple, combined with Greek suffix *–oma* used to denote tumours) are non-enveloped with icosahedral capsids which contain a single molecule of circular double-stranded DNA. The family contains 16 genera. Papillomaviruses are 55 nm in diameter (Fig. 62.1). Replication takes place in the nucleus and release of new virions occurs by lysis of the infected cell. Members of the family are resistant to lipid solvents, acid and heating at 60°C for 30 minutes. Infections, which are often persistent, usually become established early in life. Papillomaviruses are used experimentally for inserting foreign DNA into cultured cells.

Formerly, papillomaviruses were grouped with polyomaviruses in the family *Papovaviridae*. Infections with polyomaviruses are of minor veterinary importance. Although they produce no clinical effects in natural hosts, most polyomaviruses (polyoma, many tumours) are oncogenic when inoculated into newborn rodents. Budgerigar fledgling disease, caused by an avian polyomavirus, is characterized by outbreaks of acute generalized infection with high mortality in young budgerigars.

Clinical infections

These epitheliotropic, host-specific papillomaviruses cause proliferative lesions (warts) in many mammalian and avian species. Although many papillomaviruses have not been grown in cell culture, the DNA sequences of several papillomaviruses have been elucidated, allowing specific detection in lesions. In infected cells, the viral DNA is usually episomal.

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Key Points

- Non-enveloped, double-stranded DNA viruses
- Icosahedral symmetry
- Contains sixteen genera:
  - – cultivation is highly specialized requiring organotypic raft cultures of keratinocytes or epithelial xenografts in immunocompromised rodents
  - – cause papillomas and fibropapillomas in domestic animals
  - – malignant transformation of alimentary and urinary tract papillomas may occur in cattle ingesting bracken
  - – bovine papillomavirus 1 is aetiologically implicated in equine sarcoids

The clinical conditions associated with papillomavirus infections in domestic animals are presented in Table 62.1. Papillomaviruses tend to be host-specific and to produce proliferative lesions in specific anatomical sites. Infections with papillomaviruses occur in many animal species, but only those that affect humans, cattle, horses and dogs are of clinical significance. Lesions, which are most commonly observed in young animals, usually regress spontaneously after weeks or months. Regression is attributed to the development of cell-mediated immunity. Typical papillomas are composed of finger-like projections of proliferating epithelium supported by a thin core of mature fibrous tissue. In fibropapillomas, the fibrous tissue component predominates. In some host species, several papillomaviruses can cause neoplastic change. Several viral species and more than 80 genotypes have been identified in humans, while in cattle three species and up to ten genotypes are recognized. Recent changes in taxonomy (de Villiers *et al*., 2004) have resulted in the grouping of papillomavirus types into species. Three bovine papillomavirus species are recognized: bovine papillomavirus 1 (BPV genotypes 1 and 2), bovine papillomavirus 3 (BPV genotypes 3, 4 and 6) and bovine papillomavirus 5 (BPV genotype 5). The original six genotypes of bovine papillomavirus (BPV), genotypes 1, 2 and 5 (group A) and genotypes 3, 4 and 6 (group B), can be categorized on the basis of size of genome and the type and location of associated lesions. Group A viruses infect both epithelial cells and fibroblasts while Group B viruses infect epithelial cells only. The genome of BPV 5 shares similarities with the genomes of both xipapillomaviruses and deltapapillomaviruses. In addition, BPV 5 appears to be capable of giving rise to both fibropapillomas and epithelial papillomas (Bloch *et al*., 1994). As a result, BPV 5 has been placed in a separate genus, *Epsilonpapillomavirus*. Several

novel BPVs have recently been identified and at least ten genotypes are now recognized. Progression of papillomas to malignant tumours has been documented in humans, cattle and rabbits.

Figure 62.1 Papillomavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).

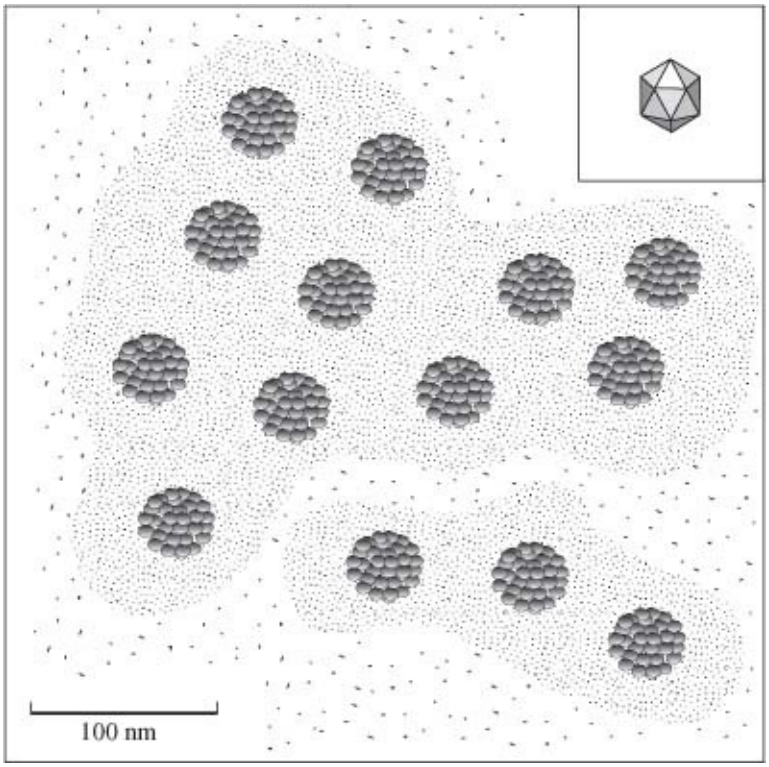

Table 62.1 Papillomaviruses of domestic animals and associated clinical conditions.

Genus	Virus	Genotype	Clinical conditions
Deltapapillomavirus	Bovine papillomavirus 1	1, 2	Fibropapillomas in young cattle; occur mainly on the head and neck and occasionally on the penis. Genotype 2 has been implicated in bladder neoplasia and enzootic haematuria. BPV 1 has been aetiologically implicated in equine sarcoids
	Ovine papillomavirus 1	1, 2	Papillomas and fibropapillomas (rare)
Xipapillomavirus	Bovine papillomavirus 3	3	Cutaneous papillomas with a tendency to persist
		4	Papillomas in the upper alimentary tract, malignant transformation may result from ingestion of bracken fern
		6	Papillomas on the teats of cows, 'frond' type
		9, 10	Papillomas on the teats of cows
Epsilonpapillomavirus	Bovine papillomavirus 5	5	Papillomas on the teats ('rice grain' type) and udder
		8	Papillomas on teats and skin
Novel genus proposed	Bovine papillomavirus	7	Papillomas on skin and teats
Zetapapillomavirus	Equine papillomavirus 1		Papillomas in young horses; occur mainly around lips and on nose
Lambdapapillomavirus	Canine oral papillomavirus		Irregularly-shaped papillomas in the oral cavity of young dogs
	Feline papillomavirus		Uncommon, skin and oral cavity papillomas, described in several cat species

Pathogenesis

Papillomaviruses infect the basal cells of squamous epithelium, usually as a result of minute abrasions. Viruses may also gain entry at vulnerable sites such as junctions between different types of epithelia. Infected cells proliferate but differentiation is delayed. Viral gene expression is restricted during this proliferative phase. Full gene expression results in the production of viral capsids only after cellular differentiation begins in the more superficial epithelial layers. The normal differentiation process is subverted by the virus, the cell nucleus is retained and synthesis of viral proteins necessary for replication and assembly proceeds. New virions can be visualized by electron microscopy in the nuclei of differentiated keratinized cells. Release of virus occurs during desquamation of infected cells from the surface of lesions.

Diagnosis

- Because the clinical appearance of papillomas (warts) is distinctive, laboratory confirmation is not usually required for papillomatous lesions.
- Histopathological examination may be required to determine the nature of some lesions, especially equine sarcoids.
- Electron microscopic examination of specimens from the epidermis may

reveal characteristic virus particles.

- Hybridization assays and PCR methods are available for the detection of papillomavirus DNA, but are not used routinely. Isolates can be typed by extraction of DNA and restriction endonuclease analysis or by Southern blotting.

Bovine cutaneous papillomatosis

In cattle, cutaneous papillomatosis is caused by several types of bovine papillomavirus. Cutaneous fibropapillomas are generally associated with BPV types 1, 2 or 5, whereas BPV types 3, 4 or 6 are commonly linked to papillomas in which the fibrous tissue component is minimal. The different gross appearances of the two types of proliferative response are exemplified by lesions produced by types of papillomavirus on teats of cows. Teat fibropapillomas, associated with BPV-5 infection, have smooth surfaces and are described as ‘rice grain’ type. In contrast, ‘frond’ type teat papillomas arise from infection with BPV-6. Predilection of viruses in this group for distinct anatomical sites is typified by these two bovine papillomaviruses which produce teat lesions.

Fibropapillomas arising from infection with BPV types 1 or 2 are often found on the head and neck of cattle under 2 years of age. Spontaneous regression of the lesions generally occurs within 1 year. Cutaneous papillomas caused by BPV-3 tend to persist. Because infection with BPV is usually self-limiting, treatment is seldom required. Surgical removal of large lesions on teats may be necessary because of interference with milking. Although inactivated autogenous vaccines are used therapeutically, their efficacy is unproven. However, inactivated vaccines can be used prophylactically.

Bovine alimentary papilloma–carcinoma complex

Papillomas of the oesophagus, rumen and reticulum are associated with BPV-4 infection. The lesions, which are often solitary and relatively small, are found incidentally at post-mortem examination. Epidemiological and experimental studies have demonstrated that there is an increased frequency in the occurrence of malignant transformation of virus-induced alimentary papillomas to squamous cell carcinomas when animals are ingesting bracken fern (Jarrett *et al*., 1978; Campo *et al*., 1994). Such malignant lesions, which are found in anatomical sites identical to those of the papillomas, may cause difficulty in swallowing, ruminal tympany and loss of condition. Nodular fibropapillomas caused by BPV-2,

which are occasionally found in similar upper alimentary tract locations, do not appear to become malignant.

Enzootic haematuria

Enzootic haematuria is encountered worldwide in cattle on poor pastures with abundant bracken fern growth. The haemorrhage originates from tumours in the bladder wall. Individual neoplastic lesions derive from either epithelial or mesenchymal tissues. Experimental studies suggest that BPV-2 and toxic compounds from bracken contribute to oncogenesis (Campo *et al*., 1992). It is probable that immunosuppression following ingestion of bracken may allow activation of latent BPV-2 in bladder tissues and this effect, together with the action of carcinogens also present in bracken, is responsible for the induction and progression of neoplastic lesions.

Equine papillomatosis

Papillomas are commonly encountered in horses between 1 and 3 years of age. Two types of equine papillomavirus have been identified based on DNA studies. Type 1 is associated with papillomas on the muzzle and legs, while type 2 is associated with papillomas of the genital tract. Spread may occur by direct or indirect contact. The lesions usually regress spontaneously after several months and recovered animals are immune to reinfection.

Equine sarcoid

The equine sarcoid, a locally invasive fibroblastic skin tumour, is the most common neoplasm of horses, donkeys and mules (Marti *et al*., 1993). Bovine papillomavirus 1 or a closely related variant is implicated in sarcoid development. Experimental inoculation of susceptible animals with these viruses results in fibromatous lesions which resemble sarcoids but which regress spontaneously. Viral DNA with a high degree of homology to BPV has been identified in tissue from sarcoids using both *in situ* hybridization (Lory *et al*, 1993) and PCR (Otten *et al*., 1993).

Lesions usually develop in horses between 3 and 6 years of age. Clusters of equine sarcoid cases have been reported in closely related horses and also in groups of horses on the same farm. However, the incidence of equine sarcoid (estimated at 0.5 to 2%) is comparatively low for a viral disease. This may

indicate that the horse is a non-permissive host (Marti *et al*., 1993).

Sarcoids can occur on any part of the body, either singly or in clusters. The most commonly affected sites are the head, ventral abdomen and limbs. They are highly variable in appearance but can be arbitrarily categorized as verrucous or fibroblastic. Clinical diagnosis should be confirmed histologically. Surgical removal is the usual form of treatment. Recurrence is common following conventional surgery and cryosurgery is more successful. Radiation therapy, CO_2 laser surgery and chemotherapy have also been used with varying degrees of success (Knottenbelt *et al*., 1995). Immunotherapy, aimed at stimulating cell-mediated immunity, may be effective in some cases. This involves intralesional injection of BCG or cell-wall extract of *Mycobacterium bovis* into horses previously sensitized to tuberculoprotein. A novel therapy involving the use of small interfering RNA (siRNA) targeting viral gene expression of the E2 protein has been shown to be effective *in vitro* (Gobeil *et al*., 2009).

Canine oral papillomatosis

Multiple transmissible papillomas in the oropharyngeal region of dogs are often encountered. This condition, which is caused by canine oral papillomavirus, is common in young dogs and is readily transmitted. While the aetiology of this oral condition is well established, the cause of papillomas occurring at other sites in the dog is uncertain (Narama *et al*., 1992).

Canine oral papillomavirus is transmitted by direct and indirect contact. The incubation period is up to 8 weeks. Lesions are usually multiple and although generally confined to the oral mucosa are sometimes found on the conjunctiva, eyelids and muzzle. The papillomas initially appear as smooth, white, raised lesions but later they become rough and cauliflowerlike. Spread may occur inside the oral cavity. There is spontaneous regression within months. Surgical removal is generally unnecessary unless the papillomas persist or cause physical discomfort. Inactivated vaccines have been used but do not appear to be effective. Live unattenuated vaccines, which are effective, may produce neoplastic lesions at the injection site (Bregman *et al*, 1987).

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Chapter 63

Adenoviridae

Adenoviruses (Greek *adenos,* gland), first isolated from expiant cultures of human adenoids, are icosahedral (70 to 90 nm in diameter) and contain a single linear molecule of double-stranded DNA. Fibres project from each of the 12 vertices of the capsid (Fig. 63.1). Agglutination of rat or monkey erythrocytes, a property of many adenoviruses, is dependent on the fibre proteins which possess type-specific immunodetermi- nants. The family *Adenoviridae* is composed of four genera, *Mastadenovirus, Aviadenovirus, Atadenovirus* and *Siadenovirus.* One further genus has been proposed, *Ichtadenovirus,* for fish adenoviruses. Mammalian adenoviruses, assigned to the genus *Mastadenovirus,* infect mammals only, share a common antigen and are serologically distinct from those that infect birds. Serogroups and serotypes are defined on the basis of neutralization assays. Haemagglutination inhibition is used to confirm serospecificity. Adenoviruses are moderately stable in the environment, in which they can survive for many weeks. They can withstand freezing, mild acid and lipid solvents. Infectivity is typically abolished by heating at 56°C for more than 10 minutes but heat sensitivity varies from genus to genus.

Adenoviruses replicate in cell nuclei. Newly assembled virions form crystalline aggregates, demonstrable as intranuclear basophilic inclusions in stained tissue sections. Adenoviruses have a natural host range generally confined to a single species or to closely related species. Infection is common in animals and humans. Fifty-one human serotypes have been recognized, grouped into six species (human adenovi- rus A to F). Most human infections appear to be subclinical or mild, although immunodeficient individuals may develop severe clinical disease. In contrast to human infections, certain animal infections result in severe illness. Genera and species belonging to the *Adenoviridae* are listed in Fig. 63.2. Adenoviruses of veterinary importance are presented in Table 63.1.

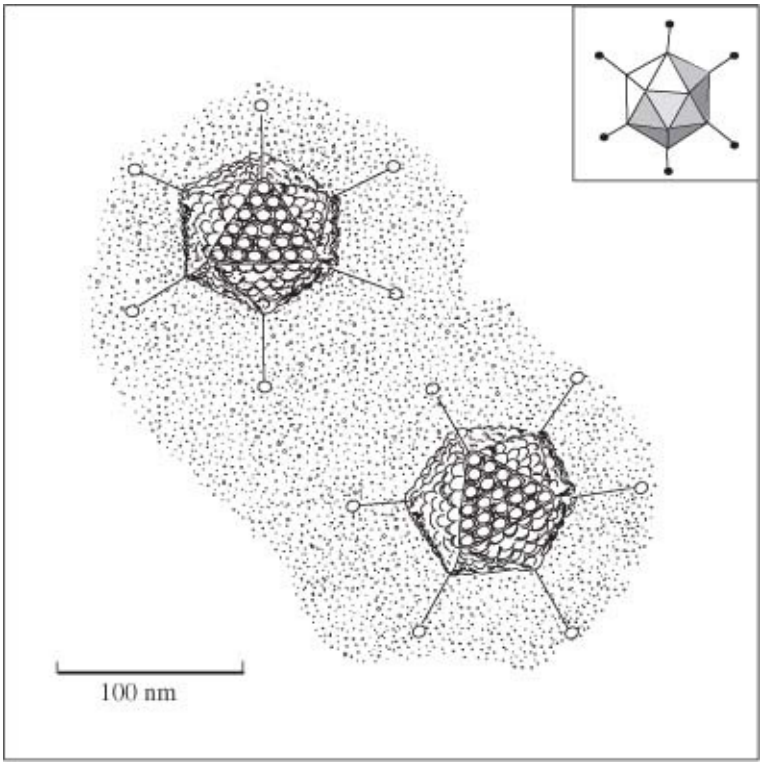
Key points

- Non-enveloped, double-stranded DNA viruses
- Icosahedral symmetry
- Replicate in nuclei, forming intranuclear inclusion bodies
- Moderately stable in the environment
- Four genera:
  - –*Aviadenovirus*, avian adenoviruses
  - –*Mastadenovirus*, mammalian adenoviruses
  - –*Atadenovirus*, viruses of vertebrates
  - –*Siadenovirus*, amphibian virus and avian virus
- Systemic and respiratory diseases in dogs
- Systemic diseases in poultry

Clinical infections

Adenovirus infections can be particularly severe in dogs and domestic fowl. Two serotypes of canine adenovirus are recognized. Infection with canine adenovirus type 1 (CAV-1) causes infectious canine hepatitis, a severe generalized disease, whereas infection with canine adenovirus type 2 (CAV-2) is commonly associated with localized respiratory disease. In other domestic mammals, adenovirus infections are associated occasionally with enteric or respiratory problems. In Arabian foals with combined immunodeficiency disease, pulmonary infection with equine adenovirus A is invariably fatal.

Figure 63.1 Adenovirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Avian adenoviruses are distributed worldwide and have been isolated from a wide range of species including chickens, turkeys, pheasants, pigeons, ducks, quail, geese, guinea fowl and budgerigars. Infection is extremely common in poultry flocks. Most of these infections are either subclinical or associated with relatively mild disease. However, severe disease may follow infection with certain avian adenoviruses, egg-drop syndrome virus and turkey adenovirus A. Fowl adenovirus A, also known as chicken embryo lethal orphan virus (CELO virus, fowl adenovirus 1), is the type species for avian adenoviruses and causes quail bronchitis. Fowl adenovirus C (serotype 4) is associated with hepatitis-hydropericardium syndrome in 3- to 6-week-old broilers.

Infectious canine hepatitis

This worldwide, generalized, viral disease of dogs, caused by CAV-1, principally affects the liver and vascular endothelium. The complete DNA sequence of CAV-1 has been determined (Morrison *et al*, 1997).

Epidemiology

Infectious canine hepatitis has become relatively uncommon because of the widespread use of effective vaccines. Although dogs are the most commonly

affected species, foxes, wolves, coyotes, skunks and bears are also susceptible (Thompson *et al*., 2010). Transmission can occur following ingestion of urine, faeces or saliva from infected animals. The immune response usually eliminates virus from host tissues by 14 days after initial infection. However, virus may persist in the kidneys and, in some instances, may be excreted in urine for more than 6 months.

Pathogenesis

Following ingestion, CAV-1 localizes in the tonsils and Peyer's patches (Fig. 63.3). As viraemia develops, replication in vascular endothelium results in rapid distribution of virus throughout the body. Virus replication also occurs in the parenchymal cells of the liver and kidneys. Clinical recovery in most dogs coincides with the production of neutralizing antibodies at about 10 days after infection. Glomerulonephritis, corneal oedema and anterior uveitis, attributable to immune complex deposition, may develop in some infected animals.

Clinical signs

The incubation period is up to 7 days. Dogs of all ages are susceptible and subclinical infection is common. Clinical disease is most frequently encountered in young dogs. The mortality rate ranges from 10 to 30% in mature dogs and up to 100% in young pups. In peracute disease, death occurs so rapidly that poisoning may be suspected. In acute disease, affected dogs present with fever, depression, anorexia, increased thirst, vomiting and diarrhoea. Abdominal palpation may elicit pain and, although hepatomegaly may be detected, jaundice is uncommon. Corneal opacity, either unilateral or bilateral, may occur within weeks of clinical recovery in about 20% of affected animals and usually resolves spontaneously. Recovered animals have life-long immunity.

Diagnosis

- A history of fever, sudden collapse and abdominal pain in young, unvaccinated dogs may suggest infectious canine hepatitis.
- Haematological examination may reveal a marked reduction in neutrophils and lymphocytes during the febrile stage. Clotting time may be prolonged.
- Basophilic intranuclear inclusion bodies in hepatocytes, Kupffer cells and endothelial cells are confirmatory.
- Viral antigen can be demonstrated by immunofluorescence in cryostat

sections of liver.

- PCR methods for detecting viral DNA in clinical specimens have been described (Kiss *et al*., 1996; Chouinard *et al*., 1998; Hu *et al*, 2001).
- Suitable specimens for virus isolation in canine kidney cells include oropharyngeal swabs, blood, urine and faeces from affected animals sampled during the febrile stage of the disease. Samples of spleen, lymph nodes and kidney obtained post mortem are also suitable for virus isolation. Because of high levels of arginase activity in hepatic tissue, liver samples are unsuitable for virus isolation as this enzyme inhibits viral replication in tissue culture.
- A rising antibody titre, detectable using virus neutralization or haemagglutination inhibition tests, is indicative of active CAV-1 infection.

Figure 63.2 Genera and species of veterinary importance in the Adenoviridae. The species name is assigned a letter if more than one adenovirus species exist in the same host.

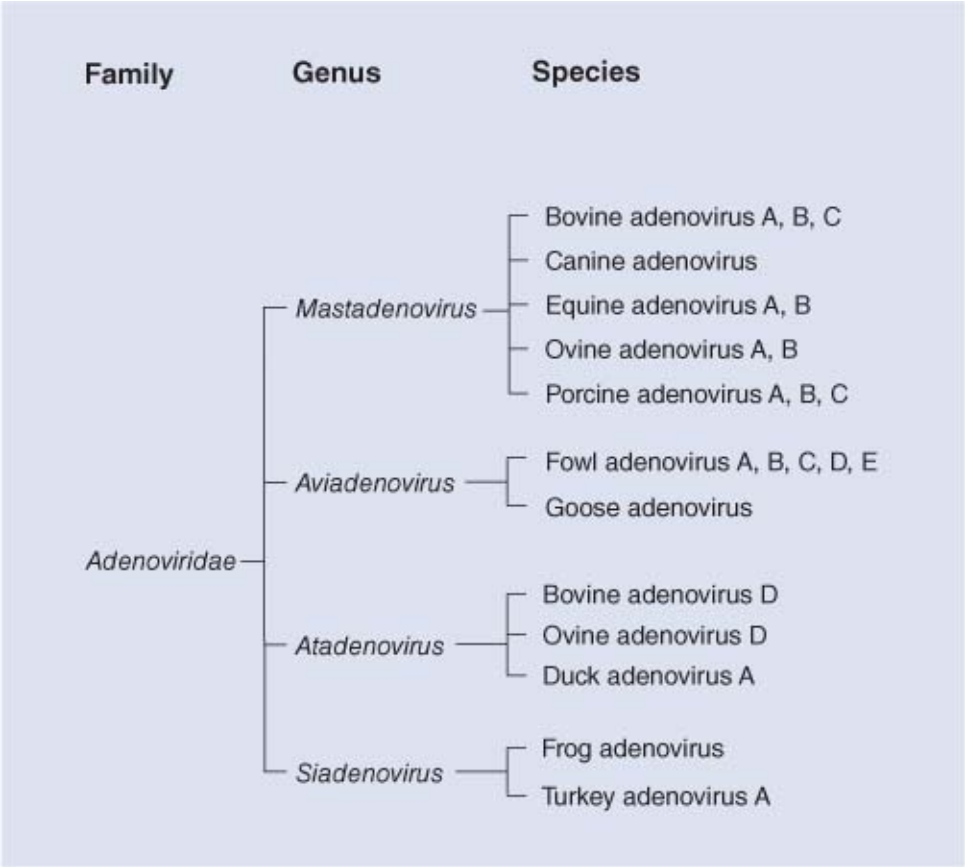
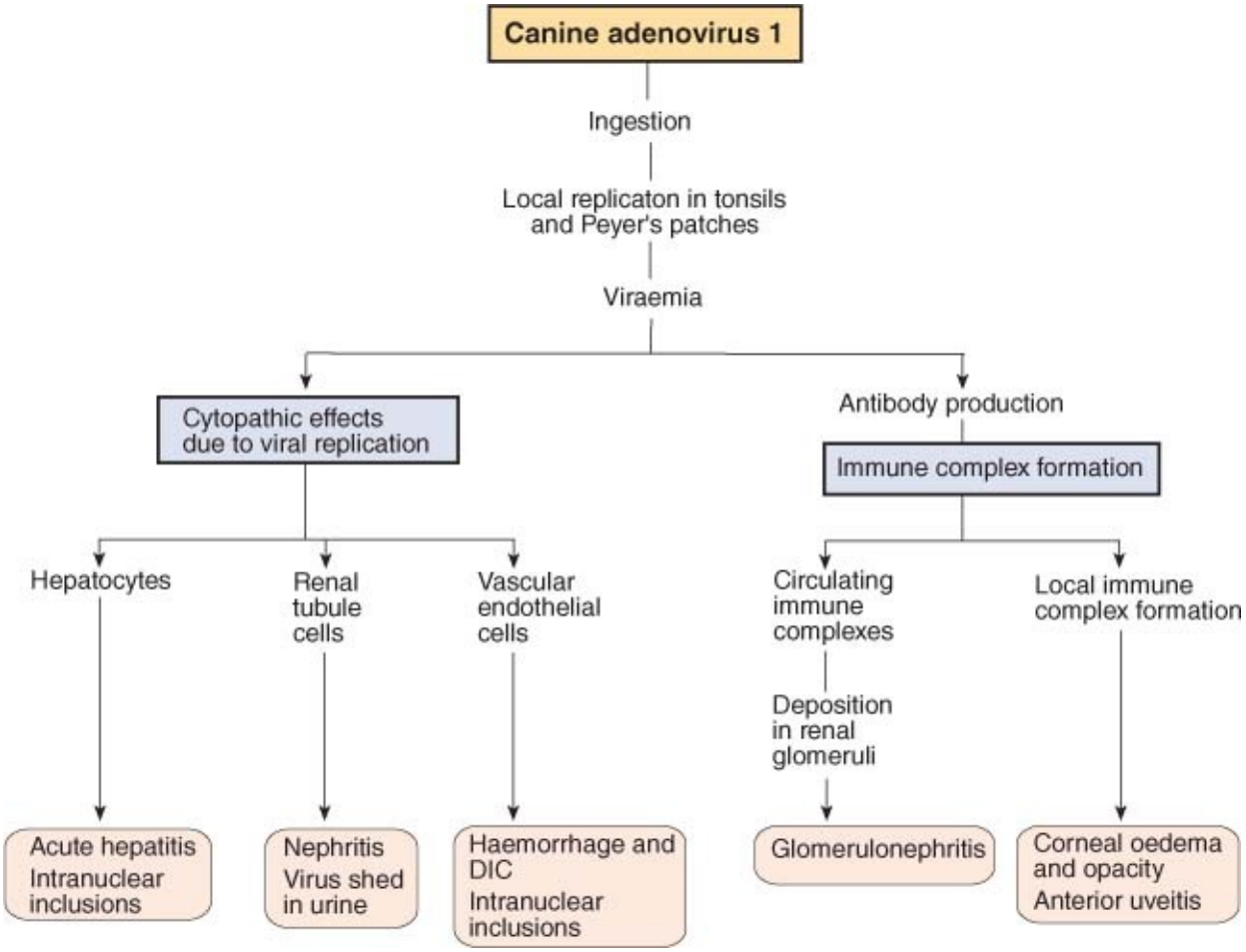


Table 63.1 Adenoviruses of veterinary importance.

Virus	Comments

Canine adenovirus	Two strains are recognized, canine adenovirus 1 and canine adenovirus 2. Canine adenovirus 1 causes infectious canine hepatitis, with lesions arising from direct cytopathic effects and immune complex formation. Canine adenovirus 2 is involved in infectious tracheobronchitis (kennel cough), a highly contagious respiratory disease
Equine adenovirus A	Usually a subclinical or mild respiratory infection in the horse population. Is associated with pneumonia, which is invariably fatal, in Arabian foals with severe combined immunodeficiency disease
Bovine adenoviruses	Associated with occasional outbreaks of respiratory and enteric disease
Ovine adenoviruses	Associated with occasional outbreaks of respiratory and enteric disease
Porcine adenoviruses	Usually subclinical infections; occasionally cause diarrhoea
Fowl adenoviruses	Frequently isolated from healthy birds or following respiratory disease. Associated with quail bronchitis, inclusion body hepatitis and hepatitis–hydropericardium syndrome
Duck adenovirus A	Causes egg drop syndrome in laying hens
Turkey adenovirus A	Causes turkey haemorrhagic enteritis (dysentery in 4 to 12-week-old poults with a mortality rate of up to 60%) and marble spleen disease in pheasants (characterized by sudden death, pulmonary oedema and splenic necrosis in 2 to 8-month-old birds)

Figure 63.3 Mechanism involved in the development of localized and generalized lesions following infection with canine adenovirus 1. DIC: disseminated intravascular coagulation.



Treatment and control

Supportive treatment may allow time for hepatocellular regeneration. Fluids should be administered intravenously to counteract dehydration and shock. In severely ill animals, blood transfusions may be required to prevent or control haemorrhage.

Inactivated and modified live vaccines are available. Occasionally, vaccination with modified live CAV-1 vaccines results in mild nephropathy with shedding of virus in urine and, in some instances, corneal opacity. These side effects do not occur with modified live CAV-2 vaccines which stimulate effective long-lasting immunity to CAV-1. Pups with unknown levels of maternally derived antibody should be vac-cinated twice, at 8 to 10 weeks and at 12 to 14 weeks of age. Booster injections, either annually or at intervals of 2 years, are recommended. Inactivated CAV-1 vaccines, which do not induce obvious side effects, require boosters at frequent intervals in order to maintain adequate antibody levels.

Infection with canine adenovirus type 2

Canine adenovirus type 2, which is readily transmitted by aerosols, replicates in both upper and lower respiratory tract. Clinical signs are generally mild. Affected dogs may present with clinical signs similar to those of canine infectious tracheobronchitis (kennel cough). Most dogs recover and are immune to subsequent challenge. Virus shedding continues for about 9 days after infection. Occasional cases of bronchopneumonia may develop due to secondary bacterial infection. Diagnosis can be confirmed by virus isolation, *in situ* hybridization and PCR (Benetka *et al*., 2006).

Inclusion body hepatitis

Although inclusion body hepatitis (IBH) affects mainly broilers, it sometimes occurs in pullets. There is uncertainty about the cause of IBH, and a number of fowl adenovirus serotypes have been aetiologically implicated. In affected flocks, a sudden increase in mortality, which may reach 30%, is a feature of the disease. Immunosuppression due to infectious bursal disease or chicken anaemia virus infection predisposes to severe infection and high mortality. Lesions include an enlarged liver with scattered haemorrhages and necrosis, intramuscular haemorrhage and anaemia. Intranuclear inclusions in hepatocytes are prominent. Diagnosis is based on characteristic hepatic lesions. Because

apparently healthy birds excrete adenoviruses and may be serologically positive, the significance of positive serological tests is questionable. As vaccines are not readily available and the aetiology of IBH is not definitively established, specific control measures cannot be formulated.

Egg drop syndrome

A disease referred to as egg drop syndrome, caused by an adenovirus prevalent in ducks and probably introduced into chickens through a contaminated vaccine, was first described in 1976. In flocks, the disease is characterized by a drop in egg production or by failure to reach peak production. Infected hens may lay abnormal eggs. Laying hens up to 36 weeks of age are most commonly affected. Inflammatory lesions are found in the oviduct, particularly the pouch shell gland. Intranuclear inclusions may be present in the epithelial cells of this gland. Samples of oviduct, including material from the pouch shell gland, are suitable specimens for virus isolation in avian cell lines, especially duck kidney or fibroblast cell lines. Virus can also be detected using ELISA, immunofluorescence or PCR. As the virus agglutinates avian red cells, haemagglutination inhibition is the preferred method for serological screening of flocks. Control of egg drop syndrome relies on the use of inactivated vaccines before laying begins. Appropriate hygienic measures combined with disinfection can be used to limit the spread of infection. Because of the high risk of cross-infection, chickens and ducks should be housed separately.

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Chapter 64

Poxviridae

The family *Poxviridae* contains the largest viruses which cause disease in domestic animals. Poxvirus symmetry is complex. The virions in this family are either brick-shaped (220 to 450nm×140 to 260 nm) with a surface membrane composed of tubular or globular proteins or ovoid (250 to 300nm × 160 to 190 nm) with a surface membrane composed of a regular spiral filament (Fig. 64.1 and 64.2). They contain more than 100 proteins including several virus-encoded enzymes. A biconcave core or nucleoid contains linear double-stranded DNA and one or two lateral bodies within a surface membrane (Fig. 64.3). A cell-derived envelope encloses some of the mature extracellular virions.

The family is divided into two subfamilies, *Chordopoxvirinae*, the poxviruses of vertebrates, and *Entomopoxvirinae*, the poxviruses of insects (Fig. 64.4). The subfamily *Chordopoxvirinae* is comprised of eight genera, namely *Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus and Yatapoxvirus*. Genetic recombination within genera results in extensive serological cross-reactions and cross-protection. Replication in the cytoplasm of host cells takes place within defined areas (viral factories) producing intracellular mature virions (IMV). Some IMVs acquire an additional double-layered envelope, derived from host cell membranes and containing host cell lipids and virus-encoded proteins such as the haemagglutinin protein of the orthopoxviruses. This form is referred to as intracellular enveloped virus (IEV). The IEVs are transported to the surface of the cell where fusion with the cell membrane results in loss of the outer envelope layer and release of the virus. Released virus may remain attached to the cell's plasma membrane as cell-associated enveloped virus (CEV) or be fully released as extracellular enveloped virus (EEV). Both the enveloped and non-enveloped forms of the virus are infectious. The CEV and EEV forms are important in cell-to-cell spread, while IMV infection becomes important following cell death and lysis of the infected cell. Virions are stable at room temperature under dry conditions but sensitive to heat, detergents, formaldehyde and oxidizing agents. The genera

differ in ether sensitivity.

Key points

- Enveloped DNA viruses
- Complex symmetry
- Replicate in cytoplasm
- Stable in the environment
- Skin lesions a prominent feature
- Individual poxviruses tend to infect particular hostspecies; some poxviruses are not species-specific

Infections with poxviruses, which can affect many vertebrate and invertebrate hosts, usually result in vesicular skin lesions (Table 64.1). Smallpox, caused by variola virus, was formerly a human disease of major international significance. The use of vaccinia virus for the prevention of smallpox, first introduced by Jenner in the late eighteenth century, eventually led to the eradication of this highly contagious disease at the close of the twentieth century. Poxviruses code for a large number of proteins that subvert immune and inflammatory pathways in the host. These proteins include inhibitors of antiviral pathways of the cell, anti-apoptotic factors and immune modulators to modulate host defences against the virus (Stanford *et al*, 2007).

Figure 64.1 Orthopoxvirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).

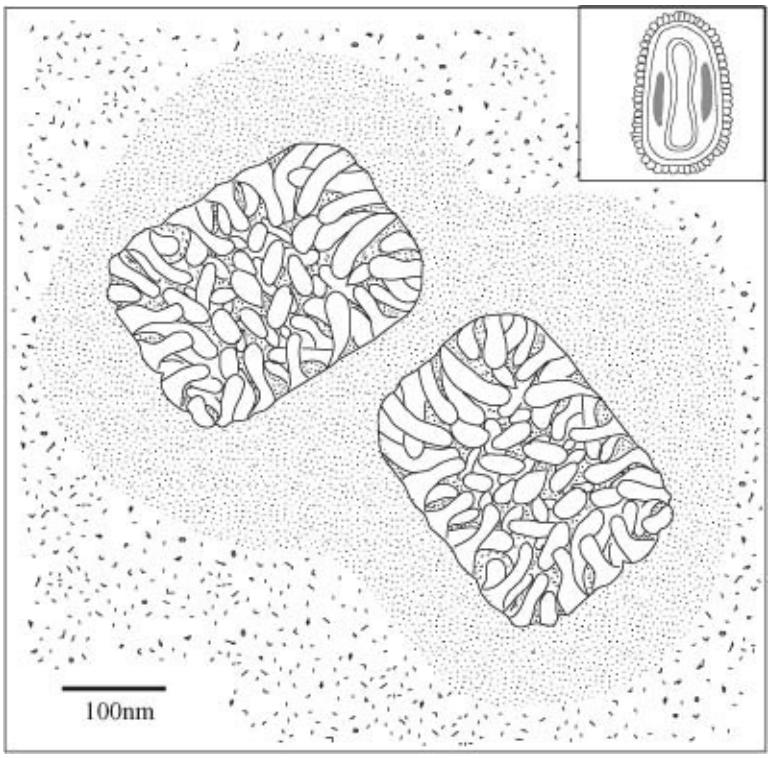


Figure 64.2 Parapoxvirus particles as they appear in an electron micrograph.

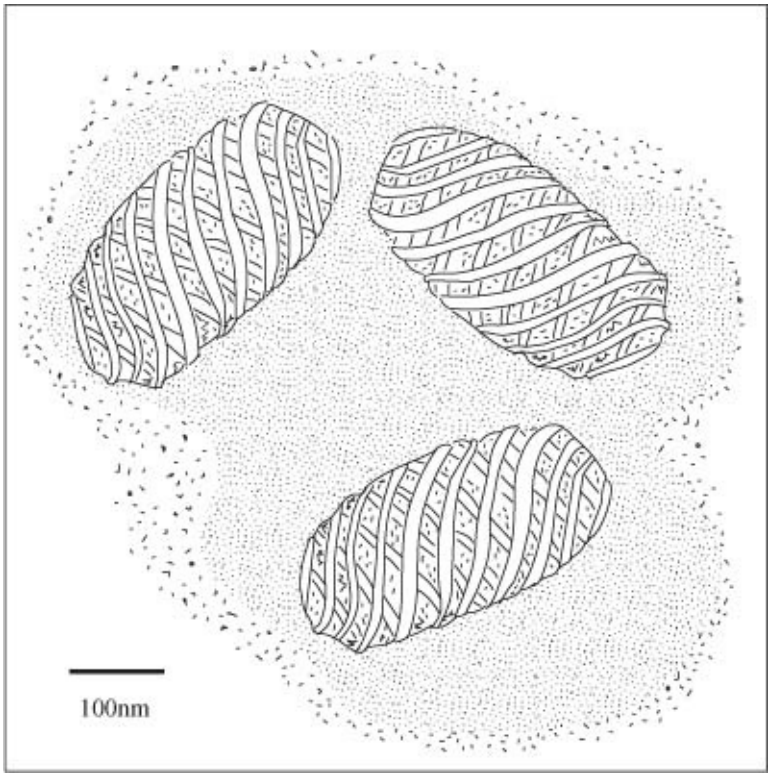
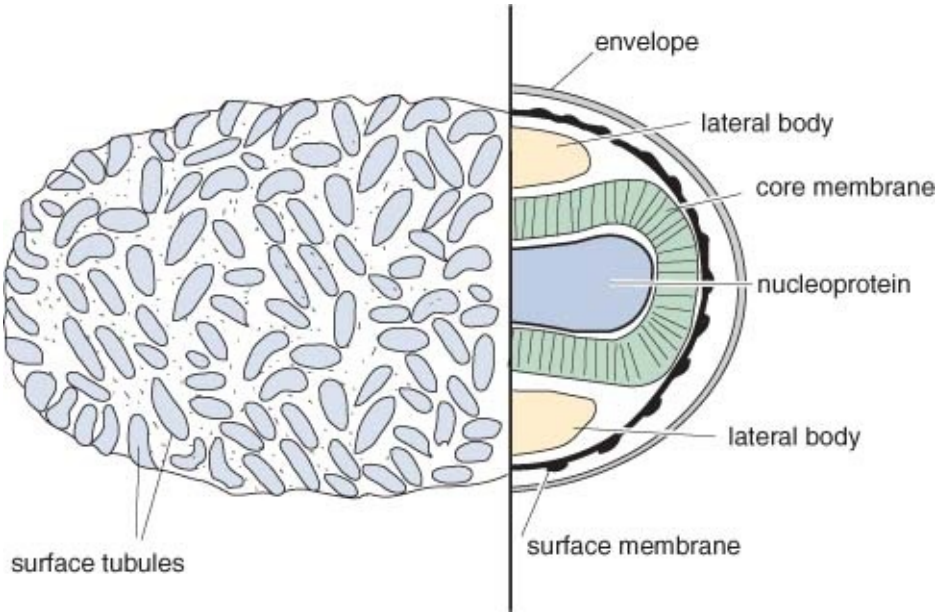


Figure 64.3 Diagrammatic representation of an orthopoxvirus displaying the surface structure of an unenveloped virion (left) and a cross-section of an

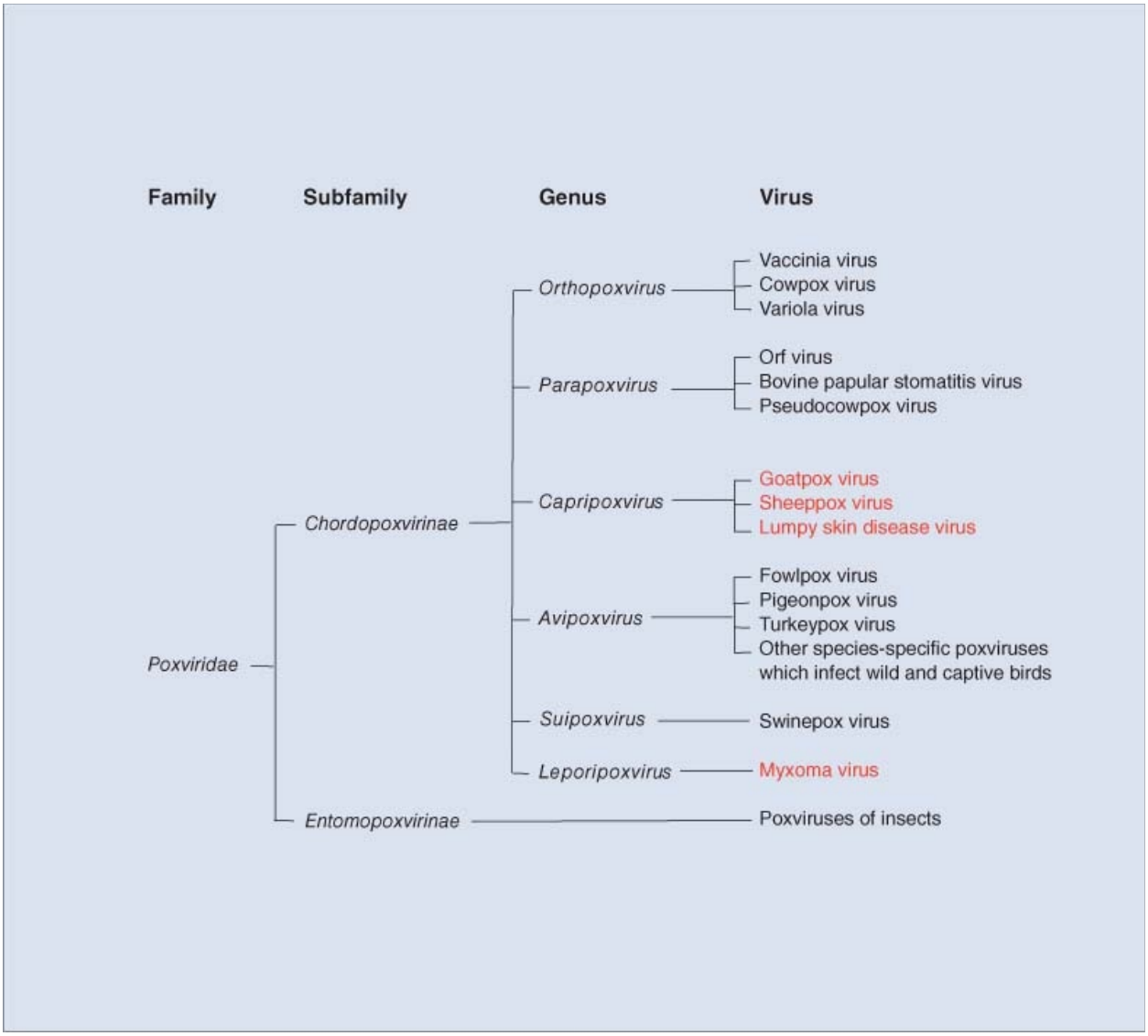
enveloped virion (right).



Clinical infections

Transmission of poxviruses can occur by aerosols, by direct contact, by mechanical transmission through arthropods and through fomites. Skin lesions are the principal feature of these infections. Several virus-encoded proteins are released from infected cells including a homologue of epidermal growth factor which stimulates cell proliferation. Typically, pox lesions begin as macules and progress through papules, vesicles and pustules to scabs which detach leaving a scar. In generalized infections, there is a cell-associated viraemia and recovered animals have solid immunity. Some localized pox infections may induce transient immunity and reinfection can occur.

Figure 64.4 Classification of poxviruses with emphasis on those that affect domestic animals. Viruses in red cause OIE-listed diseases.



Three closely related parapoxviruses, namely pseu-docowpox virus, bovine papular stomatitis virus and orf virus, infect ruminants. These viruses are transmissible to humans producing lesions which are clinically similar. Moreover, the three viruses are morphologi-cally indistinguishable and differentiation of the causal agent requires nucleic acid analysis.

Capripoxviruses are economically important viruses producing generalized infections with significant mortality in domestic ruminants. Sheeppox virus, goatpox virus and lumpy skin disease virus are closely related and share a group-specific structural protein (p32), which allows the same vaccine to be used against each virus.

Many avian species are susceptible to infection with members of the genus *Avipoxvirus*. Although antigenic relationships exist among avian poxviruses, this

relat-edness is variable. Virus species within the genus, named in accordance with their affinity for particular host species, include fowlpox virus, canarypox virus, pigeonpox virus and turkeypox virus. The type species of the genus is fowlpox virus.

Table 64.1 Members of the *Poxviridae* of veterinary significance.

Virus	Genus	Host species	Significance of infection
Vaccinia virus	*Orthopoxvirus*	Wide host range	Infections in sheep, water buffaloes, rabbits, cattle, horses and humans. Used as a recombinant virus vector for rabies vaccine
Cowpox virus	*Orthopoxvirus*	Rodents, cats, cattle	Small species of rodents are the likely reservoir hosts. Cats are the principal incidental hosts; infection results in skin lesions. Rare cause of teat lesions in cattle. Transmissible to humans
Uasin Gishu virus	*Orthopoxvirus*	Unknown wildlife reservoir, horses	Rare disease, reported in Kenya and neighbouring African countries. Causes papilloma-like skin lesions in horses
Camelpox virus	*Orthopoxvirus*	Camels	Widely distributed in Asia and Africa. Causes systemic infection with typical pox lesions; severe infection in young camels
Pseudocowpox virus	*Parapoxvirus*	Cattle	Common cause of teat lesions in milking cows; causes milker's nodule in humans
Bovine papular stomatitis virus	*Parapoxvirus*	Cattle	Produces mild papular lesions on the muzzle and in the oral cavity of young cattle. Transmissible to humans
Orf virus	*Parapoxvirus*	Sheep, goats	Primarily affects young lambs; causes proliferative lesions on the muzzle and lips. Transmissible to humans
Sheeppox/goatpox virus	*Capripoxvirus*	Sheep, goats	Endemic in Africa, Middle East and India. Causes generalized infection with characteristic skin lesions and variable mortality
Lumpy skin disease virus	*Capripoxvirus*	Cattle	Endemic in Africa. Causes generalized infection with severe lesions and variable mortality
Swinepox virus	*Suipoxvirus*	Pigs	Causes mild skin disease. Occurs worldwide. Transmitted by the pig louse, *Haematopinus suis*
Fowlpox virus	*Avipoxvirus*	Chickens, turkeys	Causes lesions on the head and on the oral mucous membrane. Occurs worldwide. Transmitted by biting arthropods
Myxoma virus	*Leporipoxvirus*	Rabbits	Causes mild disease in cottontail rabbits, the natural host, and severe disease in European rabbits. Introduced into Europe, Australia and Chile as a biological control measure
Squirrel poxvirus	Unassigned	Red and grey squirrels	Important factor in decline of native red squirrels (*Sciurus vulgaris*) in Great Britain; carried by grey squirrel (*Sciurus carolinensis*) introduced from North America

Infections caused by vaccinia virus

Although the natural host species of vaccinia virus is unknown, mild infections have been described in a wide range of species including sheep, cattle, horses and humans. Formerly, the virus was used to vaccinate against smallpox. Buffalopox virus and rabbitpox virus are considered to be subspecies of vaccinia virus. Spread of vaccinia virus among cattle and transmission to humans occurs at milking. The lesions on the teats of cows resemble those caused by cowpox

virus. A clinical condition similar to horsepox or equine papular dermatitis can be produced experimentally using vaccinia virus (Studdert, 1989). In recent years, vaccinia virus has been used as a recombinant virus vector for vaccination against several diseases including rabies, canine distemper and measles.

Infections caused by cowpox virus

Cowpox is endemic in parts of Europe. Although infection and disease have been described in cattle, cats, humans and a range of mammals in zoos, these species are considered to be incidental hosts. The reservoir hosts are probably wild rodents. There is evidence that voles and woodmice are the principal reservoir hosts in western Europe (Chantrey *et al*., 1999). In milking cows, lesions are usually confined to the teats. The domestic species in which the disease is most often recognized is the cat. Affected cats usually come from rural areas and are described as good hunters. Moreover, infections in cats tend to peak in the autumn when small rodent populations are high, suggesting rodent-derived infections. Although cat-to-cat transfer occurs, this mode of transmission is rare. Small papules on the head or forelimbs, the first recognizable signs of the infection, eventually ulcerate. Scab formation is usually followed by complete resolution in about 6 weeks. Secondary skin lesions develop in some cats and a few animals may show signs of coryza or conjunctivitis. Rarely, pneumonia and pleural exudation have been described. Diagnosis can be confirmed by histopathology, PCR, electron microscopy or virus isolation. No specific control measures are warranted. Human infections, which are usually contracted from infected cats, are uncommon.

Infections caused by pseudocowpox virus

Pseudocowpox, also known as milker’s nodule, is caused by pseudocowpox virus, a parapoxvirus with worldwide distribution. It is a common mild condition affecting the teats of lactating cows. Infection spreads slowly through milking herds with variation in the number of affected animals at any particular time. There is evidence to suggest that subclinical persistent infection occurs in lymphatic tissue in cattle (Iketani *et al*., 2002). Transmission is by direct or indirect contact. Transfer of infection can occur through teat cups and on milkers’ hands. Transmission can also occur mechanically by flies or when calves are being suckled. Small red papules on teats or udder develop into ulcers with overlying scab formation. Healing at the centre of the lesions produces

characteristic ring-shaped or horseshoe-shaped scabs. Typical parapoxvirus particles can be demonstrated in scab material using electron microscopy. Control is based on appropriate hygienic measures at milking, including the use of effective teat dips. In human infections, lesions are usually confined to the hand, forearm or face.

Bovine papular stomatitis

This mild viral disease of young cattle occurs worldwide. It is caused by a parapoxvirus, bovine papular stomatitis virus, which is transmitted by direct or indirect contact. Infection is common and usually subclinical. Mature cattle are considered to be reservoirs of infection.

Affected calves commonly develop lesions in the buccal cavity and on the muzzle. These lesions are characterized by hyperaemic foci that develop into papules with concentric zones of inflammation. Affected animals usually recover within 3 weeks. A more severe, chronic form of the disease, which may be associated with concurrent infections or immuno-suppression, has been described (Yeruham *et al*., 1994). Virions can be demonstrated in skin scrapings by electron microscopy. The virus is transmissible to humans.

Orf

This important disease of sheep, also known as contagious pustular dermatitis or contagious ecthyma, occurs worldwide and is caused by a parapoxvirus. Goats, camels and humans are also susceptible to infection.

Epidemiology

The virus is transmitted through direct or indirect contact. Under dry environmental conditions, the virus is stable and can survive in scab material for months. Infectivity is substantially reduced after exposure to adverse climatic conditions. In most flocks, infection is maintained by sheep with chronic lesions (McKeever and Reid, 1986).

Pathogenesis

The virus, which is epitheliotropic, produces proliferative wart-like lesions following entry through skin abrasions such as those acquired from thistles. The virus replicates in epidermal keratinocytes, and infected cells release a vascular

endothelial growth factor, which stimulates angiogenesis in the skin (Haig, 2006). The virus encodes a number of factors that interfere with inflammation and the local immune response including a homologue of interleukin 10, a chemokine binding protein and a granulocyte-macrophage colony stimulating factor/interleukin 2 binding protein (Fleming and Mercer, 2007). Papular lesions progress to vesicles, pustules and eventually scab formation. Proliferation of cells underlying scabs produces verrucose masses. In the absence of secondary bacterial infection, lesions usually heal within 4 weeks.

Clinical signs

The disease primarily affects young sheep. The incubation period is up to 7 days. Although lesions most often occur on the commissures of the lips and on the muzzle, they may also develop in the mouth and on the feet, genitalia and teats. Mild lesions may go unnoticed. Severely affected lambs with lesions in the buccal cavity often fail to eat, lose condition and may die. Outbreaks last for some months and vary in severity from farm to farm and from year to year. The disease does not usually recur until the birth of new susceptible lambs in the flock. Although isolates from individual flocks can differ in genotype, there is no evidence that the severity of the disease correlates with the strain of the virus involved (Gilray *et al*., 1998). Environmental management factors may influence the outcome of infection (Gumbrell and McGregor, 1997). Concurrent bacterial infection with oedema of the head and swelling of the tongue, which could be confused with bluetongue, has been described.

Immunity following natural infection may not confer complete protection, due in part to virus immunomodulator proteins that interfere with host immune responses (Haig and McInnes, 2002). However, lesions in animals previously affected are usually less severe and heal more rapidly than those developing after first exposure. In chronically infected sheep, lesions may be either mild or proliferative. Immunity to the virus requires a cell-mediated immune (CMI) response and neonatal lambs are susceptible to infection despite receipt of colostrum from previously infected ewes.

Diagnosis

Lesions of orf are readily recognized by their characteristic appearance and distribution. Virus present in lesion material can be identified by electron microscopy. Primers are available for the detection of parap-oxvirus DNA in

clinical specimens (Kottaridi *et al*., 2006).

Treatment and control

There is no specific treatment for infection with orf virus. Antibiotic therapy reduces the effect of secondary bacterial infection in young lambs.

In endemically infected flocks, control is based on the use of a fully virulent live vaccine derived from scab material or cell culture. Ewes should be vaccinated by scarification in the axilla at least 8 weeks before lambing. When close to lambing, they must be moved to a new grazing area in order to minimize exposure of lambs to infectious vaccinal scab material. Lambs should be vaccinated only if an outbreak occurs in a flock. If carried out effectively, thorough cleaning and disinfection of surfaces and equipment between periods of housing may reduce the amount of residual virus in buildings.

Humans are susceptible to infection with orf virus. Typically, a single lesion occurs on hands, forearms or face. Care should be exercised when handling infected sheep and when using live vaccines.

Sheeppox and goatpox

Both of these diseases are endemic in south-eastern Europe, the Middle East, Africa and Asia. The viruses of sheeppox and goatpox are members of the genus Capripoxvirus. A range of capripoxvirus strains have been isolated from sheep and goats and there is evidence of recombination between strains (Gershon *et al*., 1989). Although some strains are extremely pathogenic in both sheep and goats, other strains produce severe disease in only one of these species. Recent genetic studies suggest that sheeppox virus and goatpox virus are phylogenetically distinct viruses (Tulman *et al*., 2002).

Epidemiology

Virus particles are shed from skin lesions and in ocular and nasal discharges during the acute stages of the disease. Infection occurs through skin abrasions or by aerosol. Biting insects may also transmit the virus mechanically. Housing or stockading animals facilitates transmission of the virus. Following infection, capripoxvirus strains induce immunity. In endemic areas, where indigenous animals frequently have a high level of naturally acquired immunity, generalized disease and mortality are rare. In isolated flocks, outbreaks of severe disease

may occur.

Pathogenesis and pathology

The virus replicates locally either in the skin or in the lungs. Spread to the regional lymph nodes is followed by viraemia and replication in various internal organs. Skin lesions, typical of poxvirus infection, appear about 7 days post infection. Lung lesions present as multiple nodular areas of consolidation.

Clinical signs

Following an incubation period of about 1 week, infected animals develop fever, oedema of the eyelids, conjunctivitis and nasal discharge. Within a few days, macules which rapidly develop into papules appear on the skin and external mucous membranes. Scabs form over necrotic papules. The severity of the clinical signs depends on the breed, age, immune status and nutritional status of the host animal as well as the strain and virulence of the infecting capripoxvirus. Lesions in mild afebrile disease may be minimal and confined to the skin beneath the tail.

Mortality rates with infections from some strains of capripoxvirus may be up to 50% even in indigenous breeds. The disease is most severe in young animals and in imported breeds. In some European breeds, which are extremely susceptible, mortality rates may approach 100%. Secondary bacterial infection or dissemination of the virus to other sites may result in a severe form of the disease.

Diagnosis

Diagnosis can often be made solely on clinical grounds. Skin biopsies or post-mortem specimens may be used for laboratory confirmation.

- Eosinophilic intracytoplasmic inclusions may be demonstrable histologically in epidermal cells.
- Electron microscopy can be used for the rapid identification of poxvirus particles in material from lesions. Capripoxviruses can be readily distinguished from parapoxviruses.
- Virus may be isolated in lamb testis or kidney cell monolayers.
- An antigen-trapping ELISA has been developed for the detection of capripoxvirus antigen (Carn, 1995).

- Protocols for PCR-based detection of viral DNA in biopsy or tissue culture material are available (Heine *et al*., 1999). It is possible to distinguish sheeppox virus from goatpox virus by restriction enzyme analysis of PCR-amplicons of the gene encoding the major antigen P32 (Hosamani *et al*., 2004).
- Several serological methods including virus neutralization, western blot analysis, indirect ELISA and the indirect fluorescent antibody test are available.

Control

In endemic areas, control is based on annual vaccination. All capripoxviruses share a major neutralizing site, inducing good cross-protection against all field strains of virus. Several modified live vaccines are available including a Kenyan sheeppox strain used in sheep and goats, a Romanian strain used in sheep, and the Mysore strain used in goats. A subunit vaccine has also been developed (Carn *et al*., 1994). Inactivated vaccines are not recommended as they are less effective than modified live vaccines because cell-mediated immunity is the predominant protective response.

Capripoxviruses are being employed as vectors for important ruminant viral vaccines (Romero *et al*., 1993). These vector vaccines may provide protection against diseases caused by capripoxviruses as well as diseases such as rinderpest and peste des petits ruminants.

Lumpy skin disease

This acute disease of cattle, which is endemic in sub-Saharan Africa and Madagascar, is caused by lumpy skin disease virus (Neethling virus), a capripoxvirus. Outbreaks have also occurred in Egypt and Israel.

Epidemiology

Although virus is present in the saliva of infected animals and transmission may occur through environmental contamination, lumpy skin disease is not particularly contagious (Carn and Kitching, 1995-. The principal method of transmission is by mechanical transfer through biting insects (Chihota *et al*., 2001). As a consequence, disease outbreaks usually occur during the rainy season when insect activity is high and epidemics are often associated with

heavy rainfall. New outbreaks may appear in areas far removed from an initial outbreak. It is unclear how the virus persists between epidemics but subclinically infected cattle are probably important. A wildlife reservoir, possibly the African Cape buffalo, may be involved.

Pathogenesis and pathology

Virus, which is transmitted mechanically by biting insects, rapidly disseminates through a leukocyte-associated viraemia. Many cell types including keratinocytes, myocytes, fibrocytes and endothelial cells become infected. Damage to endothelial cells, which results in vasculitis, thrombosis, infarction, oedema and inflammatory cell infiltration, accounts for the nodular skin lesions.

Clinical signs

The incubation period is up to 14 days. There is a persistent fever accompanied by lacrimation, nasal discharge and a drop in milk yield. Superficial lymph nodes become enlarged and there is oedema of the limbs and dependent tissues. Circumscribed skin nodules develop particularly on the head, neck, udder and perineum. Nodules also develop on the mucous membranes of the mouth and nares. Some skin lesions may develop into ‘sit-fasts’. These structures are composed of a central plug of necrotic tissue which sloughs leaving a deep ulcer. Secondary bacterial infection or myiasis can exacerbate the condition. Recovery may take several months. Affected animals are often debilitated and pregnant cows may abort. The severity of the disease relates to the strain of virus and the breed of cattle. Domestic breeds (*Bos taurus*) are more susceptible than zebu (*Bos indicus*) cattle. Some animals have few skin lesions and no systemic reaction while others display the full spectrum of clinical signs. Although the mortality rate is usually less than 5%, the economic impact of the disease can be considerable.

Diagnosis

- Generalized skin nodules in cattle in an endemic area are highly suggestive of lumpy skin disease.
- Intracytoplasmic inclusions may be demonstrable histologically in recently developed lesions.
- Capripoxvirus particles in biopsy material or desiccated crusts can be identified using electron microscopy.

- The virus can be isolated in lamb testis cell monolayers.
- An antigen trapping ELISA is available for the detection of capripoxvirus antigen (Carn, 1995).
- Viral DNA can be amplified in biopsy samples using capripox virus-specific primers (Tuppurainen *et al*., 2005).
- Serological assay methods include virus neutralization, western blot analysis, the indirect fluorescent antibody test and indirect ELISA.

Control

In endemic regions, vaccination is the method of control. Two modified live vaccines have been used specifically for the control of lumpy skin disease, one based on a South African strain of lumpy skin disease virus and the other on a Kenyan strain of sheeppox virus. A recombinant vaccine providing protection against lumpy skin disease and rinderpest has been developed (Romero *et al*., 1993). Imported cattle should be vaccinated before introduction into high-risk areas. Surveillance and eradication policies are appropriate control measures in countries bordering on endemic regions (Yeruham *et al*., 1995).

Swinepox

This disease, which occurs worldwide, is mild and often goes unrecognized. Swinepox virus is the sole member of the genus Suipoxvirus. The virus is transmitted mechanically by the pig louse, *Haematopinus suis*. Following an incubation period of about 1 week, infected animals display a slight fever and rash. Papules and pustules with scab formation resolve within 3 to 4 weeks. These skin lesions are similar to those which occur in pigs infected with vaccinia virus. Virus particles can be demonstrated in material from the lesions by electron microscopy. A vaccine is not available although there is interest in the use of swinepox virus as a vaccine vector. Control within a herd can be achieved by improved hygiene together with louse eradication.

Fowlpox

This disease, which affects domestic poultry, including chickens and turkeys, is caused by infection with fowlpox virus. The infection is slow-spreading and characterized by proliferative skin lesions and diphtheritic lesions in the upper digestive and respiratory tracts. Fowl pox has a worldwide distribution.

Epidemiology

Fowlpox, pigeonpox and turkeypox viruses are closely related and are not strictly host-specific. Several avian species are susceptible to infection with fowlpox virus. Transmission occurs by contact and by mechanical transfer on the mouthparts of biting arthropods, particularly mosquitoes. Virus enters the body through abrasions on unfeathered skin, on oral mucosa or on respiratory mucosa. Aerosols generated from scab material may result in transmission by inhalation. There is evidence that the virus may persist in some birds with reactivation occurring as a result of stress or immunosuppression.

Pathogenesis

Virus multiplication occurs at the site of introduction and may be confined to that site when the strain of infecting virus is of low virulence. Infections caused by virulent strains result in viraemia with replication in internal organs. The route of introduction influences the distribution and severity of lesions. Factors such as malnutrition, debilitation and stress may contribute to the severity of the disease.

Clinical signs

The incubation period is up to 14 days. Two forms of fowlpox have been described, a cutaneous form (dry pox) and a diphtheritic form (wet pox). In the cutaneous form, nodular lesions develop on the comb, wattles and other unfeathered areas of skin. Progression to vesicle formation is followed by ulceration and scab formation. Healing occurs within 2 weeks. In severely affected birds, lesions may involve both feathered and unfeathered areas of skin and involvement of the eyelids may lead to complete closure. In the diphtheritic form of the disease, yellowish necrotic lesions (cankers) develop on the mucous membranes of the mouth, oesophagus and trachea. Oral lesions may interfere with eating. Tracheal involvement may lead to laboured breathing and rales.

The mortality rate, which is higher in birds with the diphtheritic form than the cutaneous form, may approach 50% in severe outbreaks, particularly when accompanied by secondary bacterial or fungal infection. Economic losses are largely due to a transient drop in egg production in laying birds and reduced growth in young birds.

Diagnosis

- Large intracytoplasmic inclusions (Bollinger bodies) containing small elementary bodies (Borrel bodies) may be demonstrable in epithelial cells. Immunofluorescence and immunoperoxidase techniques can be used to identify viral antigen in intracyto-plasmic inclusions.
- Typical poxvirus particles can be demonstrated by electron microscopy in material from lesions.
- Virus may be isolated on the chorioallantoic membrane of 9 to 12-day-old embryonated eggs.
- Nucleic acid probes can be used for diagnosis. Alternatively, viral DNA can be amplified by PCR using specific primers (Lee and Lee, 1997).
- Suitable serological tests include ELISA, virus neutralization, agar gel precipitation and passive haemagglutination.

Treatment and control

There is no specific treatment. Control of secondary bacterial infection is desirable. In endemic areas, improved management and hygiene along with regular vaccination have reduced the effect of the disease on commercial poultry production. Modified live fowlpox or pigeonpox virus vaccines, produced in tissue culture or chick embryo, are available commercially. Chickens are usually vaccinated at about 1 month of age. Recombinant vaccines employing fowlpox and canarypox viruses are being developed for use not only in birds but also in mammalian species. Although fowlpox virus can enter mammalian cells and expresses proteins, it is unable to replicate.

Myxomatosis

This severe generalized disease of European rabbits is caused by myxoma virus, the type species of the genus *Leporipoxvirus.*

Epidemiology

The natural hosts of myxoma virus are New World species of rabbits, *Sylvilagus brasiliensis* in South America and *S. bachmani* in California. Infection has long been endemic in South America and western North America. In natural hosts, myxoma virus infection causes a benign cutaneous fibroma. In contrast, infection in the European rabbit (*Oryctolagus cunicu- lus*) is lethal. During the

1950s South American isolates of myxoma virus were introduced into populations of *O. cuniculus* in Europe, Chile and Australia as a method for controlling rabbit numbers. More than 99% of infected rabbits died and the disease is now endemic in these regions. Both attenuated virus strains and resistant rabbit populations have emerged. Virus is transmitted mechanically on the mouthparts of mosquitoes and fleas. Epidemics, which may occur annually, relate to the presence of arthropod vectors and large numbers of young susceptible rabbits.

Pathogenesis and pathology

Virus replicates at the site of inoculation and in regional lymph nodes and the subsequent viraemia is mainly cell-associated, with most virus particles in lymphocytes. Gelatinous, myxoma-like swellings are evident in the skin about 1 week after infection.

Clinical signs

Subcutaneous gelatinous swellings are particularly prominent in the head and anogenital areas. Blepharoconjunctivitis develops, accompanied by an opalescent ocular discharge. Affected animals are febrile and listless, and some may die within 48 hours. Activation of *Pasteurella multocida* infection can result in nasal discharge. The mortality rate, which ranges from 25% to 90%, is influenced by the genetic resistance of the rabbit population and the virulence of the virus strain. Low ambient temperatures increase the severity of the disease.

Diagnosis

The clinical signs are characteristic. Isolation of virus or detection of poxvirus particles in exudate or in material from lesions by electron microscopy is confirmatory.

Control

Commercial and laboratory rabbit stocks can be protected by vaccination with modified live myxoma virus or with rabbit fibroma (Shope fibroma) virus, a related *Leporipoxvirus*. Control of flea infestation in colonies and insect proofing of accommodation to reduce transmission of infection by other arthropods may be necessary in endemic areas.

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Chapter 65

Asfarviridae

African swine fever virus (ASFV), formerly assigned to the family *Iridoviridae*, has been reassigned to a newly created family, *Asfarviridae*, containing the single genus *Asfivirus*. African swine fever virus is the type species of this genus and is the only known DNA virus transmitted by arthropods. The virus has similarities in genome structure and method of replication to poxviruses but is different in other respects. Virions are 175 to 215 nm in diameter and consist of a membrane-bound nucleoprotein core inside an icosahedral capsid surrounded by an outer lipid-containing envelope (Fig. 65.1). This complex virus contains more than 50 proteins, including a large number of structural proteins and several virus-encoded enzymes required for transcription and post-translational modification of mRNA. The genome consists of a single molecule of linear double-stranded DNA. Following replication in the cytoplasm of host cells, virus is released either by budding through the plasma membrane or following cellular disintegration. African swine fever virus is stable in the environment over a wide range of temperature (4°C to 20°C) and pH values. The virus may persist for months in meat. Infectivity can be destroyed by heating and by treating with lipid solvents and some disinfectants such as orthophenylphenol, formalin and halogen compounds.

African swine fever

African swine fever (ASF) is an economically important viral disease of pigs, characterized by fever, haemorrhages in many tissues and a high mortality rate. It is endemic in sub-Saharan Africa, Madagascar and Sardinia. Outbreaks have occurred in Belgium, Italy, The Netherlands, Russia, Malta, Brazil, Cuba, Haiti and the Dominican Republic. The Iberian Peninsula was declared free of the disease in 1995, almost 30 years after initial introduction into the region, but ASFV reappeared in 1999 in Portugal. It has been eradicated from South America and the Caribbean.

Key points

- Enveloped DNA virus
- Icosahedral symmetry
- Replicates in cytoplasm of host cells and in soft ticks of the *Ornithodorus* species
- Causes African swine fever

Epidemiology

Domestic and wild pigs are the only species susceptible to infection. In Africa, ASFV is maintained in a sylvatic cycle involving soft ticks of the genus *Ornithodorus* and inapparent infection of warthogs and bushpigs (Fig. 65.2). Adult warthogs with persistent inapparent infection rarely develop viraemia. In contrast, young warthogs develop viraemia and are a major source of virus for soft ticks. Replication of virus occurs in the ticks and both transovarial and transstadial transmission have been described. Soft ticks feed for short periods on hosts before dropping off and sheltering in crevices in walls or cracks in the ground. As ticks are able to maintain and transmit the virus to pigs for years, the presence of ticks in a particular region makes the eradication of ASF difficult. The principal tick species involved in transmission are O. porcinus porcinus (*O. moubata*) in Africa (Kleiboeker *et al*., 1998) and *O. erraticus* in Spain and Portugal. Experimentally, several other *Ornithodorus* species support virus replication. Virulent strains of ASFV, producing high mortality in infected animals, are widely distributed in Africa. Many isolates from other parts of the world are less virulent and mortality rates are usually below 50%.

Following infection of domestic pigs with virulent virus, body fluids and tissues contain large quantities of virus until death or recovery occurs. Ingestion of uncooked meat from infected warthogs or domestic pigs is a major method of transmission. Spread can also occur by direct contact usually through oral or nasal secretions. Occasionally, animals become infected by contact with blood shed as a result of fighting. Indirect transmission can occur through contaminated transport vehicles, fomites and footwear. Feeding uncooked swill is an important mechanism of spread of ASF internationally, with outbreaks often starting in herds close to airports and ports.

Pigs which have recovered from clinical disease may remain infected for long periods. Carrier pigs are considered to be important sources of virus dissemination. Although recovered pigs are clinically unaffected by challenge

with genotypically related ASFV, the challenge virus may replicate and spread to other pigs.

Figure 65.1 African swine fever virus particles as they appear in an electron micrograph and a diagrammatic representation (inset).

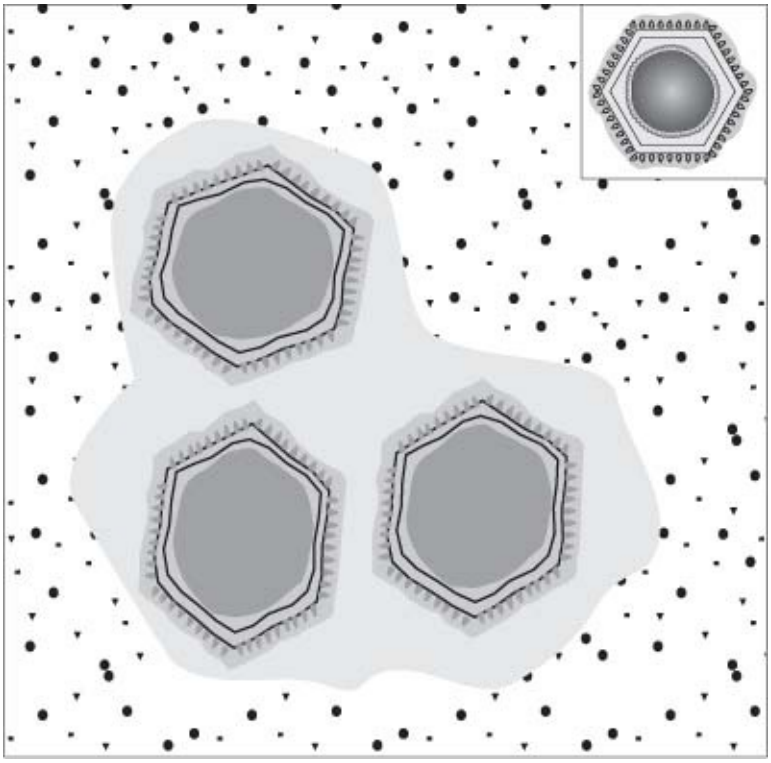
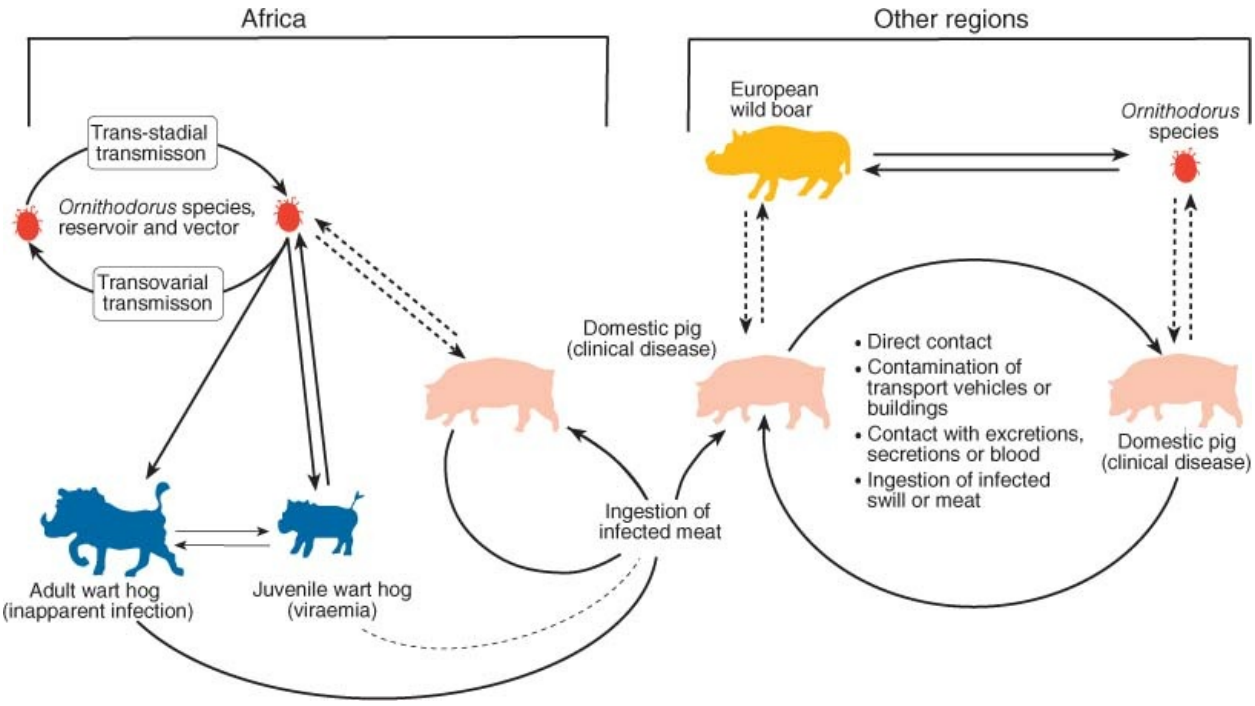


Figure 65.2 The maintenance and transmission of African swine fever virus in wild and domestic pig populations and in tick vectors.



Pathogenesis and pathology

Infection in domestic pigs is usually acquired via the oronasal route. The virus replicates initially in pharyngeal mucosa, tonsils and the regional lymph nodes. Infection then spreads by the bloodstream to other lymph nodes, bone marrow, spleen, lung, liver and kidneys. Secondary replication in these sites results in prolonged viraemia. Although the virus replicates primarily in cells of the lymphoreticular system, it can also infect megakaryocytes, endothelial cells, renal epithelial cells and hepatocytes. Lesions include splenic enlargement, swollen haemorrhagic gastrohepatic and renal lymph nodes, subcapsular petechiation in the kidneys, petechial and ecchymotic haemorrhages on serosal surfaces, oedema of the lungs and hydrothorax. The widespread haemorrhages result from disseminated intravascular coagulation, endothelial damage and destruction of megakaryocytes (Rodriguez *et al*., 1996). Leukopenia is marked. Because the virus does not appear to replicate in T and B lymphocytes, it has been suggested that the lymphopenia and necrosis of lymphoid organs follows apoptosis of lymphocytes induced by proinflammatory cytokines (Carrasco *et al*., 1996; Salguero *et al*, 2005). Lesions in chronic disease include pneumonia, fibrinous pleuritis and pericarditis, pleural adhesions and hyperplasia of lymphoreticular tissues.

Clinical signs

The clinical signs of ASF, which range from inapparent to peracute, relate to the challenge dose and virulence of the virus and to the route of infection. The incubation period, which may extend from 4 to 19 days, is typically 5 to 7 days in acute cases. Animals with peracute disease die suddenly without premonitory clinical signs. Fever, inappetence, depression and recumbency are features of acute disease. Cutaneous hyperaemia and, in some cases, haemorrhages may be evident. Other signs include dyspnoea, conjunctivitis, diarrhoea, bleeding from the nose and rectum, and abortion. The mortality rate is high. Subacute disease has a course of 3 to 4 weeks. Clinical signs include pneumonia, swollen joints, emaciation, depression and inappetence. Mortality rates, which are variable, depend on the age and general health of infected pigs. Animals may recover clinically or may develop a chronic form of the disease, which usually occurs in regions where ASFV is endemic. The immune mechanisms responsible for recovery and protection from ASFV are poorly understood. Neutralizing antibodies are not demonstrable in the sera of recovered animals. Cell-mediated immunity is considered to be an important component of the immune response.

Diagnosis

Laboratory confirmation of ASF is mandatory because the clinical signs and lesions, which occur in some other important pig diseases such as classical swine fever, erysipelas and septicaemic salmonellosis, are similar.

- Suitable samples for laboratory examination include blood, serum, tonsil, spleen and lymph nodes.
- The most convenient and frequently used test for detection of ASFV is the PCR. Direct immunofluorescence, which is fast and economical, can be carried out on impression smears or cryostat sections. However, the sensitivity of the test is only 40% in pigs with subacute or chronic ASF because of the blocking action of bound antibody in antigen-antibody complexes. Most field strains of ASFV induce haemadsorption. Pig erythrocytes adhere to the surface of infected monocytes and macrophages, forming a characteristic rosette. The haemadsorption test can be carried out by employing leukocytes from blood samples of pigs under investigation or by inoculating primary blood leukocyte cultures with blood or homogenized tissue from suspect pigs.
- The polymerase chain reaction is used to detect DNA from ASFV in tissues

(Aguero *et al*., 2003; King *et al*., 2003).

- Antibodies persist for long periods in recovered animals. Serological testing may be the only means of detecting animals infected with strains of low virulence. Techniques for detecting antibodies to ASF include ELISA and immunoblotting.

Control

A successful vaccine is not yet available. Inactivated vaccines do not induce protection. Although live attenuated vaccines induce protection against challenge with homologous virus strains in some pigs, a proportion of these animals become carriers and may develop chronic lesions.

Countries maintain disease-free status by prohibiting importation of pigs and pig products. Waste food scraps from aircraft and ships must be boiled before inclusion in pig feed. In the face of an outbreak of ASF in countries free of infection, an eradication policy is implemented. The occurrence of low virulence strains renders eradication difficult.

Restriction of pig movement, serological monitoring of carrier pigs and prevention of contact between domestic pigs and warthogs or ticks are important control measures in countries where the disease is endemic. Eradication of tick species which act as vectors of ASFV is an essential part of a control programme.

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Chapter 66

Parvoviridae

Viruses belonging to the family *Parvoviridae*, referred to as parvoviruses, are small (Latin *parvus*, small) and range in size from 18 to 26 nm in diameter (Fig. 66.1). These non-enveloped, icosahedral viruses possess a linear genome of single-stranded DNA. There are two sub-families: *Parvovirinae*, which includes viruses of vertebrates, and *Densovirinae*, viruses of arthropods. Of the five genera within the subfamily *Parvovirinae* (Fig. 66.2), the genus *Parvovirus* contains viruses which are of particular veterinary importance.

Parvoviruses replicate only in the nuclei of dividing host cells, a feature which determines the tissues targeted. After entering a cell, the virion is uncoated and its single-stranded DNA genome is converted to double-stranded DNA by cellular DNA polymerases in the nucleus. Following viral replication, cell lysis occurs as virions are released.

Parvoviruses are stable in the environment. They are resistant to many factors including lipid solvents, a wide range of pH values (pH 3 to 9) and heating at 56°C for more than 60 minutes. They are inactivated by formalin, beta-propiolactone, sodium hypochlorite and oxidizing agents. With the exception of Aleutian mink disease virus and goose parvovirus, parvoviruses of vertebrates agglutinate erythrocytes. Hae-magglutination inhibition (HAI) by specific antisera is widely used for the identification of these viruses. Mink enteritis virus, canine parvovirus and racoon parvovirus are considered to be host-range mutants of feline panleukopenia virus.

Key points

- Small, non-enveloped, single-stranded DNA viruses
- Icosahedral symmetry
- Replicate in the nucleus, form intranuclear inclusion bodies
- Require rapidly dividing cells for replication
- Stable in the environment
- Resistant to heat, solvents, disinfectants and pH changes
- *Genus Parvovirus*:

– Many have haemagglutinating activity

– Shed in large numbers in faeces

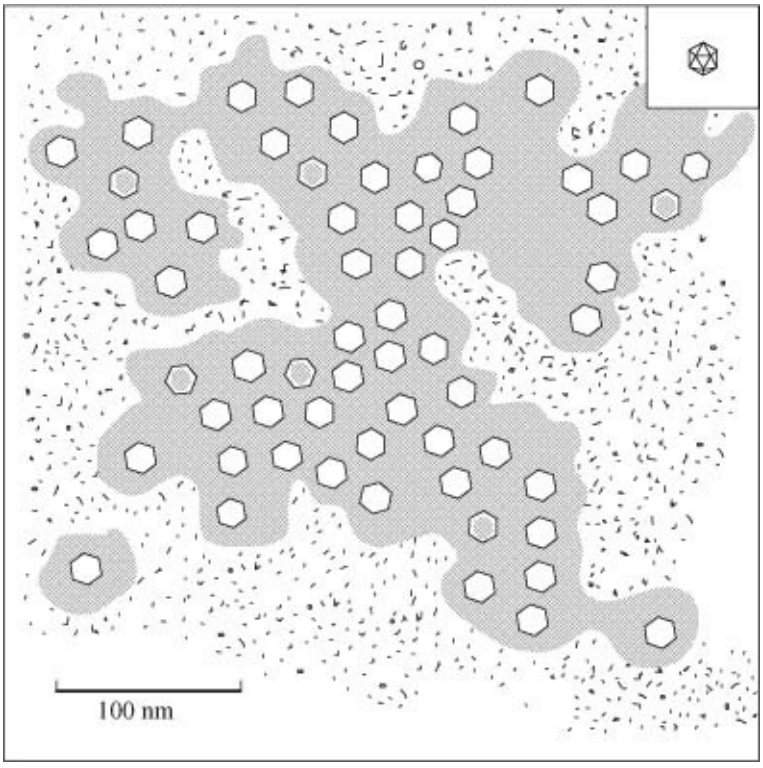
– Enteric and systemic diseases in dogs and cats

– Reproductive failure, SMEDI syndrome in pigs

Clinical infections

Parvoviruses can infect many domestic and wild animals (Table 66.1). Although most members of the group produce acute systemic diseases, some such as canine minute virus and bovine parvovirus are of uncertain pathogenic significance. Two distinct parvoviral diseases of mink, Aleutian mink disease and mink enteritis, are recognized. Mink enteritis, first described in the 1940s, affects kits and clinically resembles feline panleukopenia. Aleutian mink disease is a persistent infection, primarily affecting animals which are homozygous for pale coat colour. The disease is characterized by B lymphocyte stimulation leading to plasmacytosis, hypergammaglobulinaemia and immune complex-related lesions in the kidneys and other organs. Aleutian mink disease can also occur in domestic ferrets (Welchman *et al*., 1993). Goose parvovirus infection, which causes hepatitis and myositis in goslings, is highly contagious and often fatal. A related virus, duck parvovirus, causes a similar disease in Muscovy ducks. Both viruses are related to adeno-associated viruses but do not require a helper virus for productive replication. The most important parvoviral diseases of domestic mammals are feline panleukopenia, canine parvovirus infection and porcine parvovirus infection.

Figure 66.1 Parvovirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Feline panleukopenia

Feline panleukopenia, also known as feline infectious enteritis or feline distemper, is a highly contagious generalized disease of domestic and wild cats caused by feline panleukopenia virus. Only one serotype of this virus has been identified. The disease, which is worldwide in distribution, is one of the most common feline viral infections.

Epidemiology

Most species of *Felidae* are highly susceptible to infection, which is generally endemic in unvaccinated cat populations. Some species of *Mustelidae, Procyonidae* and *Viverridae* can also become infected but they seldom develop clinical disease. Although cats of all ages are susceptible to infection, disease occurs predominantly in young, recently weaned kittens as maternally derived antibody levels wane. Many infections are subclinical, particularly in older cats and in kittens partially protected by maternally-derived immunity. The disease may have a cyclical or seasonal pattern which is related to the births of kittens. Transplacental transmission of infection occurs in fully susceptible queens.

Figure 66.2 The family *Parvoviridae*, subfamilies *Parvovirinae* and

Densovirinae and the genera of *Parvovirinae* which can infect mammalian species and some avian species.

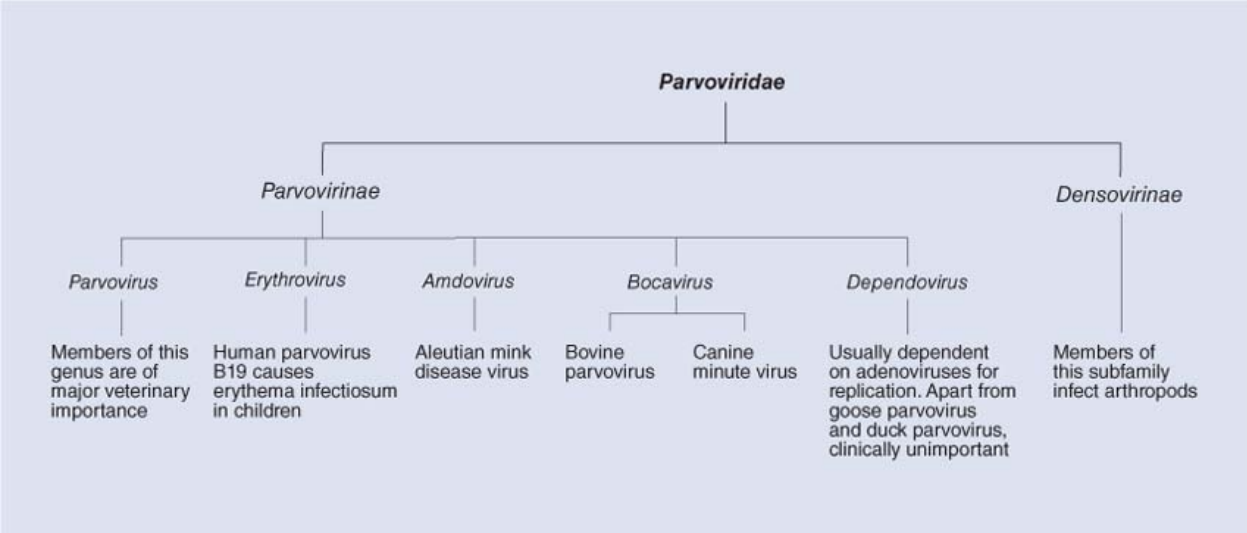


Table 66.1 Parvoviruses of veterinary importance.

Virus	Genus	Hosts	Consequences of infection
Feline panleukopenia virus	*Parvovirus*	Domestic and wild cats	Highly contagious systemic and enteric disease most common in weaned kittens, manifested by depression, vomiting, diarrhoea. Intrauterine infection: abortion or cerebellar ataxia in neonatal kittens
Canine parvovirus (canine parvovirus 2)	*Parvovirus*	Dogs	Highly contagious enteric disease with depression, vomiting, dysentery and immunosuppression. Intrauterine or perinatal infection: myocarditis in pups (rare)
Porcine parvovirus	*Parvovirus*	Pigs	Major cause of stillbirths, mummified foetuses, embryonic deaths and infertility (SMEDI syndrome)
Mink enteritis virus	*Parvovirus*	Mink	Generalized disease of mink kits, analogous to feline panleukopenia
Aleutian mink disease virus	*Amdovirus*	Mink, ferrets	Chronic progressive disease of mink homozygous for pale coat colour. Persistent viraemia, plasmacytosis, hypergammaglobulinaemia and immune complex-related lesions
Goose parvovirus (goose plague virus)	*Dependovirus*	Geese	Highly contagious, fatal disease of 8 to 30-day-old goslings (Derzsy's disease): hepatitis, myositis, including myocarditis
Duck parvovirus	*Dependovirus*	Ducks	High mortality, clinical signs and lesions similar to goose parvovirus
Canine minute virus (canine parvovirus 1)	*Bocavirus*	Dogs	Role of virus in disease is uncertain; serological surveys suggest the virus is widespread
Bovine parvovirus	*Bocavirus*	Cattle	Associated with sporadic outbreaks of diarrhoea in calves

High rates of virus excretion occur during the acute stage of the disease, mainly in faeces but also in saliva, urine, vomitus and blood. Faecal shedding usually continues for some weeks following clinical recovery. Although long-term, low-grade shedding of virus by some subclinical carriers may occur, the stability of the virus in the environment is of greater significance in its persistence and spread. In cool, moist, dark environments, infectivity may last for more than a year. Fleas and humans may act as mechanical vectors.

Pathogenesis and pathology

Following ingestion or inhalation, replication occurs in the mitotically active lymphoid tissues of the oropharynx and associated lymph nodes. Viraemia develops within 24 hours, producing infection of mitotically active cells in other tissues, particularly the cells of the intestinal crypts and the lymphopoietic cells of the bone marrow, thymus, lymph nodes and spleen. Destruction of these target tissues results in panleukopenia and villous atrophy. The crypts of Lieberkühn are dilated and contain necrotic epithelial cells. Intranuclear inclusions are sometimes evident in crypt cells. Intestinal villi become blunted and may fuse. The effects of transplacental infection on foetuses generally relate to the stage of gestation at which viral infection occurs. Infection early in gestation usually leads to abortion or resorption; viral invasion later in gestation may result in cerebellar hypoplasia, retinal dysplasia or early neonatal death.

Clinical signs

The incubation period of feline panleukopenia ranges from 2 to 10 days but is typically 4 to 5 days. Subclinical infection is common and results in a mild fever and leukopenia, usually followed by life-long immunity. Subacute disease presents as depression, fever and diarrhoea lasting 1 to 3 days, followed by rapid recovery. The disease is most severe in young, unvaccinated kittens between 6 and 24 weeks of age and is characterized by sudden onset of pronounced depression, anorexia and fever. Vomiting, sometimes accompanied by diarrhoea or dysentery, follows within 2 days and can result in severe dehydration and electrolyte imbalance. Abdominal pain may be evident. The mortality rate ranges from 25 to 90%, with most deaths occurring within 3 to 5 days of the onset of illness. Animals which survive require several weeks for full recovery. Immunity is strong and long-lasting. Subnormal temperatures are followed by death within 24 hours. Although intrauterine infection of the developing foetuses often occurs, infected pregnant queens usually show no signs of illness. Foetal infections early in gestation may result in resorption or abortion. Because of developing foetal immune competence, infections after mid-gestation are usually less severe. However, stillbirths, early neonatal death and teratological changes such as cerebellar hypoplasia and retinal dysplasia may occur in the litters of queens infected during late pregnancy. Kittens with cerebellar hypoplasia exhibit cerebellar ataxia manifested as hypermetria, incoordination and, frequently, intention tremors, conditions which persist for life.

Diagnosis

- Feline panleukopenia should be considered when unvaccinated cats present with diarrhoea.
- A white cell count of less than 7×10^9/litre is often encountered in acutely affected animals. Neutropenia is more common than lymphopenia. Cell counts return to normal after a few days in cats which survive.
- Specimens for virus isolation in primary feline cell lines include oropharyngeal swabs, faeces, spleen, mesenteric lymph nodes and ileum.
- Large numbers of virus particles may be demonstrated by electron microscopy in faecal samples from cats with acute disease.
- Typical histopathological changes may be present in sections of the ileum and jejunum. Intranuclear inclusion bodies may be detected in crypt cells.
- Viral antigen can be detected in faeces using latex agglutination, ELISA or haemagglutination employing pig or rhesus monkey red cells. Commercially available canine parvovirus kit sets can be used for the detection of feline panleukopenia virus antigen (Addie *et al*., 1998).
- The polymerase chain reaction assay has been shown to be useful for the detection of viral DNA in faeces (Schunck *et al*., 1995) and in tissues (Meurs *et al*., 2000).
- A rising antibody titre may be detected in serum samples by a number of tests including the haemagglutination-inhibition (HAI), ELISA, indirect immunofluorescence or virus neutralization (VN) tests.

Treatment

Specific antiviral treatment is not available.

- Intensive supportive therapy is usually necessary:
  – Appropriate fluid therapy for dehydration should be given.
  – Whole blood or plasma from immune donors may be beneficial in cats with anaemia or hypoproteinaemia.
  – Parenterally administered broad–spectrum antibiotics can be used to combat secondary bacterial infections.
- Affected animals should be housed in a clean, warm environment and maintained on an optimal diet supplemented with B complex vitamins.

Control

Immunity following natural infection is strong and long-lasting. Vaccination is the principal control measure.

- Modified live and inactivated vaccines are commercially available:

  – Inactivated vaccines are less effective than modified live vaccines and require booster inoculations. They are safe for pregnant queens and might be considered for vaccination of Siamese and Burmese kittens which can have adverse reactions to modified live vaccines (Carwardine, 1990).

  – Modified live vaccines can be used to immunize kittens at 8 to 10 weeks of age, with a booster dose at 12 to 14 weeks of age. A booster vaccination 12 months after the primary vaccination course is recommended followed by subsequent boosters at 3-year intervals. These vaccines should not be used in pregnant queens because replicating virus may cause cerebellar hypoplasia in developing foetuses.

  – Cats should have completed a vaccination schedule or should be given a booster injection at least 2 weeks before introduction to premises where feline panleukopenia has recently occurred.

- Clinical infections cause heavy environmental contamination. Premises should be thoroughly disinfected with 1% sodium hypochlorite or 2% formalin (Scott, 1980). Cats on such premises should be vaccinated without delay.

Canine parvovirus infection

Infection with canine parvovirus (CPV) emerged in the late 1970s as a worldwide disease in dogs with high morbidity and mortality. Acute or subacute heart failure in pups infected *in utero* or during the perinatal period was a common manifestation of the disease. With the gradual development of immunity in the adult dog population as a consequence of both natural exposure and vaccination, the clinical pattern of the disease changed. The most common clinical presentation currently encountered is acute enteric disease in young dogs between weaning and 6 months of age. Since the appearance of CPV in 1978, further mutations affecting the genome and antigenicity of the virus have occurred and three subtypes of the virus are currently recognized, CPV-2a, CPV-2b and CPV-2c (Truyen, 2006). Infection or vaccination with one subtype generally confers immunity against the other subtypes, but there have been calls to incorporate recent field strains in the vaccines (Decaro *et al*., 2009). Canine parvovirus is believed to have evolved from FPV as a result of five or six amino

acid changes in the capsid protein gene, which permitted the virus to bind to the transferrin receptor on canine cells. Canine parvovirus infections in cats appear to be rare. Canine parvovirus is now classified as a strain of FPV.

Epidemiology

Many canine species are susceptible to infection and transmission is predominantly by the faecal–oral route. Infected dogs shed large numbers of viruses in their faeces. The number of viruses in faeces may be 10^9/g from the fifth or sixth day after infection. Persistent shedding is uncommon and the continued presence of the disease in dog populations depends mainly on the stability of the virus in the environment. The low dose of virus required to establish infection and the ease with which mechanical transfer can take place are important additional factors contributing to the spread of infection.

Pathogenesis and pathology

The virus replicates initially in pharyngeal lymphoid tissues and Peyer's patches. Once viraemia develops, the main target tissues are those with rapidly multiplying cell populations. During the first 2 weeks of life there is active division of cardiac myocytes allowing viral replication with resultant necrosis and myocarditis. In older pups, the virus invades the actively dividing epithelial cells of the crypts in the small intestine. Loss of cells from the intestinal crypts leads to blunting of villi, with resultant reduced absorptive and digestive capacity, leading to diarrhoea. There may be extensive haemorrhage into the intestinal lumen in severely affected pups. Destruction of lym-phoid tissues of the intestinal mucosa and mesenteric lymph nodes contributes to immunosuppression which allows proliferation of Gram-negative bacteria with secondary invasion of damaged intestinal tissues. Endotoxaemia, leading to endotoxic shock, may follow in some instances.

Clinical signs

The age and the immune status of the animal largely determine the form and severity of the disease. After a short incubation period of 4 to 7 days, animals with enteric disease show sudden onset of vomiting and anorexia. Depression and fever may also be observed. Diarrhoea, often blood-stained, develops within 48 hours, and in severe cases there may be frank haemorrhage. The faeces have a foetid smell. Concurrent intestinal parasitism and viral or bacterial infections

may exacerbate the condition. Affected dogs deteriorate rapidly due to dehydration and weight loss. Prolonged illness is uncommon; severely affected animals die within 3 days. Animals which survive the disease develop a long-lasting immunity.

In the myocardial form of the disease, which is now rare, affected pups usually show signs of acute heart failure before 8 weeks of age. Some pups may develop congestive heart failure months after the initial infection as a result of extensive fibrosis following myocardial necrosis.

Diagnosis

- Samples for laboratory examination should include faeces, blood and other tissues, particularly affected portions of intestines and myocardium.
- The nature and distribution of the gross and microscopic enteric lesions may point to a parvoviral infection. Immunocytochemical staining can confirm the presence of viral antigen in tissue sections.
- The presence of basophilic intranuclear inclusions in cardiac myocytes is confirmatory.
- A leukopenia may be detected, particularly in severely affected animals.
- Definitive diagnosis in clinically affected animals early in the course of the disease relies on the demonstration of virus or viral antigen in faeces:

  – Numerous virus particles can be demonstrated by electron microscopy.

  – ELISA or HA may be used to demonstrate viral antigen. The virus will haemagglutinate pig or rhesus monkey red cells. Commercial assays are available.

  Virus can be isolated in a number of suitable canine and feline cell lines.
- Polymerase chain reaction protocols are available for the detection of viral DNA in faeces (Mochizuki *et al*., 1993; Uwatoko *et al*., 1995) and paraffin-embedded tisues (Truyen *et al*., 1994).
- Serological tests, including HAI, VN, ELISA and indirect immunofluorescence, may confirm the diagnosis.

Treatment

Specific antiviral treatment is not available.

- Intensive supportive therapy including antiemetics and fluid administration is required for the treatment of parvoviral enteritis.

- Broad-spectrum antibiotics, administered parenterally, reduce the risk of secondary bacterial infections.
- Dogs with subacute or chronic heart failure may improve with rest and diuretic therapy.

Control

Vaccination alone usually cannot control the cycle of endemic parvovirus infection in kennels and therefore it is important to minimize exposure of pups to the virus until they reach 20 weeks of age (Pollock and Coyne, 1993). Non-protective, low levels of maternal antibody can interfere with the efficacy of some modified live vaccines. In many pups, these critical levels last until 8 to 12 weeks of age, but in some instances interference with vaccination may continue until 18 weeks of age (O'Brien, 1994).

- Inactivated and modified live vaccines are commercially available:

  – Inactivated vaccines usually provide protection for up to 1 year and are safe to use in pregnant bitches.

  – Although modified live vaccines generally give good, long-lasting protection, annual boosters may be required. These vaccines should not be used for pregnant bitches. Modified live vaccines vary in their degree of viral attenuation. The less attenuated strains of vaccinal viruses can replicate in pups despite the presence of residual maternal antibodies (Churchill, 1987; Burtonboy *et al.*, 1991) and vaccination of pups at 12 weeks of age is often claimed by vaccine manufacturers to be protective.
- Thorough disinfection of premises must be carried out following a disease outbreak:

  – Effective disinfectants include 1% sodium hypochlorite and 2% formalin.

  – Fumigation with formaldehyde gas, where feasible, is the most efficient disinfection procedure.

Porcine parvovirus infection

Porcine parvovirus is an important cause of reproductive failure in pigs. The virus, which occurs as a single serotype, is found worldwide and infection is endemic in many conventional pig herds.

Epidemiology

On farms where the disease is endemic, many sows are immune. They remain seropositive for up to 4 years and transmit passive protection through colostrum to their piglets. Maternally-derived immunity usually persists for about 4 months, but it can persist in some pigs until they are 6 to 9 months of age. During this period, the maternally-derived antibodies may interfere with the development of active immunity and consequently some gilts can be seronegative and susceptible to infection at mating. Infected pigs shed virus in their faeces and other secretions, including semen, for only a few weeks. However, pens may remain contaminated for several months because of the exceptional stability of the virus.

Pathogenesis

After infection by the oronasal route and occasionally through semen, local replication of virus is followed by viraemia. The virus has a predilection for the mitotically active cells of foetal tissues. Transplacental infection in pregnant sows occurs 10 to 14 days after exposure to the virus. The major damage to foetuses arises before onset of immunological competence, at about 60 to 70 days of gestation (Huysman *et al.*, 1992). Infection of embryos in the first weeks of life results in death and resorption. When infection occurs later in gestation, but before day 70, foetuses die and become mummified. Infection after 70 days of gestation usually results in the birth of healthy seropositive piglets.

Clinical signs

Porcine parvovirus infection is a major cause of SMEDI, an acronym used to describe porcine reproductive failure in which stillbirths, mummified foetuses, early embryonic death and infertility (SMEDI) occur. Abortion and neonatal deaths have been reported occasionally. Generally, small litters with mummified piglets of different sizes are produced following transplacental infection and subsequent sequential exposure of foetuses by intrauterine spread. If the number of viable embryos is reduced below four, the entire litter is typically lost. Infection with porcine parvovirus does not appear to damage the male reproductive tract.

Diagnosis

- When reproductive failure is detected in young or recently introduced sows, particularly if associated with mummified foetuses, infection with porcine parvovirus must be considered.
- Several foetuses should be submitted for laboratory examination.
- Demonstration of viral antigen in cryostat sections of foetal tissues by immunofluorescence, particularly in lung tissue, is reliable and sensitive.
- Agglutination of guinea-pig erythrocytes by homogenates of foetal tissue indicates the presence of viral haemagglutinin.
- Swine kidney cell lines may be used for virus isolation. However, viral infectivity is gradually lost following death of the foetus, and isolation from mummified tissues may be unsuccessful.
- PCR assays have been used for the detection of viral DNA in foetal tissues (Gradil *et al*., 1994).
- Serological techniques include HAI and VN tests. Antibodies may be detected in sera or body fluids of older foetuses or aborted piglets. However, serological testing of the sow's serum is usually of little diagnostic value in infected herds.

Control

Control in herds in which the disease is endemic is based on exposure of gilts and susceptible sows to porcine parvovirus prior to mating, thereby inducing immunity. Vaccination can be used to enhance immunity in herds with endemic disease. It can also be used for male or female breeding stock introduced into these herds.

- Natural exposure can be achieved by increasing the contact between susceptible gilts and older seropositive sows. Methods of stimulating immunity include exposing animals to contaminated faeces or to placental or foetal tissue from infected sows.
- Modified live and inactivated vaccines against the single serotype of porcine parvovirus have been developed experimentally, but only inactivated vaccines are available commercially. Gilts and susceptible seronegative sows and boars should be vaccinated 2 to 4 weeks before mating. Vaccination prevents intrauterine infection for a limited period. Most vaccination strategies rely on subsequent natural exposure to the virus to reinforce immunity (Huysman *et al*., 1992).

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Chapter 67

Circoviridae

Viruses in the family *Circoviridae* cause disease in vertebrate animals and plants. Circoviruses (17 to 22 nm in diameter) are non-enveloped with icosahedral symmetry (Fig. 67.1). They are stable in the environment at pH 3 to pH 9 and are resistant to heating at 60°C for 30 minutes.

The genome consists of a molecule of circular single- stranded DNA. Replication occurs in the nuclei of dividing cells. Genetic sequencing studies suggested that circoviruses fell into three groups (Niagro *et al.*, 1998) and this is reflected in recent classification changes. Chicken anaemia virus (22nm in diameter), the type species, has been assigned to the newly- created genus, *Gyrovirus.* The animal circoviruses (17 nm in diameter), porcine circovirus and beak and feather disease virus, have been assigned to the genus *Circovirus*. The third grouping contains plant viruses which have now been assigned to the family *Nanoviridae.*

Clinical infections

Circoviruses, which are host-specific and have a worldwide distribution, infect cells of the haemolym- phatic system. Infections with chicken anaemia virus and with porcine circovirus are of veterinary interest. Beak and feather disease virus is associated with a debilitating, immunosuppressive disease of young psittacine birds, particularly cockatoos.

Key points

- Small, non-enveloped, single-stranded DNA viruses with icosahedral symmetry
- Replicate in the nucleus of dividing cells
- Stable in the environment
- Circoviruses cause disease in chickens, psittacine birds and pigs

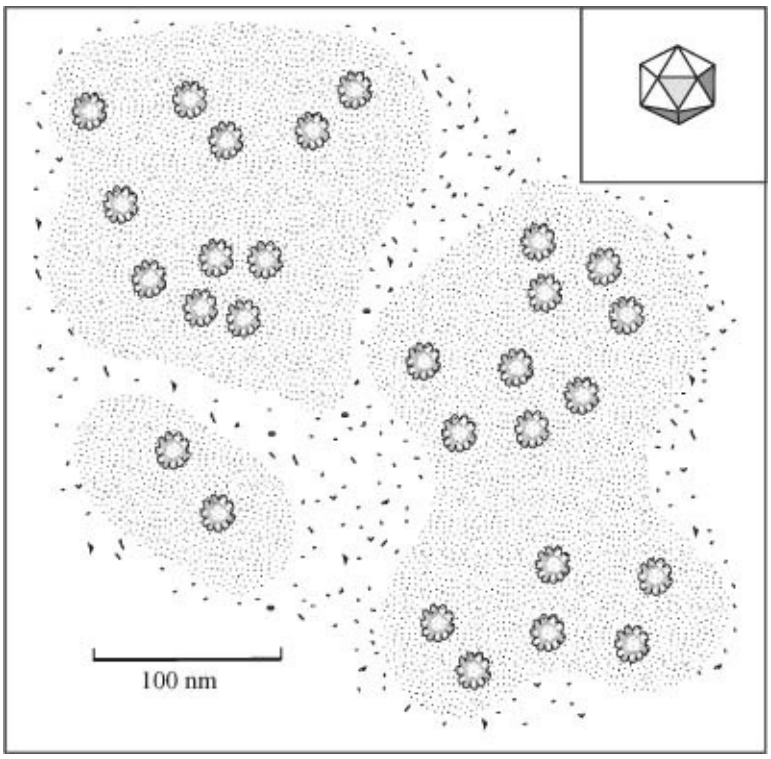
Chicken anaemia virus infection

Young birds infected with chicken anaemia virus (CAV) develop aplastic anaemia and generalized lym- phoid atrophy. This virus, which infects chickens only, is present in poultry flocks worldwide. All of the field isolates of the virus, which appear to be equally pathogenic, belong to a single serotype.

Epidemiology

Both horizontal and vertical transmission occur. Infection is by the faecal-oral route.Vertical transmission through the egg occurs during the 1 to 3- week viraemic period following infection of laying hens. Once infection is established in a breeder flock, most birds develop antibodies before laying begins. Maternally-derived antibodies do not prevent chicks from becoming infected and shedding the virus. However, they prevent the development of clinical disease. An age resistance to disease but not to infection develops in chicks at about 2 weeks of age. In breeding flocks containing many serologically positive adult birds, subclinical infection is common in chickens. Age resistance and the protective effect of maternally-derived antibodies do not prevent clinical disease if immunosuppressive viruses such as infectious bursal disease virus or gallid herpesvirus 2 are present in the flock.

Figure 67.1 Circovirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Pathogenesis and pathology

Viraemia develops following infection of susceptible day-old chicks and virus can be recovered from most organs and from faeces for about 3 or 4 weeks. The principal target cells are precursor T cells in the thymus and haemocytoblasts in the bone marrow. Destruction of these cells results in immunosuppression and anaemia. Gross post-mortem findings include atrophy of thymic and bursal tissues, a pale bone marrow and haemorrhages in skeletal muscle and at subcutaneous sites.

Clinical signs

Chickens develop clinical signs at about 2 weeks of age. Affected birds are depressed, anorexic and pale. The mortality rate, which is usually about 10%, may be up to 50%. Birds which survive the acute phase of the disease recover slowly. Subclinical infection in broilers from breeder flocks can adversely affect weight gains.

Diagnosis

A presumptive diagnosis is based on the clinical signs and gross lesions at post mortem. Laboratory confirmation relies on detection of viral antigen by immu-

nocytochemical techniques. Viral DNA can be demonstrated in bone marrow and thymus by *in situ* hybridization, by dot-blot hybridization or by PCR. Virus isolation, although possible, is exacting and expensive. Serum antibodies can be detected using virus neutralization, indirect immunofluorescence and ELISA. A commercial ELISA is available and can be used to identify seronegative breeder flocks before laying begins.

Control

Because infection with CAV is common, it is difficult to maintain breeder flocks free of infection. Breeding birds should be exposed to infection before laying commences. Seronegative flocks can be deliberately exposed to virus by transfer of old litter from CAV- positive farms or by the addition of crude tissue homogenates from affected birds to drinking water but such exposure is unreliable and inherently unsafe.

Commercial live vaccines are available and are designed to prevent vertical transmission of the virus from breeder hens. Vaccination does not prevent economic losses in broilers due to subclinical infection. Control of other immunosuppressive viruses in CAV- positive flocks is essential due to the additive effects of these combined infections.

Pig circovirus infection

Porcine circovirus was first described in 1974 as a picornavirus-l ike contaminant of the continuous pig kidney cell line PK/15. Experimental challenge suggests that this virus, termed porcine circovirus 1, is non-pathogenic. An antigenically and genomically distinct circovirus, porcine circovirus 2 (PCV 2), is consistently isolated from piglets with post-weaning multisystemic wasting syndrome (PMWS). Sero- epidemiological studies indicate that infection with PCV 2 is widespread in pig populations worldwide. The virus has also been linked to porcine dermatitis and nephropathy syndrome (PDNS) and also to reproductive problems. Three genotypes are recognized, PCV-2a, PCV-2b and PCV-2c.

Post-weaning multisystemic wasting syndrome

Post-weaning multisystemic wasting syndrome (PMWS), a progressive wasting condition with lesions in several organ systems, was first described in Canada in

1991 in specific pathogen-free herds (Allan and Ellis, 2000).

Epidemiology

Although molecular methods indicate differences between isolates of PCV-2, it is unclear if these differences correlate with pathogenicity. The oro-nasal route is considered to be the principal transmission pathway with efficient horizontal transmission to in-contact animals. Viral shedding is thought to occur in nasal, salivary and ocular secretions as well as in urine and faeces.

Pathogenesis

As pigs infected with PCV-2 do not always develop PMWS, co-factors appear to be necessary for the development of the full clinical disease. It is thought that immune stimulation may be an important trigger, possibly by increasing the number of permissive cells entering the S phase of the cell cycle. A generalized depletion of lymphocyctes is a consistent feature of the disease and the destruction of thymic lymphocytes is believed to play a central role in its pathogenesis (Darwich *et al*., 2004). The virus is also capable of inducing hepatitis.

Clinical signs

Affected animals are usually 2 to 4 months of age and present with weight loss, dyspnoea, enlarged lymph nodes, diarrhoea and, sometimes, icterus. Morbidity rates in affected herds tend to range from 4% to 30% with a mortality rate up to 80% in affected pigs.

Diagnosis

Diagnosis of PMWS is based on clinical signs and pathological findings. The histopathological changes in lymphoid tissues, which are characteristic, include granulomatous inflammation, depletion of lymphocytes and intracytoplasmic inclusions in macrophages. Antibodies to PCV 2 may be detected using indirect immunofluorescence, immunoperoxidase monolayer assay or ELISA. Virus isolation in pig cell lines is also indicative of infection. A definitive diagnosis requires demonstration of PCV 2 antigen or viral nucleic acid in association with lesions, which can be achieved by the use of immunohistochemistry and *in situ* hybridization (Kim and Chae, 2004).

Control

Due to the widespread nature of the virus, control is largely directed at eliminating co-factors and predisposing and contributory factors that may be present on individual farms. Control is based on good husbandry, rapid removal of affected animals and the elimination of other infectious agents. Commercial inactivated and subunit vaccines are available. The vaccines have been used in piglets or alternatively in sows and gilts to provide passive immunity to piglets via colostrum.

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Chapter 68

Retroviridae

Retroviruses (Latin *retro*, backwards) are labile, enveloped RNA viruses, 80 to 100 nm in diameter. The family is divided into two subfamilies, *Orthoretrovirinae* and *Spumaretrovirinae*. Seven genera are currently assigned to the family: *Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, Len- tivirus* and *Spumavirus* (Fig. 68.1). The family name refers to the presence in the virion of a reverse tran- scriptase, which is encoded in the viral genome.

The envelope, which is acquired from the plasma membrane of the host cell, surrounds an icosahedral capsid containing two linear, positive-sense, single strands of RNA and core proteins including the enzymes reverse transcriptase and integrase (Fig. 68.2). The nucleocapsid is eccentric in the case of betare- troviruses, concentric for alpharetroviruses, gamma-retroviruses, deltaretroviruses and spumaviruses, and rod- shaped or truncated cone- shaped in the case of lentiviruses (Fig. 68.3 and 68.4). Historically, based on electron microscopy, retroviruses were categorized as A-type, B-type, C-type and D-type particles.

Reverse transcriptase acts as an RNA-dependent DNA polymerase, which transcribes from RNA to DNA. The RNA genome of replication-competent members of the *Retroviridae* contains a minimum of three major genes or open reading frames *gag*, *pol* and *env*. The *gag* (group-specific antigen) gene encodes structural proteins. The *pol* (polymerase) gene encodes the enzymes reverse transcriptase and integrase. The env (envelope) gene encodes the envelope glycoprotein which is transported to the Golgi apparatus and cleaved into separate surface (SU) and transmembrane (TM) subunits. A fourth essential gene, the *pro* gene, is expressed in different ways in different retroviruses and may be separate or fused in frame to the *gag* or *pol* gene. The *pro* (protease) gene encodes the enzyme protease, which is responsible for cleavage of precursor polyproteins and is the target of protease-inhibiting drugs used in humans in highly active antiretroviral therapy (HAART) for acquired

immunodeficiency syndrome (AIDS).

Key points

- Enveloped, spherical labile viruses
- Diploid, containing two linear positive-sense single strands of RNA
- Icosahedral capsid surrounds helical nucleocapsid
- Members of this family are unique in possessing a reverse transcriptase which transcribes viral RNA to double-stranded DNA
- Double-stranded DNA is inserted as a provirus into the host genome
- Mutation and recombination occur with high frequency
- The family is composed of two subfamilies and seven genera:
  - – Lentivirus contains viruses which usually cause immunodeficiency diseases
  - – Spumavirus contains viruses which cause cell vacuolation *in vitro* but do not produce disease
  - – Viruses in the remaining five genera can induce neoplastic change in specific cell types

Figure 68.1 Classification of retroviruses with emphasis on those that produce disease in animals or induce cellular changes *in vitro*. Viruses in red cause OIE-listed diseases.

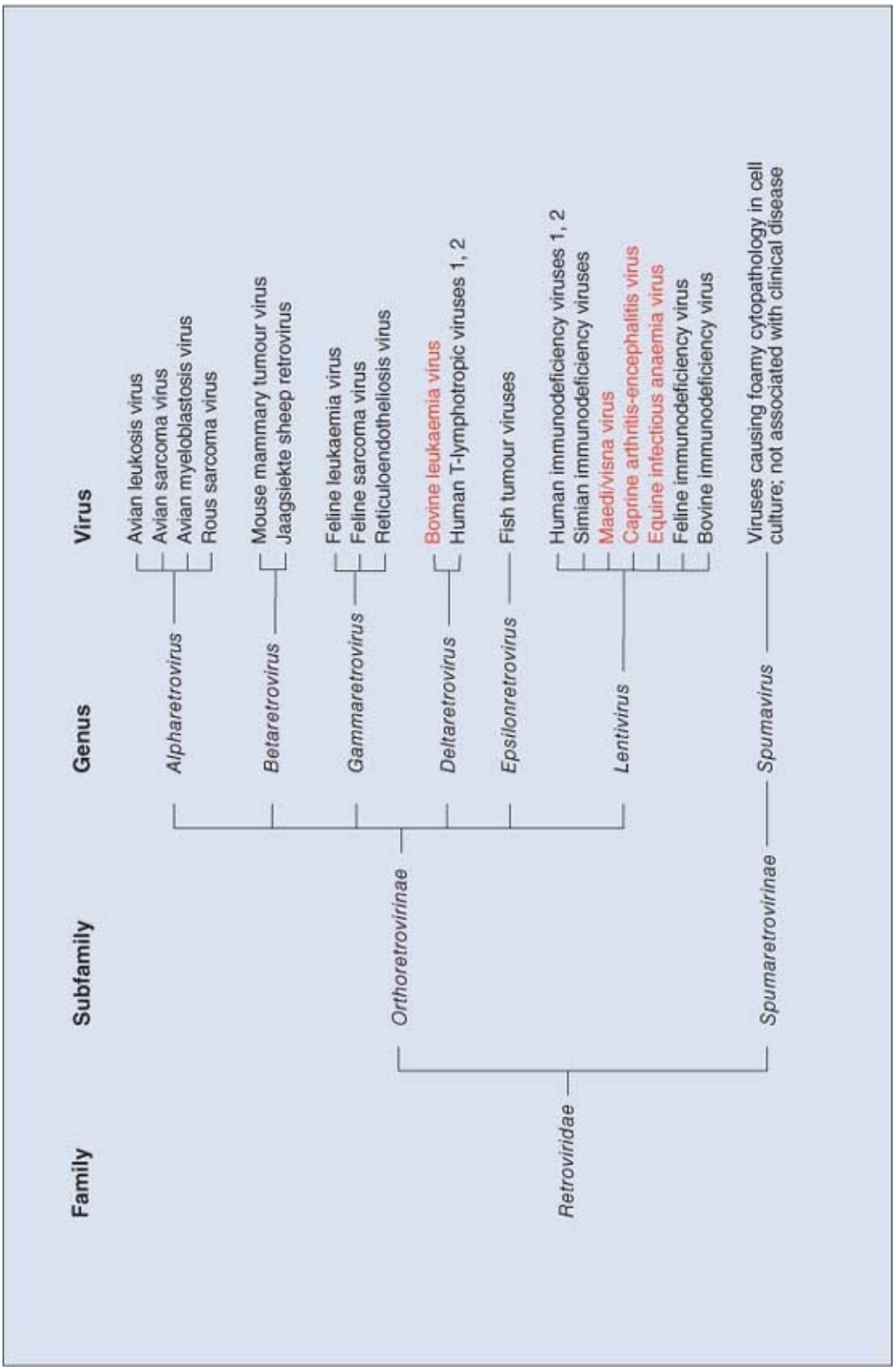


Figure 68.2 Schematic representation of the structures and components of a

retrovirus virion.

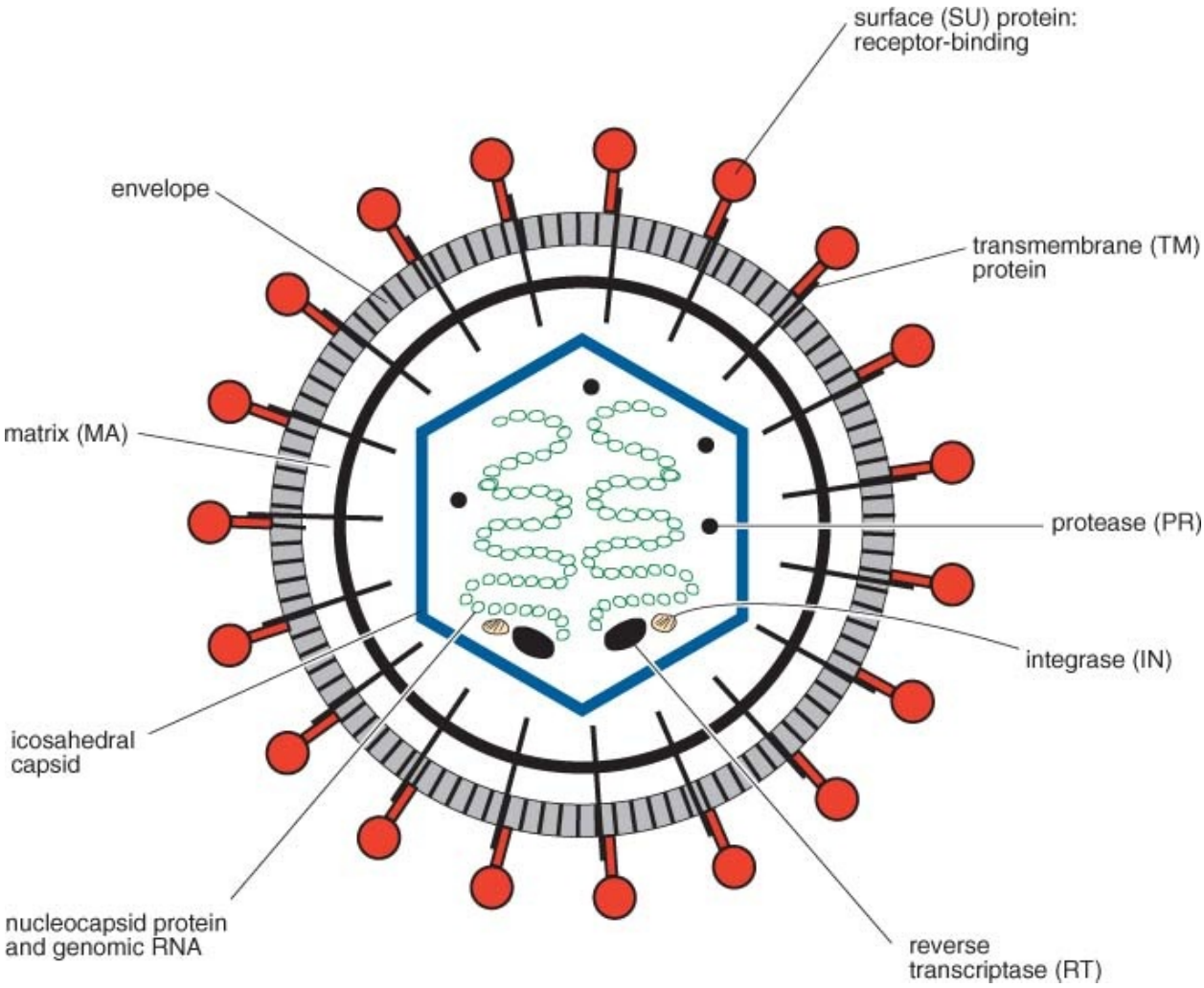


Figure 68.3 A, Budding from a cell membrane of a typical type C retrovirus with crescent-shaped nucleocapsid; B, mature, extracellular type C retrovirus virions.

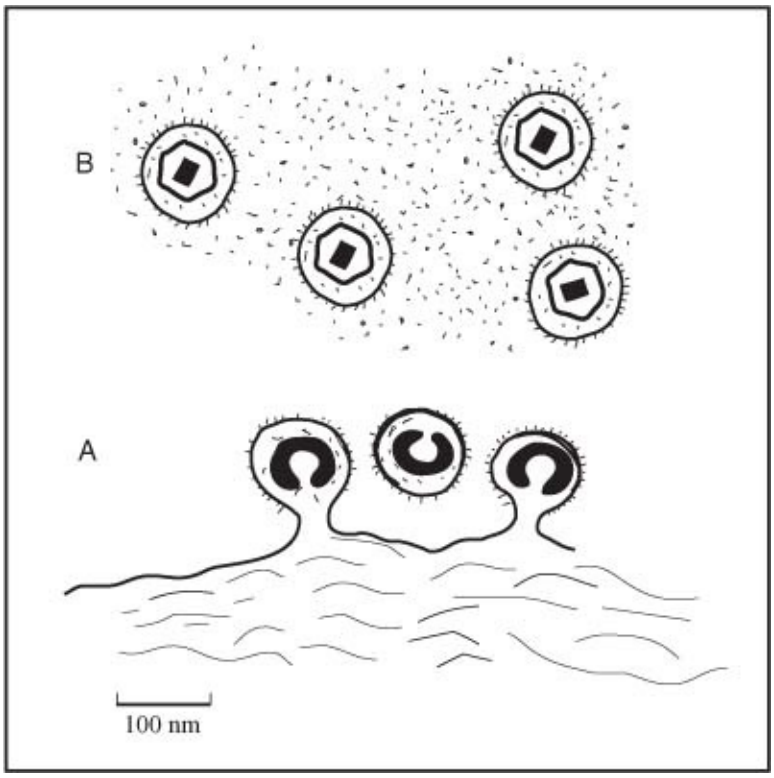
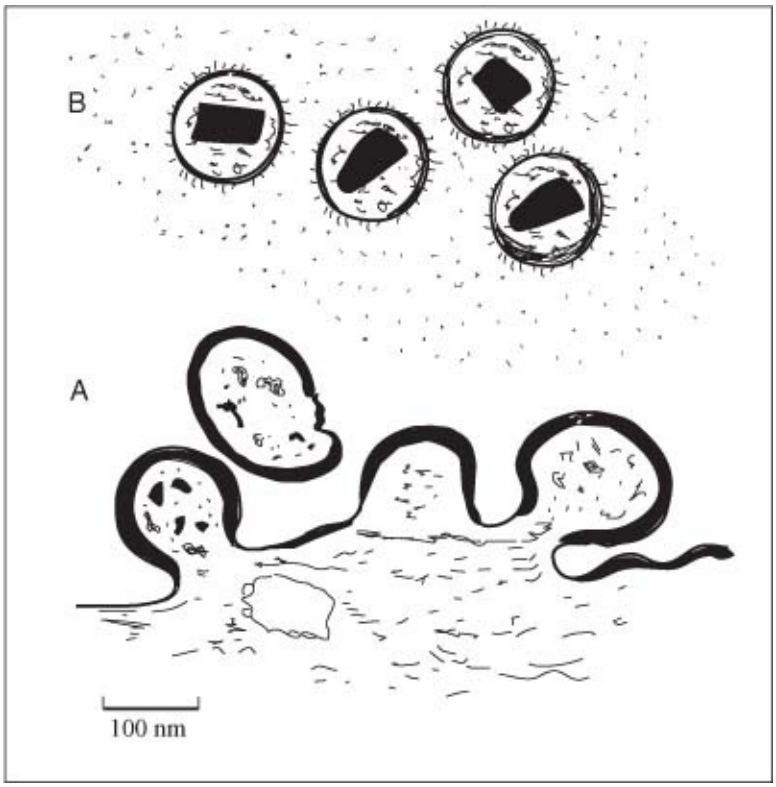


Figure 68.4 A, Budding of lentivirus particles from a cell membrane; B, mature, extracellular lentivirus virions.



Cell entry involves a complex interaction between the envelope glycoprotein

and a specific cell receptor. It appears that the SU subunit makes the initial contact. This is followed by significant conformational changes in the envelope protein. The TM subunit is thought to induce fusion of the viral and cellular membranes resulting in internalization of the virion core into the cytoplasm. Under the influence of the reverse transcriptase, double-stranded DNA copies of the viral genome are synthesized in the cytoplasm of the host cell. During this process, repeat base sequences, containing several hundred base-pairs and called long terminal repeats (LTRs), are added to the ends of the DNA transcripts. Transcripts are integrated into the chromosomal DNA, as provirus, at random sites through the action of viral integrase. The sites of proviral integration determine the extent and nature of cellular changes. The LTRs contain important promoter and enhancer sequences, which are involved in the transcription of mRNA and virion RNA from provirus. Release of mature virions often occurs by budding from cell membranes. If the provirus of certain retroviruses is inserted close to the host genes which regulate cell division, the proviral LTRs may increase the rate of mitosis with a risk of inducing neoplasia (insertional mutagenesis).

Because errors are relatively frequent during reverse transcription, a high mutation rate is a feature of retroviral replication. In addition, recombination between retroviral genomes in doubly-infected cells can occur because reverse transcriptase can transfer from the RNA template of one virus to that of another. Consequently, antigenically-different retroviruses (quasispecies) frequently emerge and classification of species and subtypes often proves difficult.

Retroviruses can be categorized as endogenous or exogenous. Endogenous retroviruses occur widely among vertebrates, the result at some time in the distant past of infection of germline cells. They are transmitted only as provirus in germ cell DNA vertically, from parent to offspring. They are regulated by cellular genes and are usually silent. The gradual accumulation of these retroviral sequences in the host cell genome indicates that they may confer some advantage on the host. It has been suggested that endogenous retroviruses act as restriction factors, which block the replication of related, pathogenic, exogenous retroviruses. However, the relationship between endogenous and exogenous retroviruses is complex. Endogenous retroviral genomes may contribute env genes to produce recombinant feline leukaemia viruses and avian leukosis viruses. Occasionally, endogenous retroviruses can be activated by irradiation, mutagens or carcinogens with the production of new virions. Endogenous retroviruses of pigs may be potentially dangerous for humans receiving

xenotransplants.

Retroviruses are sensitive to heat, lipid solvents and detergents. Because of their diploid genomes, they are relatively resistant to UV light.

Clinical infections

Retroviruses in the genera *Alpharetrovirus, Betare- trovirus, Gammaretrovirus, Deltaretrovirus* and *Epsilon- retrovirus* are frequently referred to as oncogenic retroviruses because they can induce neoplastic transformation of cells which they infect.

On the basis of the interval between exposure to the virus and tumour development, exogenous oncogenic retroviruses are designated either as slowly transforming (*cis*-activating) viruses or as rapidly transforming (transducing) viruses. Slowly transforming retroviruses induce B cell, T cell or myeloid tumours after long incubation periods. For malignant transformation to occur, the provirus must be integrated into the host cell DNA close to a cellular oncogene (*c-onc*, proto-oncogene), resulting in interference with the regulation of cell division (insertional mutagenesis). Most insertions are innocuous and even if a host gene is disrupted, the mutation may be recessive in nature. Rarely the provirus insertion creates a dominant- acting mutation that may result in the activation of a proto-oncogene or, alternatively, the inactivation of a tumour-suppressor gene. In contrast, rapidly transforming retroviruses, which can induce tumour formation after short incubation periods, contain viral oncogenes (v-*onc*). Viral oncogenes are considered to be cellular oncogenes acquired by recombination during virus evolution. These oncogenes are very diverse in sequence and function and include genes encoding intracellular tyrosine kinases (v-*src*, v-*fps*, v-*fes*, v-*abl*), growth factors (v-*sis*), growth factor receptors (v-*erbB*), transcription factors (v-*myc*, v-*erbA*) and members of the G protein family (v-*ras*). Such genes exert a strong influence on mitogenic signalling pathways, on the control of the cell cycle and on antiapoptotic pathways. If the oncogene is integrated into the viral genome without loss of replicative virus genes, as in Rous sarcoma virus, the retrovirus is described as replication-competent. Frequently, as a consequence of cellular oncogene integration, existing trans-acting viral sequences necessary for replication are deleted. Such replication-defective retroviruses rely on helper viruses to multiply and are rarely transmitted under normal field conditions. Occasionally, they may cause rapidly developing neoplastic disease. A third method of tumour induction is

exemplified by bovine leukaemia virus which depends on the *tax* gene encoding for a protein capable of up-regulating both viral LTRs and cellular promoter sequences, even when the provirus is integrated into a different chromosome (*trans*-activation). A schematic representation of the important genes in various categories of oncogenic viruses is presented in Fig. 68.5.

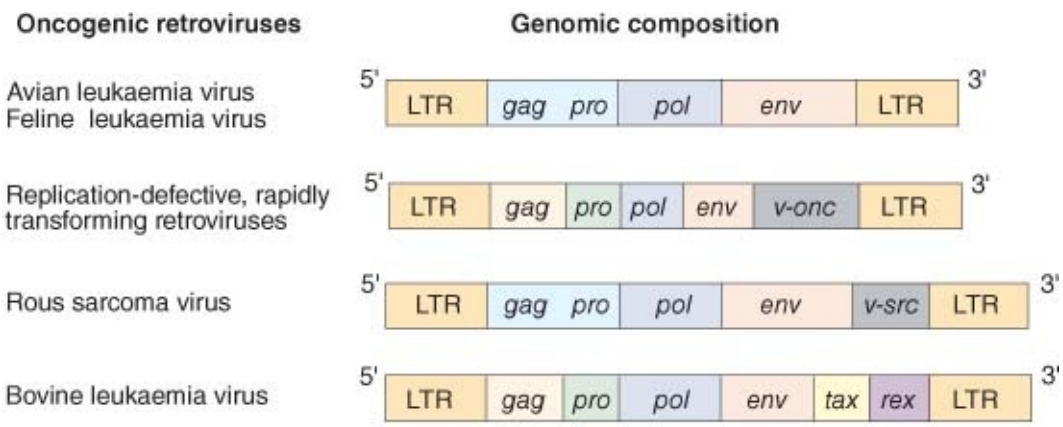
The newly established *Epsilonretrovirus* genus contains viruses associated with neoplasia in fish. The oncogenic retroviruses of poultry are presented in Table 68.1 and those of domestic mammals in Table 68.2.

Lentiviruses (Latin *lentus*, slow) cause diseases with long incubation periods and insidious protracted courses. Important animal and human diseases caused by lentiviruses include AIDS, feline immunodeficiency, equine infectious anaemia and maedi/visna. Lentiviruses of domestic animals are presented in Table 68.3. Although spumaviruses (Latin *spuma*, foam) cause vacuolation of cultured cells, they are not associated with clinical disease. Spumaviruses have been identified in primates, cats, horses and cattle.

Avian leukosis

The avian leukosis virus (ALV) group includes both replication-competent and replication-defective retro- viruses. Neoplastic conditions in chickens including the lymphoid, erythroid and myeloid leukoses, fibrosarcoma, haemangiosarcoma and nephroblastoma are caused by viruses in the group. Lymphoid leukosis, a B cell lymphoma and the most common of these neoplastic conditions, is economically the most important.

Figure 68.5 Schematic representation of important genes present in oncogenic retroviruses and their encoded proteins. LTR, long terminal repeat sequences.



Gene	Encoded protein
gag	Nucleocapsid
pro	Protease
pol	Enzymes: reverse transcriptase, integrase
env	Envelope glycoproteins
v-onc	Oncoprotein
v-src	Oncoprotein (tyrosine phosphokinase)
tax	Transcriptional activator
rex	Post-transcriptional activator

Table 68.1 Oncogenic retroviruses of poultry.

Genus	Virus	Hosts	Comments
Alpharetrovirus	Avian leukosis virus	Chickens, pheasants, partridges, quails	Endemic in commercial flocks. Exogenous and endogenous transmission of virus can occur. Causes lymphoid leukosis in birds between 5 and 9 months of age
Gammaretrovirus	Reticuloendotheliosis virus	Turkeys, ducks, chickens, quails, pheasants	Infection usually subclinical. Sporadic disease may present with anaemia, feathering defects, impaired growth or neoplasia. Disease outbreaks have occurred following use of vaccine contaminated with reticuloendotheliosis virus

Table 68.2 Oncogenic retroviruses of domestic mammals.

Genus	Virus	Hosts	Comments
Betaretrovirus	Jaagsiekte sheep retrovirus (ovine pulmonary adenocarcinoma virus)	Sheep	Causes jaagsiekte, a slowly progressive neoplastic lung disease of adult sheep which is invariably fatal. Occurs worldwide with the exception of Australasia
	Enzootic nasal tumour virus (ENTV)	Sheep, goats	Two distinct viruses, ENTV-1 in sheep and ENTV-2 in goats; closely related to jaagsiekte sheep retrovirus. Infection results in transformation of secretory epithelial cells of the ethmoid turbinate resulting in an adenocarcinoma of low-grade malignancy which affects the nares
Gammaretrovirus	Feline leukaemia virus	Cats	Important cause of chronic illness and death in young adult cats. Causes immunosuppression, enteritis, reproductive failure, anaemia and neoplasia. Worldwide in distribution
Deltaretrovirus	Bovine leukaemia virus	Cattle	Causes enzootic bovine leukosis in adult cattle. A small percentage of infected cattle develop lymphosarcoma

Table 68.3 Lentiviruses of domestic mammals.

Genus	Virus	Hosts	Comments
Lentivirus	Feline immunodeficiency virus	Cats	Causes life-long infection with persistent viraemia and immunosuppression in cats over 5 years of age. Worldwide distribution
	Equine infectious anaemia virus	Horses, mules, donkeys	Causes life-long infection with recurring febrile episodes. Anaemia is a prominent clinical sign
	Maedi/visna virus	Sheep	Causes life-long infection with progressive respiratory disease and indurative mastitis in older sheep. Clinical signs develop in a small percentage of infected animals. Some infected sheep develop progressive neurological disease
	Caprine arthritis-encephalitis virus	Goats	Causes life-long infection. Associated with polyarthritis and indurative mastitis in adults and progressive nervous disease in kids. Common in dairy goat herds. Worldwide distribution
	Bovine immunodeficiency virus	Cattle	Widely distributed; pathogenicity uncertain at present
	Jembrana disease virus	Cattle	Closely related to bovine immunodeficiency virus but disease described only in Bali cattle in Indonesia. Acute disease which occurs within days of infection is characterized by fever, anorexia, enlarged lymph nodes and, in some instances, death. Animals which recover remain viraemic

On the basis of differences in viral envelope glycoproteins, avian leukosis viruses are divided into ten subgroups (A to J). Isolates from chickens belong to subgroups A, B, C, D, E and J; viruses in the other subgroups infect other avian species. Most isolates from outbreaks of disease in chickens belong to subgroup A. Endogenous avian leukosis viruses, which are commonly present in chickens and are transmitted vertically in the germline cells, usually belong to subgroup E. Members of subgroup J, a group recently recognized in broilers, are associated with myeloid leukosis and have arisen from recombination of a novel family of endogenous viruses (ev/J) and exogenous avian leukosis viruses (Benson *et al*., 1998).

Because of the time required for the genetic events to occur that lead to transformation of cells and malignancy, there is usually an incubation period of months to years between natural infection with ALV and the development of neoplasia. Neoplastic conditions associated with ALV include lymphoid leukosis, myeloid leukosis, sarcomas and renal tumours. Avian leukosis virus is also associated with osteopetrosis. One of the final steps in oncogenesis can be the generation of recombinant, rapidly transforming viruses, which have incorporated a cellular oncogene into their genome. Viruses isolated from such tumours include avian erythroblastosis virus, avian myeloblastosis virus and Rous sarcoma virus, all of which rapidly cause tumours when inoculated experimentally into susceptible chickens. These viruses are usually defective and require a helper ALV for replication. Rous sarcoma virus is exceptional in that it has an oncogene (*src*) in addition to a complete ALV genome, and consequently is replication-competent as well as capable of rapid cell transformation *in vivo* and *in vitro*. This type of acutely transforming virus is very seldom, if ever, transmitted under natural conditions. The endogenous ALVs carried by chickens in most flocks do not directly cause tumours.

Epidemiology

Exogenous ALV is transmitted both vertically, through virus present in egg albumen, and horizontally by close contact. Chicks which hatch from infected eggs are usually immunotolerant and exhibit persistent viraemia. They are the principal source of virus in a flock. Virus is transmitted in saliva and faeces to incontact birds. Viral shedding into oviducts results in transmission to chick embryos. Chicks infected after hatching develop a transient viraemia before they produce neutralizing antibodies. Some of these birds may become carriers, shed virus intermittently and produce infected chicks if exposed early in life in the absence of maternal antibodies. Natural exposure of adult birds to infection does not usually result in virus shedding. Neoplasms develop in persistently viraemic birds, particularly congenitally-infected chicks. Virus-neutralizing antibodies are passed in the yolk sac from antibody-positive hens to their chicks, providing passive immunity to infection for the first few weeks of life.

Pathogenesis

Following infection, virus spreads throughout the body, replicating in most tissues. Avian leukosis virus transforms B cells after integration of provirus close

to the *c-myc* gene which induces cellular replication under the influence of the viral LTR promoter. Less commonly, ALV has been associated with erythroblastosis when the *c-erbB* gene in an erythroid cell is activated. Subgroup J isolates are associated with late- onset myeloid leukosis in broilers (Benson *et al*., 1998). Rapidly transforming viruses are formed rarely in individual birds by transfer of a *c-onc* (proto-oncogene) into the ALV provirus during its integration. Multiple insertions of the provirus into the host cell genome result in exaggerated gene expression and overproduction of a transformation-associated protein. More than a dozen different oncogenes have been identified in transforming avian retroviruses. The protein products of oncogenes may act as hormone or growth factor receptors, transcription control factors and kinases in signal transduction pathways. Although a particular virus strain may be capable of producing neoplasia of more than one cell type, usually one cell type predominates in an affected animal.

Clinical signs

The incubation period for lymphoid leukosis is usually more than 4 months. Disease is generally sporadic in infected flocks but occasional epidemics have been described. Affected birds become inappetent, weak and emaciated. They have pale wattles and the liver and bursa of Fabricius may be enlarged. Osteopetrosis, in which the long bones of the legs become visibly thickened, sometimes accompanies lymphoid leukosis. Subclinical infections with ALV are associated with depressed egg production and fertility, decreased hatchability and growth rate and increased death rates. Economic loss from lymphoid leukosis is mainly due to deaths in egg-laying and breeding birds between 5 and 9 months of age.

Diagnosis

- Post-mortem findings and histopathological determination of tumour type are usually diagnostic.
- Differentiation from Marek’s disease is important and is based on the age of affected birds, the presence of bursal tumours, absence of thickening of peripheral nerves and histological assessment of neoplastic cell types.
- Virus isolation, which is difficult, is not usually attempted.
- Commercial ELISA kits for the detection of ALV group-specific antigen are available.

- The presence of flock infection can be demonstrated by detecting antibodies in serum or egg yolk. Suitable assays include virus neutralization, ELISA and indirect immunofluorescence.
- The polymerase chain reaction has been adapted for the detection of ALV nucleic acid (Cavanagh, 2001). Specific primers or sequencing of the amplicon can be used to determine the infecting subgroup or to distinguish endogenous from exogenous virus (Silva *et al*., 2007).

Control

The eradication of ALV infection with exogenous subgroup A has been successfully achieved in most commercial chicken flocks. The cycle of vertical transmission is interrupted by hatching and rearing infection-free chicks in isolation. Ongoing monitoring for the presence of infection is essential. Because autosomal genes encode subgroup-s pecific cell surface receptors through which ALV gains entry to the cell, genetically resistant birds with decreased numbers of specific cell surface receptors can be bred. Attention has focussed largely on resistance to subgroup A viruses. Selection for genetic resistance is an ongoing process because mutant viruses capable of overcoming host resistance frequently arise. Control of ALV infection in commercial flocks is based on high standards of hygiene and effective management aimed at reducing levels of infection. Birds from disease-free or genetically resistant stock should be used for breeding. Because the virus is labile, all-in all-out management systems along with thorough washing and disinfection programmes between batches of birds are effective for preventing transfer of infection. Chicks of uncertain status should not be mixed with disease-free chicks. Chick rearing should take place remote from older birds. Vaccination with inactivated or modified live ALV vaccines has not been successful. Recombinant avian leukosis and fowlpox viruses expressing subgroup A envelope glycoproteins have been shown to have potential as effective vaccines.

Feline leukaemia and associated clinical conditions

Infection with feline leukaemia virus (FeLV) not only results in feline leukaemia but is also associated with a variety of other clinical conditions. On the basis of differences in the gp70 envelope glycoprotein of FeLV, isolates of this gammaretrovirus are assigned to four subgroups (A, B, C and T). Feline leukaemia virus A (FeLV-A), the predominant subgroup, can be isolated from all

FeLV-infected cats. Viruses of subgroup B, which arise through recombination between the *env* genes of FeLV-A and endogenous FeLV-related proviral DNA, are present in about 50% of isolates. FeLV-B is transmitted only in association with FeLV- A. In a proportion of cats exposed to a mixture of FeLV-A and FeLV-B, the FeLV-B component is lost. Therefore, the continuing survival of FeLV-B depends upon the generation of new recombinants in cats persistently infected with FeLV-A. Cats that are infected with both FeLV-A and FeLV-B have a higher risk of developing tumours than those infected with FeLV-A alone. Each FeLV-C isolate is unique, arising *de novo* in a FeLV- A infected cat through mutations in the receptor-binding region of the FeLV-A *env* gene. Once generated, FeLV-C viruses rapidly cause a fatal anaemia and consequently are not transmitted to other cats. Conversion of FeLV-A to FeLV-T requires a combination of an insertion and single amino acid changes to the envelope protein, giving rise to a T cell tropic, cytopathic virus capable of inducing immunodeficiency. In order to enter and infect host cells, FeLV-T requires a soluble cofactor coded for by an endogenous retroviral sequence (Cheng *et al*, 2007).

Like the ALVs, FeLV causes tumours by several means, including insertional mutagenesis and recombination with a variety of cellular proto-oncogenes to produce acutely transforming, replication-defective viruses. Examples of the latter are FeLVs isolated from thymic lymphomas, and feline sarcoma viruses (FeSV) that are isolated from rare multicentric fibrosarcomas in young cats. These viruses are not transmitted under natural conditions.

Epidemiology

Infection with feline leukaemia virus, which occurs in domestic cats worldwide, is an important cause of mortality. Close contact is required for transmission of this labile virus, and the prevalence of infection is related to population density, ranging from less than 1% in individually kept cats to more than 20% in catteries and multicat households. Large amounts of virus are shed in saliva with smaller quantities present in tears, urine, milk and faeces. Infection is usually acquired by licking, grooming and through bite wounds. Young kittens are more susceptible to infection than adults. Although maternally-derived antibody is protective in kittens up to 6 weeks of age, a significant proportion of those exposed before 14 weeks of age become persistently infected. Such animals constitute the main reservoir of FeLV and they are prone to develop FeLV-related disease. Most cats exposed after 4 months of age develop immunity and eliminate the virus. Kittens born to persistently-infected queens develop

persistent infection, acquired either transplacentally or through ingestion of milk.

Pathogenesis

Following oronasal exposure, virus replicates in the lymphoid tissues of the oropharyngeal region. The virus spreads in infected mononuclear leukocytes to other lymphoreticular tissues and bone marrow. In most cats, cell-mediated immunity and neutralizing antibodies to the gp70 envelope glycoprotein are produced at this stage, usually resulting in virus elimination. However, a latent bone marrow infection, which is eliminated after several months, is present in about 50% of cats. Failure to contain the infection results in extensive virus production in the bone marrow and persistent viraemia. The virus, present in both leukocytes and plasma, is disseminated to glandular and mucosal epithelia. Large quantities of virus are shed from the salivary glands and the upper respiratory tract. Because the production of virus particles requires cellular DNA synthesis, tissues with high mitotic activity such as bone marrow and epithelia are targeted. Prolonged periods of viral replication in haemo-lymphatic tissues can lead to depletion of lymphoid and myeloid cells, producing immunosuppression and anaemia. Severe immunosuppression is caused by infection with FeLV-T. Isolates of FeLV-C are associated with severe non-regenerative anaemia.

Neoplastic changes in lymphoid or myeloid cells follow insertion of provirus close to a cellular oncogene with activation or deregulation of the gene, or generation of acutely transforming viruses by the acquisition of a cellular oncogene such as *myc* by recombination.

Clinical signs

The incubation period ranges from months to years. The majority of persistently-infected cats die within 3 years of infection. About 80% of these cats die from non-neoplastic FeLV-associated disease; the remaining 20% of infected cats succumb to neoplasia, particularly lymphosarcoma. During the early phase of infection, cats may develop fever, malaise and lym-phadenopathy, which may not be detected clinically. A variable period as asymptomatic carrier cats follows. Clinical signs, which are often non-specific and chronic, are usually seen in adult cats between 2 and 4 years of age. Anaemia, reduction in reproductive performance, fading kitten syndrome, enteritis and a variety of secondary infections due to the immunosuppressive effects of the virus are important

features of the disease. Immune complex formation, initiated by circulating antigen, may give rise to glomerulonephritis.

Lymphosarcoma, the most commonly occurring feline tumour, is usually linked to infection with FeLV. Thymic, alimentary, multicentric and leukaemic forms of lymphosarcoma are described and clinical signs relate to the anatomical sites involved. Fibrosarcoma and myeloid tumours, which have also been associated with FeLV infection, occur less frequently than lymphosarcoma.

Diagnosis

Detection of viral antigen in blood or saliva is the method commonly used for the laboratory diagnosis of feline leukaemia. Virus isolation, which is expensive and time-consuming, is used as a confirmatory test.

- Commercial ELISA and rapid immunomigration tests, designed to detect the major capsid protein (p27), are available. Cats giving positive results to these tests should be retested some months later as their immune response may clear the viraemia up to 16 weeks later.
- The immunofluorescent antibody test, which is generally employed as a confirmatory test, can be used to detect viral antigen in the cytoplasm of leukocytes in blood smears.
- Conventional PCR (Jackson *et al*., 1996; Miyazawa and Jarrett, 1997) and real - time PCR (Hofmann - Lehmann *et al*., 2001) assays are available for the detection of proviral FeLV DNA in peripheral blood samples. However, cats which have overcome FeLV viraemia may continue to be provirus positive. Detection by RT-PCR of high virus loads in saliva, plasma or faeces is a reliable indicator of viraemia (Gomes-Keller *et al*., 2006).
- Serological testing for antibodies is not used for diagnosis since both viraemic and recovered cats can have anti-FeLV antibodies. However, the demonstration of a high titre of virus neutralizing antibodies generally indicates that a cat is immune and resistant to infection.
- An antigen termed feline oncovirus - associated cell membrane antigen (FOCMA) is expressed in all FeLV and FeSV-transformed cells. The development of antibodies to FOCMA provides protection against FeLV-associated neoplasia.

Treatment and control

Supportive treatment is important for FeLV-viraemic cats. Aggressive courses of

antibiotics to combat secondary infections may be needed. Treatment of viraemic cats with feline interferon omega has been shown to improve clinical signs and extend survival time. The antiviral drug AZT has also been shown to be useful but requires careful monitoring due to possible side effects. A test and removal policy has been shown to be effective in eradicating infection from catteries. The status of individual cats should be confirmed by retesting after 12 weeks. Infected cats, which must be separated from susceptible animals, should be excluded from breeding programmes. Serological testing for antigen at intervals of 6 months is recommended and cats about to be introduced into a cattery should be isolated until test results are known. Several commercial vaccines, including killed whole virus, recombinant canarypox virus, and subunit and recombinant subunit types, are available. Vaccination does not result in elimination of the virus in persistently- nfected cats. Accordingly, prior to vaccination, cats should be tested. Vaccination does induce significant protection against persistent viraemia as well as helping to reduce proviral and viral loads. However, vaccination does not provide complete protection or prevent infection and other appropriate control measures should be implemented also.

Enzootic bovine leukosis

This retroviral disease of adult cattle is characterized by persistent lymphocytosis, the presence of circulating antibody to the causal agent, bovine leukaemia virus (BLV) and the development of B cell lymphosarcoma in a number of infected animals. Enzootic bovine leukosis (EBL) has a worldwide distribution. Some countries, including Ireland and most EU Member States, have eradicated the disease; other countries are embarking on eradication programmes.

Epidemiology

Transmission, which can occur by direct contact or transplacentally, usually takes place through transfer of blood or secretions such as colostrum and milk containing infected lymphocytes. Infection with BLV is lifelong. The labile virus is intimately cell-associated.

Less than 10% of calves born to infected dams are infected at birth. Calves are protected from contact infection for several months by maternally-derived antibody. Animals are usually infected between 6 months and 3 years of age (Hopkins and DiGiacomo, 1997). Iatrogenic transmission is important and has

been linked to reuse of needles, multidose injectors, contaminated surgical instruments and rectal examination procedures. While biting flies may transmit the virus mechanically, their importance as vectors is uncertain. The prevalence of infection is higher in dairy cattle than in beef cattle. Susceptibility to infection is influenced by genotype and related to the bovine major histocompatibility antigen type.

Pathogenesis

The primary target cell is the B lymphocyte. Bovine leukaemia virus does not possess an oncogene. Nucleic acid sequences at the 3′ end of the *env* gene, termed the X region, encode for the regulatory proteins Tax and Rex which are central to neoplastic transformation. The Tax protein interacts with cellular transcription factors resulting in transactivation of the promoter in the LTR of integrated BLV provirus. Up-regulation of some cellular genes including those coding for IL-2 and its receptor may also occur.

Clinical signs

Although infections are lifelong, most animals remain subclinically infected. About 30% of infected animals develop persistent lymphocytosis, an increase in lymphocyte numbers in the blood without clinical signs of disease. A small percentage of BLV-seropositive cattle eventually develop lymphosarcoma. Clinical disease usually occurs in adult animals between 4 and 8 years of age. The presenting signs, which relate to the sites of tumour formation, include enlargement of superficial lymph nodes, digestive disturbance, inappetence, weight loss and general debility.

Diagnosis

Enzootic bovine leukosis (EBL) must be differentiated from sporadic bovine leukosis which usually affects calves and young adult cattle. Formerly, blood lymphocyte counts were employed for laboratory diagnosis of infected animals and for the eradication of EBL. However, all infected cattle do not develop lymphocytosis. Serological testing for virus-specific antibody is now used for diagnosis and also for eradication.

- Several serological tests including AGID and ELISA are suitable for the detection of antibodies to BLV. Antibodies detected in calves less than 6 months of age may be colostral in origin.

- Although virus can be isolated by cultivation of peripheral blood lymphocytes, this technique is not performed routinely.
- The polymerase chain reaction has been employed as a sensitive research tool for the detection of provirus in peripheral blood lymphocytes.

Control

A commercial vaccine is currently not available. Test and removal strategies have been successfully used for both national and individual herd eradication programmes. Serological testing at 6-month intervals is recommended (Brunner *et al*., 1997). In countries in which the prevalence of BLV infection is too high to permit removal of all seropositive animals from herds, management practices aimed at reducing the spread of infection should be adopted. Such practices include separating infected and susceptible animals, rearing calves on milk from non-infected cows and serological testing of replacement animals.

Jaagsiekte

This disease, also called ovine pulmonary adenomatosis, is a slowly progressing neoplastic disease of adult sheep. It is caused by jaagsiekte sheep retrovirus (JSRV), also known as ovine pulmonary adenocarcinoma virus. Jaagsiekte is an Afrikaaner word meaning ‘panting sickness’. With the exception of Australia, New Zealand and Iceland, jaagsiekte has a wide geographical distribution. Goats appear to be less susceptible to infection than sheep and the disease occurs rarely in goats. Multiple copies of endogenous retroviruses related to JSRV have been found in the genomes of both sheep and goats. Jaagsiekte sheep retrovirus is not considered to be an endogenous retrovirus.

Epidemiology

Respiratory exudates from affected sheep are infectious and transmission occurs by the respiratory route. Close contact facilitates spread of infection with the incidence of disease highest in housed animals. Within an infected flock, disease incidence, which may be up to 20%, is influenced by breed and type of flock management. Susceptibility to jaagsiekte appears to be age-related with a longer incubation period in older animals.

Pathogenesis

The virus replicates principally in two types of pulmonary cells, type II alveolar cells and non-ciliated bronchial cells. Hyaluronidase 2 (Hyal2) has been shown to be the cellular receptor for virus attachment before cell entry. Tumours arising from these cell types progressively replace normal lung tissue leading to death from asphyxia. The excess production of surfactant proteins by the neoplastic cells results in large amounts of pulmonary fluid. About 10% of the tumours metastasize to regional lymph nodes. Metastasis to heart or skeletal muscle occurs rarely. The presence of an oncogene has not been demonstrated in the viral genome and the envelope of the virus has been shown to be capable of inducing cell transformation (Caporale *et al.*, 2006). Multiple copies of closely related endogenous betaret-roviruses (enJSRV) are present in the genome of sheep and goats. They play a role in protecting their host from JSRV infection by blocking particular steps in the viral replication cycle (Varela *et al.*, 2009). They are expressed in the placenta and are required for placental development, leading to speculation that this may account for the apparent immunological tolerance in mature animals to the disease-producing, exogenous JSRV (Palmarini *et al.*, 2004).

Clinical signs

The incubation period may range from several months up to 2 years. Tumour nodules have been detected in lambs as early as 10 days after experimental inoculation. Affected animals are usually 3 to 4 years of age and in poor bodily condition. They display respiratory distress and mouth breathing, particularly after exercise. Using the so-called ‘wheelbarrow test’, when the hind legs are raised, a clear fluid flows from the nostrils. Moist rales may be heard. Often, only a single animal in a flock may be clinically affected at a particular time. The course of the disease may extend over weeks or months. Secondary pasteurellosis is a frequent complication.

Diagnosis

The characteristic clinical signs may be masked in individual animals by secondary infection. Histo-pathological confirmation is desirable. Attempts to culture the virus in monolayers have been unsuccessful. It is possible to detect virus in lung exudates or washings by ELISA and viral nucleic acid may be detected by PCR. Virus is also demonstrable in lymphoid tissue and peripheral blood leukocytes. As infected animals do not appear to develop a specific

humoral immune response (Ortin *et al*., 1998), it is not possible at present to confirm infection by serology.

Control

Jaagsiekte was successfully eradicated from Iceland in 1952 after drastic depopulation procedures. The incidence of disease in a flock can be reduced by strict isolation, particularly during the rearing of lambs (Voigt *et al*., 2007), and by elimination of suspect animals immediately after clinical or laboratory confirmation.

Feline immunodeficiency virus infection

This condition was first reported in 1987 and infection with feline immunodeficiency virus (FIV), a lentivirus, is now recognized worldwide as an important cause of disease in cats. The infection in cats is sometimes referred to as 'feline AIDS' on account of similarities to acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus. Five subtypes or clades of FIV, designated A to E, have been identified based on diversity in the envelope gene amino acid sequences. This diversity may account for differences in the pathogenesis and clinical progression of the disease associated with different isolates. The majority of isolates belong to subtypes A or B.

Epidemiology

Infection with FIV occurs in domestic cats. Related lentiviruses have been isolated from a number of wild *Felidae*, including pumas, spotted hyenas and lions. Seroprevalence in cats varies from region to region with reported estimates of 1 to 14% in normal cats and up to 44% in cats showing clinical signs of chronic infections. Animals remain infected for life. Virus is shed mainly in the saliva and transmission usually occurs through bites. Accordingly, infection rates are highest in free-roaming, adult male cats. Nonaggressive intimate contact may also be important in transmission under natural conditions. Queens may transmit infection to kittens *in utero*, during parturition or in milk, particularly during the acute phase of infection.

Pathogenesis

The virus replicates principally in $CD4^+$ (helper) T lymphocytes. Replication

also occurs in macrophages, dendritic cells, astrocytes and microglial cells. Infected cats remain persistently viraemic. The level of viraemia increases rapidly after infection and peaks at 8 to 12 weeks before gradually declining and increasing again during the terminal stage of the disease. Humoral responses are normal, or occasionally enhanced, with antibodies appearing 2 weeks after infection. However, there is progressive deterioration in cell-mediated immunity due to depletion of $CD4^+$ T lymphocytes. Lymphocyte depletion is attributed to the cytopathic effect of virus in addition to decreased production of lymphocytes and apoptosis. Other immunological abnormalities associated with the infection include reduced interleukin-2 responsiveness and production, impaired lymphocyte blastogenesis in response to mitogens and reduced antibody response to T cell-dependent antigens. Reduction in $CD4^+$ lymphocyte numbers, increased production of virus, the emergence of variants with increased virulence, and infection with opportunistic pathogens contribute to the development of clinical disease.

Clinical signs

The prevalence of clinical disease is highest in cats over 6 years of age. The course of the disease may be divided into an acute phase, an asymptomatic phase, a phase characterized by vague clinical signs and a terminal phase with marked immunodeficiency (Hartmann, 1998). The acute phase, which may last several weeks or months, is manifested by pyrexia, generalized lymphadenopathy and neutropenia. A prolonged period during which infected cats appear clinically normal follows. The third phase is marked by recurrent fever, leukopenia, anaemia, weight loss, lymphadenitis, chronic gingivitis and behavioural changes. Opportunistic infections are frequent in the terminal phase of the disease. Chronic stomatitis is a common finding. Other manifestations include chronic respiratory, enteric and skin infections. Neurological signs, usually due to direct viral damage, develop in a small number of infected cats. Concurrent FeLV infection may exacerbate the immunodeficiency and accelerate the appearance of clinical signs. An increased incidence of neoplasia, particularly B cell lymphomas, is recorded in FIV-infected cats. Some infected cats do not develop FIV-related clinical disease during their lifetime.

Diagnosis

- Serological testing for antibodies to FIV is the principal method for

confirming infection. Commercial ELISA and immunoconcentration kit-sets are available. Alternative tests include immunoblotting and indirect immunofluorescence. Some cats fail to produce antibodies for several months following infection; antibody levels may become undetectable in terminally ill cats. The kittens of infected queens may remain seropositive for up to 5 months due to ingestion of colostral antibodies.

- Although virus isolation from blood or saliva is possible, it is not considered realistic for routine diagnostic purposes.
- Proviral DNA can be detected using the polymerase chain reaction. However, these tests are not reliable in all cases and may be inferior to serological tests (Crawford *et al*., 2005). In general, these tests detect virus belonging to subtype A in a reliable manner; they are considered to be less reliable for detecting other subtypes of this virus.

Treatment and control

Treatment is aimed primarily at the control of secondary infections. A number of antiviral drugs such as azidothymidine (AZT), directed against viral reverse transcriptase, have a beneficial effect in clinically ill cats but do not eliminate infection. Unfortunately, AZT-resistant mutants of FIV can arise within months of commencing treatment. Both feline interferon omega and human interferon alpha have been used with varying success. A commercial, killed, whole virus (subtypes A and D) vaccine is available and has been shown to protect against some heterologous virus strains (Hosie and Beatty, 2007). It is difficult to distinguish serologically between vaccinated and infected cats, although one approach has been to use a dualsubtype vaccine (Kusuhara *et al*., 2007). Control is based on prevention of exposure by separating infected and non-infected cats in multicat households, by preventing cats from roaming freely, by using seronegative queens for breeding and by screening all cats before introduction into seronegative populations.

Equine infectious anaemia

This disease, also called swamp fever, affects horses, mules and donkeys in many countries worldwide. It is caused by a lentivirus, equine infectious anaemia virus (EIAV). Although infected *Equidae* remain viraemic for life, such animals often bring the infection under control with limited viral replication evident and an absence of clinical signs.

Epidemiology

Virus is transmitted mechanically by haematophagous insects, particularly *Tabanus* and *Stomoxys* species. The virus survives for only short periods on the mouth-parts of biting flies. These haematophagous insects usually obtain a complete blood meal from a single host. If interrupted during feeding, they may transfer virus to another host when they resume feeding. Transmission occurs most often in the summer, during periods of high insect activity, in low-lying swampy areas close to woodlands (the preferred habitat of tabanids). The risk of transmission is related to the levels of virus in the blood, being highest during febrile episodes. Iatrogenic transmission can occur through contaminated needles or surgical instruments. Although in utero transmission can occur, it is uncommon.

Pathogenesis

Virus replicates in macrophages, monocytes and Kupffer cells. A cell-associated viraemia develops with dissemination throughout the body (Oaks *et al*., 1998). Although EIAV infects monocytes, viral expression appears to occur only following macrophage differentiation in the tissues, a mechanism referred to as the ‘Trojan horse’. Despite mounting a strong immune response, infected horses fail to eliminate the virus and become persistently infected following insertion of provirus into the genome of host cells. With the continuous production of virus particles, many target cells become infected. In the course of further provirus production by reverse transcription in infected cells, mutations frequently arise due to errors during the transcription process. This can result in the emergence of new virus strains or quasispecies exhibiting antigenic variation in envelope glycoproteins (antigenic drift). Febrile episodes and marked immune stimulation signal the emergence of these new strains. Non-neutralizing antibodies produced against virus early in the course of infection lead to the formation of immune complexes. Such immune complexes activate complement contributing to the initiation of fever, anaemia and thrombocytopenia, and later, glomerulonephritis. Haemolysis, enhanced erythrophagocyto- sis and depressed erythropoiesis are responsible for the anaemia in chronically affected horses. In most animals, clinical episodes eventually cease, probably as a consequence of a broad-based immune response involving cytotoxic T lymphocytes and neutralizing antibodies against a wide range of viral epitopes.

Clinical signs

The majority of infected horses display mild signs, which may go undetected. Most of the clinical signs are attributed to the host's immune response rather than to direct viral damage. Following an incubation period of up to 3 weeks, infected animals may present with fever, depression and petechiae on mucous membranes and conjunctivae. However, following this phase of the disease, most horses appear clinically normal for several weeks until the recrudescence of clinical signs. Rarely, severe epistaxis and ventral oedema may be followed by death. The number and severity of recurring disease episodes vary widely. Most occur during the first year after infection and decline in number thereafter. Many horses that appear clinically normal remain carriers. Some exhibit a chronic form of the disease characterized by weight loss, anaemia, ventral oedema and debilitation, leading eventually to death.

Diagnosis

Laboratory confirmation of infection is based on the demonstration of serum antibodies to the core virus protein p26.

- The serological test recognized for international trade is the AGID test (Coggins test). Although the ELISA is a suitably sensitive assay, positive results should be confirmed by the more specific AGID test. Results can also be confirmed by immunoblotting.
- Antibodies may not be detectable early in the course of the disease.
- False-positive results may be encountered in foals up to 6 months of age owing to the presence of colostral antibodies.
- The presence of viraemia can be demonstrated by inoculation of a susceptible horse with blood from a suspect animal.
- Virus can be isolated in leukocyte cultures prepared from the blood of susceptible horses. Because of the time and expense involved, virus isolation is rarely attempted.
- Proviral DNA may be detected by PCR and viral RNA can be detected using RT-PCR.

Control

Commercial vaccines are not available in western countries. Envelope antigenic variation among strains of EIAV presents a significant obstacle to the

development of an effective vaccine. Control measures are aimed at reducing the risk of infection. In many countries, legislation requires certification of freedom from EIA prior to importation of horses. Restriction of animal movement is also used to minimize the risk of disease spread. Management practices, including detection and removal of seropositive animals, insect control and testing of animals prior to introduction on to a property, are worthwhile control measures. Care should be taken to ensure that chemicals used routinely for disinfecting surgical instruments also inactivate EIAV.

Small ruminant lentivirus group

Two distinct lentiviruses have been described in small ruminants. While maedi/visna virus (MVV) primarily affects sheep and caprine arthritis-encephalitis virus (CAEV) principally affects goats, some strains of these closely-related viruses are transmissible to both sheep and goats with the production of persistent infections. Genomic analyses of these ovine and caprine lentivirus isolates suggest that they evolved from a common ancestral genotype. The current view is that they comprise a heterogeneous group with a variable host range and different pathogenic capabilities (Pasick, 1998; Shah *et al*., 2004).

Maedi/visna

This lentiviral disease, also referred to as ovine progressive pneumonia, la bouhite and zwoegersiekte, occurs in many countries. Maedi/visna virus (MVV) causes a life-long infection in sheep and is associated with chronic progressive disease in adult sheep. Maedi and visna, Icelandic words meaning ‘laboured breathing’ and ‘ wasting’, respectively, refer to the clinical features encountered in the respiratory form and the rare nervous form of the disease. Maedi/visna caused significant losses in Icelandic sheep prior to its eradication in 1965. Apart from Iceland, Australia and New Zealand, ovine lentiviruses are present in most countries.

Epidemiology

Infection is frequently subclinical. The clinical severity of disease is influenced by viral virulence, the age of the host when exposed and other host factors. Although virus is widely distributed in tissues of infected animals, it is transmitted principally in pulmonary exudates, colostrum and milk. Horizontal transmission occurs by inhalation of aerosols and is facilitated by close

confinement of animals during winter housing. In flocks with endemic disease, lambs may be exposed to infection by lactogenic transmission if born to seropositive ewes or to infection from aerosols. Transmission may occur transplacentally or by iatrogenic exposure but such routes are probably of minor importance.

Pathogenesis

Clinical signs take several months or years to develop. The chronic, progressive inflammation is characterized by mononuclear cell infiltration and lymphoproliferation, particularly in the lungs and mammary glands. Lesions are also present in synovial membranes and in the brain. Persistent infection and the interaction between viral antigens and cellular and humoral components of the immune system are responsible for the development of lesions. Provirus integrated into the genome of monocytes and their precursors is activated only when the monocytes develop into macrophages. Restricted virus production in the monocytes permits insidious spread of virus around the body with minimal immune stimulation ('Trojan horse' mechanism). The occurrence of antigenic variants during the course of an infection, as a result of genetic instability, also facilitates persistence. For some weeks after infection, there is a period of viraemia in most animals. A vigorous immune response, sufficient to restrict virus production to low levels, does not eliminate infection. Both humoral and cell-mediated responses occur in infected sheep but neither is fully effective and may, in fact, contribute to the pathogenesis of the disease. The period from infection to seroconversion is usually up to 8 weeks but may take several months or years. This delay in antibody response reflects a low level of viral antigen production (Brodie *et al*., 1998).

Clinical signs

Clinical disease develops slowly and is rarely observed in animals less than 2 years of age. It is influenced by the prevalence of infection in the flock and by breed. Concurrent infection with jaagsiekte sheep retrovirus in the flock results in severe disease. Only about 30% of infected animals develop clinical signs. Respiratory distress, which becomes more severe as the disease progresses, is the most common clinical presentation. Affected sheep are usually afebrile until the later stages of the disease when secondary bacterial infection often occurs. Death is due to anoxia or to secondary infection. Indurative mastitis with

reduction in milk production, which results in poor growth of lambs, is a relatively common finding. Lameness and swelling of one or more joints, particularly the carpus and tarsus, have been described in some flocks. Neurological signs, which are relatively rare, may occur in association with other signs of the disease.

Diagnosis

A presumptive clinical diagnosis of maedi/visna can be confirmed serologically and should be conducted on a flock basis. The time required for seroconversion may be long and unpredictable but, once antibody production is initiated, it continues. Commonly used serological assays include AGID, ELISA and western blotting. Virus isolation is possible but time-consuming and expensive. Viral nucleic acid can be detected in peripheral blood and in tissues by PCR but the procedure is not widely used on account of the wide strain variation and the low virus load *in vivo* (de Andres *et al*., 2005).

Control

Eradication of MVV infection requires regular serological monitoring and removal of seropositive animals. Newborn lambs should be removed at birth from infected dams and reared in isolation. Colostrum and milk from certified MVV-free ewes should be used for feeding lambs. An effective vaccine is not available at present.

Caprine arthritis-encephalitis

This lentivirus disease of goats, characterized by polyarthritis in adults and, rarely, by leukoencephalomyelitis in kids, occurs worldwide. It is caused by persistent infection with caprine arthritis-encephalitis virus (CAEV).

Epidemiology

Although infection is common among dairy goats in most countries, clinical disease is uncommon. The virus is usually acquired by kids during the neonatal period through ingestion of colostrum or milk from infected does. Close contact between susceptible and infected goats over an extended period is required for horizontal transmission.

Pathogenesis

The pathogenesis is similar to that of maedi/visna with persistent infection and non-protective immune mechanisms responsible for lesion development. Virus production occurs after infected monocytes develop into macrophages. Viral antigens provoke a vigorous cell-mediated reaction which is responsible for the characteristic changes in target tissues.

Clinical signs

The most common presentation in adult animals is arthritis which is insidious in onset and slowly progressive. Swelling of affected joints, particularly the carpus, occurs but the degree of lameness is variable. Affected animals gradually lose weight. Reduced milk production relates to chronic mastitis. A rapidly progressive neurological disease, which can affect kids up to 4 months of age, manifests as posterior paresis progressing to quadriplegia.

Diagnosis

Laboratory confirmation relies on detection of virus-specific antibodies. The most commonly used assays are AGID and ELISA. Virus isolation can be achieved by co-cultivating leukocytes from blood or milk with synovial membrane cells. Nucleic acid detection by PCR is also possible.

Control

This is based on test and segregation programmes. Infected flocks should be tested regularly and seropositive animals should be culled; kids should be separated from their dams at birth and reared on heat-treated colostrum or pasteurized milk. An effective vaccine is not currently available.

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Chapter 69

Reoviridae

Viruses in the family *Reoviridae* were originally isolated from **r**espiratory and **e**nteric sources without any associated disease, namely **o**rphan. These icosahedral viruses, 60 to 80 nm in diameter, are non-enveloped and possess a layered capsid which is composed of one to three concentric protein shells (Fig. 69.1). The family contains 15 genera, which can be divided into two groups based on their appearance: 'spiked' viruses, so called because they possess turret-shaped projections situated at the 12 icosahedral vertices, and 'smooth' viruses, which have a spherical appearance. It has been proposed to create two subfamilies, *Spinareovirinae* and *Sedoreovirinae*, in recognition of these two morphological forms. The genome of the virion is composed of 10 to 12 segments of double-stranded RNA. Genetic reassortment readily takes place in cells co-infected with viruses of the same species. Replication occurs in the cytoplasm of host cells often with the formation of intracytoplasmic inclusions. Members of the genera *Orthoreovirus* and *Rotavirus* infect animals and humans (Fig. 69.2). Members of the genera *Orbivirus*, *Coltivirus* and *Seadornavirus* infect both arthropod vectors and vertebrate hosts. The genera *Fijivirus*, *Phytoreovirus* and *Oryzavirus* contain viruses of plants that are transmitted by arthropod vectors (leafhoppers). The genera *Idnoreovirus*, *Cypovirus*, *Dinovernavirus* and *Cardoreovirus* contain viruses of arthropods, while members of the genus *Aquareovirus* infect fish. Members of the genus *Mycoreovirus* infect fungi and the genus *Mimoreovirus* contains a virus which infects algae. Viruses in the family are moderately resistant to heat, organic solvents and non-ionic detergents. Orthoreoviruses and rotaviruses are stable over a wide range of pH values unlike orbiviruses which lose infectivity at low pH values. In some genera, the action of proteases such as trypsin on the outer capsid shell is essential for the development of infectivity; the modified virions are termed infectious or intermediate subviral particles (ISVPs).

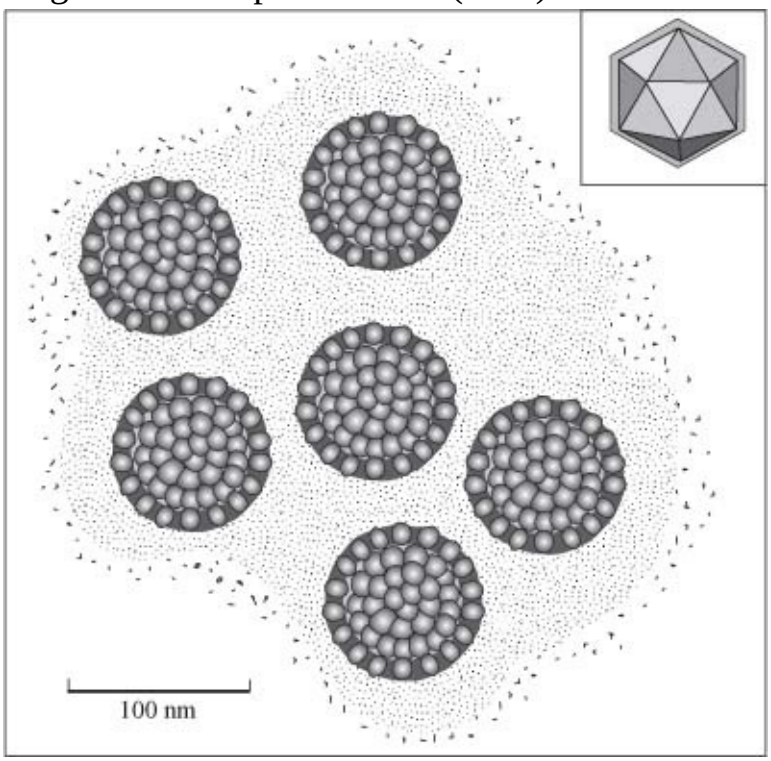
Key points

- Non-enveloped viruses with double or triple-layered capsid and icosahedral structure
- Segmented double-stranded RNA
- Replicate in cytoplasm
- Three genera of veterinary importance, *Orthoreovirus*, *Orbivirus*, *Rotavirus*:
  - – Orthoreoviruses cause arthritis and tenosynovitis in poultry
  - – Rotaviruses cause enteritis in neonatal farm animals
  - – Orbiviruses are arthropod-borne infections that cause African horse sickness in horses and blue-tongue in sheep and in other domestic and wild ruminants

Clinical infections

Orthoreoviruses, which are widespread in nature, have beenm isolated from many animal species (Table 69.1). Mammalian and avian orthoreoviruses possess distinct group antigens. Avian orthoreovi-ruses have been implicated in arthritis, tenosynovitis, chronic respiratory disease and enteritis. Transmission of orthoreoviruses is by the enteric or respiratory routes. Rotaviruses cause acute diarrhoea in young intensively reared farm animals. Transmission of rotaviruses is through contact with contaminated faeces.

Figure 69.1 Rotavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Within the 21 currently recognized serogroups (species) of orbiviruses, there are defined serotypes and, in addition, antigenic complexes. The main serogroup-specific antigen is the immunodominant core protein VP7. Individual serotypes are distinguished by serum neutralization assays utilizing antibodies against outer capsid proteins. African horse sickness and bluetongue are particularly important diseases caused by orbiviruses. Epizootic haemorrhagic disease of deer and Ibaraki disease in cattle, both caused by closely related strains/serotypes of epizootic haemorrhagic disease virus, have clinical effects in these species similar to those of bluetongue in sheep. Infection with equine encephalosis virus has been recognized only in southern Africa and Israel. Serological evidence suggests that although this infection is widespread, acute disease occurs sporadically. African horse sickness, bluetongue, epizootic haemor-rhagic disease and equine encephalosis virus are transmitted by arthropods, especially by *Culicoides* species.

Diseases caused by avian orthoreoviruses

Figure 69.2 Viruses of animal and human importance in the family *Reoviridae*. Viruses in red cause OIE-listed diseases.

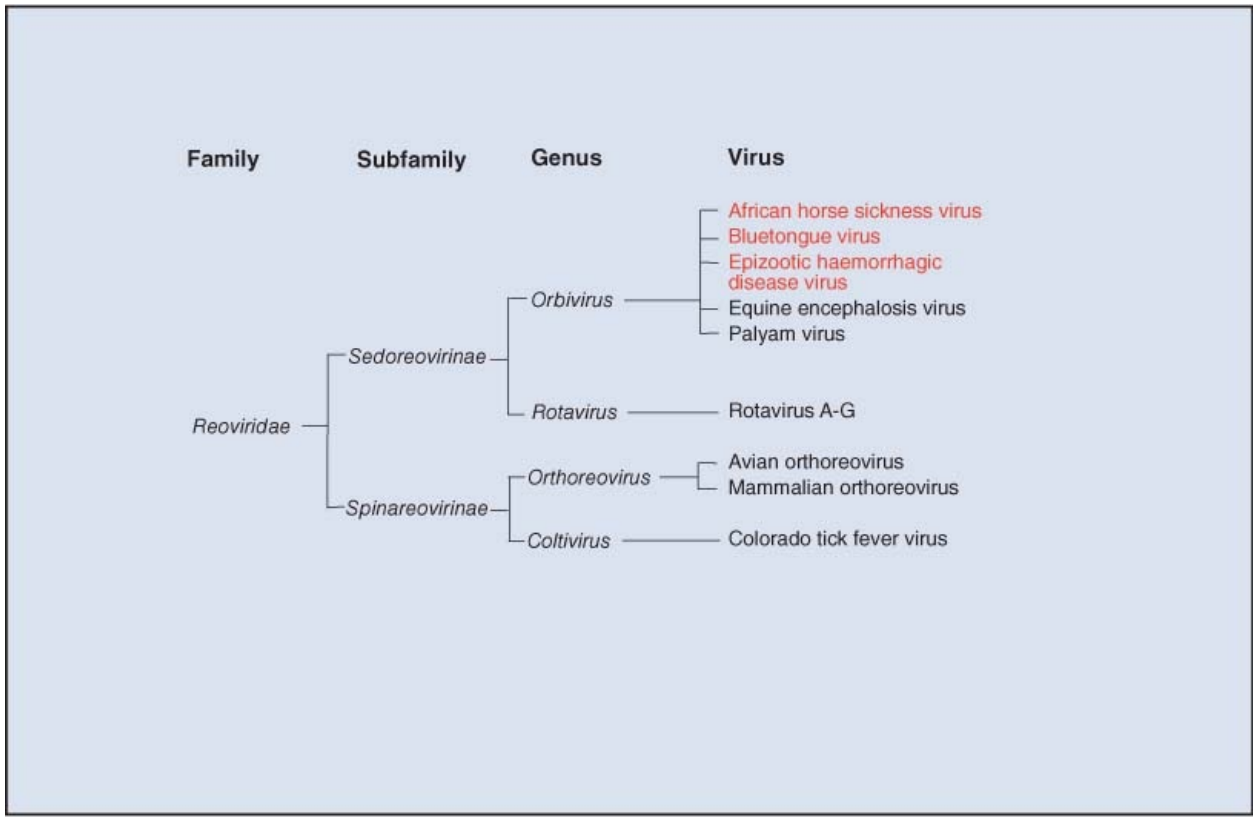


Table 69.1 Viruses of animal and human importance in the family *Reoviridae*.

Genus	Virus	Comments
Orbivirus	African horse sickness virus	Arthropod-borne infection of *Equidae*, principal vector *Culicoides* species. Endemic in Africa. High mortality rate
	Bluetongue virus	Arthropod-borne infection of sheep, cattle, goats and wild ruminants. Principal vector *Culicoides* species. Severe disease in some species of deer. Teratogenic effects. Although clinical disease is uncommon in cattle, serotype 8 can cause acute disease in cattle
	Epizootic haemorrhagic disease virus	Arthropod-borne infection of deer, cattle and buffalo. Principal vector *Culicoides* species. Clinical signs similar to bluetongue. Important disease of deer in North America. Usually causes subclinical infection in cattle. Ten serotypes recognized within the serogroup, including Ibaraki virus which is present in south-east Asia and causes acute disease in cattle
	Equine encephalosis virus	Reported in southern Africa and Israel. Transmitted by *Culicoides* species. Seven serotypes are recognized. Majority of infections subclinical. Sporadic cases of acute fatal disease occur; cerebral oedema, fatty liver and enteritis are prominent features
	Palyam virus	Arthropod-borne disease of cattle. Causes abortion and teratogenic effects. Recorded in southern Africa, south-east Asia and Australia. Multiple serotypes of the virus occur
Rotavirus	Rotavirus A, B, C, D, E, F, G	Mild to severe diarrhoea in intensively reared neonatal animals and chicks; severity of infection influenced by virulence of infecting strain, age, availability of colostrum and management factors
Orthoreovirus	Avian orthoreoviruses	Important cause of viral arthritis/tenosynovitis in chickens. Multiple serotypes described. Turkeys and other avian species susceptible
	Mammalian orthoreoviruses	Associated with mild enteric and respiratory disease in many species, severity dependent on secondary infections. Four serotypes recognized
Coltivirus	Colorado tick fever virus	Rodent species act as reservoirs. Arthropod - borne, mainly by ticks and also mosquitoes. Primarily of significance in humans; may cause encephalitis in children

Diseases c aused by a vian o rthoreoviruses

Infections caused by avian orthoreoviruses are usually inapparent. In certain circumstances, however, they may either cause primary disease or contribute to the severity of mixed infections. Using serum neutralization tests, at least nine serotypes are recognized. Although transmission is mainly by the faecal–oral route, transfer to developing chicks *in ovo* can occur. Arthritis/tenosynovitis, caused by orthoreoviruses in chickens between 4 and 16 weeks of age, has been reported worldwide. Lameness is a prominent feature of this disease and rupture of the tendon of the gastrocnemius muscle may occur. Affected birds have limited mobility and may die of starvation. Morbidity is usually less than 10%. The synovial lesions resemble those caused by infection with *Mycoplasma synoviae* or with *Staphylococcus aureus*. Orthoreovirus involvement can be confirmed by virus isolation. Specimens suitable for virus isolation are affected articular cartilage and tendon sheaths. Synovial fluid is not a reliable source for virus isolation. Suspensions of macerated tissues are inoculated into the yolk sac of embryonated eggs or on to monolayers of chick embryo liver cells. Avian orthoreoviruses induce syn-cytium formation. Viral antigen may be detectable by immunofluorescence in cryostat sections of tissues. RT-PCR protocols are available for the detection and differentiation of avian orthoreoviruses (Caterina

et al., 2004; Liu *et al*., 2004). Although serological testing is not particularly useful because of the high prevalence of subclinical infections, it may be employed to determine the immune status of a flock. Both inactivated and modified live vaccines have been used in parent flocks to stimulate high levels of maternally-derived antibody in chicks. However, vaccines may induce protection only against homologous serotypes (Meanger *et al*., 1997). Control measures include total depopulation at the end of a production cycle followed by thorough cleansing and disinfection of premises.

Enteric disease caused by rotaviruses in young animals

Rotaviruses cause diarrhoea in intensively-reared young farm animals worldwide. Isolates are divided into seven antigenically distinct serogroups (A to G), also termed species, based on reactions with the major capsid protein, VP6. The host range varies depending on the viral species. Rotavirus E has been isolated only from pigs to date, while rotavirus D, F and G are associated only with avian species. Most isolates belong to serogroup A. Fifteen serotypes (G1–G15) are recognized within serogroup A on the basis of the antigenicity of VP7, an outer capsid glycoprotein, which is highly immunogenic and induces type-specific neutralizing antibodies. Field infections with rotaviruses are considered to be species-specific. However, virus isolates from one species can be transmitted experimentally to other species. Human infection with animal rotaviruses appears to be uncommon (Cook *et al*., 2004).

Epidemiology

High titres of virus (10^{9} virus particles per gram of faeces) are excreted by clinically affected animals. Horizontal transmission occurs following ingestion of contaminated feed. Because the virus is stable in the environment, premises may be heavily contaminated and intensively-reared animals are those most often affected. Buildings can remain contaminated for long periods if thorough cleansing and disinfection procedures are not implemented.

Pathogenesis

The severity of infection is largely determined by the virulence of the infecting viral strain, the amount of virus ingested and the level of maternally-derived

immunity. Other factors that influence the outcome of infection include age of the animal at the time of exposure, overcrowding and the presence of other enteric pathogens. Rotavirus diarrhoea has malabsorption and secretory components (Ramig, 2004). The virus, which can survive gastric acidity, passes through the stomach and infects enterocytes at the tips of villi in the small intestine. For many years a crypt-cell invasion hypothesis has been accepted as the explanation for the diarrhoea associated with rotavirus infection in young animals. Infection is thought to result in destruction of mature enterocytes located on the upper two-thirds of the villi of the small intestine. Because the rate of enterocyte replacement is relatively slow in young animals, affected villi become stunted and covered by cuboidal cells. These immature replacement cells have reduced levels of disaccharidases and defective glucose-coupled sodium transport. Undigested lactose provides an ideal substrate for bacterial proliferation in the intestinal lumen. In addition, it exerts an osmotic effect which results in the retention of fluid in the lumen and, along with impaired fluid absorption, contributes to the development of a malabsorption type of diarrhoea. However, the correlation between histological lesions and disease signs is not absolute; it is possible for diarrhoea to occur in the absence of visible tissue damage. The non-structural protein NSP4 has been shown to function as an entero-toxin, specifically inhibiting the Na^{+}–D-glucose symport activity of the sodium-glucose linked transporter protein, SGLT1 (Lorrot and Vasseur, 2007).

Clinical signs

The incubation period is short, usually less than 24 hours. Affected animals are anorexic and depressed, and produce light-coloured, semi-liquid or pasty faeces. In uncomplicated cases, animals frequently recover within 4 days without treatment. Concurrent infection with other enteric pathogens such as *Escherichia coli*, *Salmonella* species and *Cryptosporidium* species may add to the severity of the diarrhoea and deaths may occur.

Diagnosis

- Specimens suitable for laboratory examination include faeces and intestinal contents.
- Although negative-contrast electron microscopy is rapid, large numbers of virus particles (10^6 per gram of faeces) must be present for reliable confirmation. Immunoelectron microscopy increases the sensitivity of the

procedure. Mixed viral infections can be detected by negative-contrast electron microscopy.

- Viral antigen can be demonstrated in faeces by ELISA and latex agglutination. The antiserum employed in these tests is usually specific for sero-group A rotaviruses. Reagents for these assays are commercially available. Immunofluorescence can be used to detect viral antigen in smears or in cryostat sections of affected small intestines.
- Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) has been used successfully to demonstrate RNA segments of rotaviruses in clinical samples. The sensitivity of this procedure is comparable with electron microscopy. The electrophoretic patterns permit differentiation of rotavirus serogroups.
- Rotaviruses are difficult to isolate in tissue culture from clinical samples. The addition of low concentrations of trypsin to the growth medium facilitates viral uncoating and improves viral replication.

Treatment

Oral electrolyte solutions may be beneficial in some cases. Intravenous fluid replacement and antibiotic administration are required in severe cases complicated by bacterial infection.

Control

Measures aimed at reducing the levels of virus challenge in young animals are essential. These must be combined with management procedures which ensure that neonatal animals receive adequate amounts of colostrum. Local immunity is more important than circulating antibody; ingestion of colostrum provides protective antibodies in the intestinal lumen. Vaccination of pregnant dams enhances antibody levels in mammary secretions. Oral vaccination of newborn animals using modified live vaccine is of questionable value. A modified live pentavalent vaccine has been developed by reassorting a bovine rotavirus isolate (strain WC3) with five human rotavirus A serotypes and appears promising in human infants. Stressful environmental conditions should be minimized.

African horse sickness

This is a non-contagious disease of horses, mules and donkeys caused by

African horse sickness virus (AHSV). Nine serotypes of this orbivirus, which can be distinguished by neutralization tests, constitute the African horse sickness serogroup. The disease is endemic in subtropical and tropical Africa, where the zebra is considered to be the natural vertebrate host and reservoir of the virus. Although serious outbreaks of AHSV serotype 9 have occurred in the Middle East, India and Pakistan, the disease has not persisted in these regions. Outbreaks of serotype 4 have been recorded in Spain, Portugal and Morocco in recent years. African horse sickness is classified as a listed disease by the Office International des Epizooties.

Epidemiology

The virus is transmitted by haematophagous insects. The principal biological vector is *Culicoides imicola*, a species of Afro-Asian midge, which remains infected for life. This midge prefers a warm climate; it aestivates at temperatures below 10°C, while replication of the virus in the midge ceases at temperatures below 15°C (Mellor *et al*., 1998). With increased temperatures associated with climate change in recent years, the distribution of *Culicoides imicola* has extended northwards into southern Europe as far as latitude 46°N. Endemic disease occurs only in regions where *C. imicola* is constantly present. Epidemics of African horse sickness occur periodically outside these regions following climatic conditions which allow windborne transfer of infected midges for up to 700 km. Outbreaks of the disease are seasonal, usually occurring in late summer. The virus may be isolated from clinically normal maintenance hosts such as the zebra and African donkey. Although dogs are susceptible to infection following ingestion of infected horsemeat, they do not appear to have any role in the epidemiology of the disease.

Pathogenesis and pathology

The primary sites of viral replication are believed to be regional lymph nodes, spleen and lungs. Viraemia persists throughout the febrile period, typically 4 to 8 days. In zebras and donkeys, viraemia may last up to 4 weeks. Endothelial cells are important sites of secondary viral replication, resulting in increased vascular permeability, oedema, haemorrhage and intravascular coagulation. Post-mortem findings include diffuse pulmonary oedema, hydrothorax, ascites and hydropericardium.

Clinical signs

The incubation period is usually less than 9 days. Four forms of this febrile disease, all of which can occur in a particular outbreak, are recognized. A peracute pulmonary form is characterized by depression and nasal discharge with rapid progression to severe respiratory distress. Mortality rate may approach 100%. A subacute cardiac form manifests as conjunctivitis, abdominal pain and progressive dyspnoea. Subcutaneous oedematous swellings of the head and neck are most obvious in the supraorbital fossae, palpebral conjunctiva and intermandibular space. Colic is often a feature of this form of the disease, which has a mortality rate of up to 70%. A third form of African horse sickness, the mixed form, presents with both cardiac and pulmonary features. A mild or subclinical form, termed horse sickness fever, is the only form observed in zebras and African donkeys.

Diagnosis

- Characteristic clinical signs, such as oedema of the supraorbital fossae, may allow a clinical diagnosis. Post-mortem findings, including pericardial and pleural effusions, are consistent with a diagnosis of African horse sickness.
- Suitable samples for laboratory examination include blood, lymph node and spleen. Inoculation of cell cultures may be used to demonstrate the presence of virus. Intracerebral inoculation of newborn mice may also be used for this purpose. Virus can be identified by immunofluorescence and typed using virus neutralization with monovalent antiserum or competitive ELISA.
- Viral antigen can be detected in samples by sandwich ELISA.
- Viral RNA can be detected by RT-PCR (Zientara *et al*., 1998). This test may provide results within 24 hours and can be used to distinguish the nine serotypes (Sailleau *et al*., 2000).
- Suitable serological methods include ELISA and serum neutralization tests. In acute disease, infected animals may die before antibodies are produced. Seroconversion in donkeys, used as sentinel animals outside endemic areas, confirms the presence of the disease.

Control

Vector control, quarantine of affected animals and vaccination are the main

methods for preventing outbreaks of disease. Insect vector control includes the use of repellents and insecticides, the elimination of insect breeding areas and housing of animals in insect-proof buildings at dawn and at dusk when insect activity is highest. Attenuated vaccines, both monovalent and polyvalent containing up to four serotypes, are available. However, these vaccines fail to prevent viraemia and may cause teratogenic effects in pregnant mares. Moreover, the vaccine virus may revert to a virulent state and be transmitted by vectors. In addition, vaccinated animals cannot be differentiated serologically from those with field infections. Inactivated vaccines, based on serotype 4, are effective in preventing clinical disease. A polyvalent vaccine must be used if there is a risk of exposure to different serotypes. Protective immune responses may be generated using recombinant-expressed structural proteins as subunit vaccines (Roy and Sutton, 1998). Such vaccines should be safe and permit differentiation of vaccinated from infected animals. Adequate bio-containment facilities are mandatory for vaccine production.

Bluetongue

This non-contagious viral disease of sheep and other domestic and wild ruminants is transmitted by biting insects, principally *Culicoides* species. Isolates of the causal agent, bluetongue virus (BTV), belong to a distinct serogroup in the *Orbivirus* genus. Twenty-five serotypes of BTV have now been described; the most recent serotype, Toggenburg orbivirus, was isolated from goats in Switzerland. Bluetongue (BT) is of greatest significance in sheep and deer. The severity of the disease is influenced by the serotype of the virus, the breed of sheep and prevailing environmental conditions. Bluetongue is classified as a listed disease by the Office International des Epizooties.

Epidemiology

Bluetongue is widely distributed between latitudes 53°N and 40°S, reflecting the distribution of *Culicoides* species. *Culicoides imicola* is the principal vector in Africa, the Mediterranean Basin and the Middle East. In Australia, *C. fulvus*, *C. wadai* and *C. brevitarsis* are involved in transmission. Other *Culicoides* species of importance in transmission are *C. varipennis* var. *sono-rensis* in North America and *C. insignes* in South America. Since 2006, BTV-8 has caused a severe epizootic in Northern Europe. Indigenous European *Culicoides* species including *C. dewulfi* and *C. obsoletus* complex appear to be capable of

maintaining the epizootic from one vector season to the next (Saegerman *et al*., 2008). Clinical disease in cattle has been a feature of this epizootic with clinical signs similar to but generally milder than those observed in sheep. Female midges feeding on viraemic animals become infected and virus replicates in their tissues. *Culicoides* species can transmit virus in saliva within 7 to 10 days and they remain infected for life. Temperatures of 18°C to 29°C along with high humidity favour insect activity, while temperatures greater than 12°C are required for virus replication within the vector. These factors account for the seasonal occurrence of the disease in many parts of the world. *Culicoides* species are most active at dawn and dusk. In localized areas within endemic regions, there may be an increased frequency of BT outbreaks. These areas are particularly suitable for the breeding of *Culicoides* species because of the accumulation of animal faeces in marshland. Extension of disease to contiguous areas occurs through the movement of viraemic animals or insect vectors. Although the flight range of *Culicoides* species is limited, they may be transported over long distances by wind movement resulting in BT outbreaks in susceptible ruminant populations outside endemic regions. Such events may precipitate epidemics which are usually self-limiting unless the climate is suitable for vector activity throughout the year. It is considered that four potential routes may operate for the virus to overwinter in ruminant populations, permitting viral recrudescence in spring: vertical transmission in ruminants (dam to offspring), prolonged subclinical virae-mia in certain infected individual animals, vertical transmission in the vector with survival of infected offspring through the winter, and survival of infected adult midges (Wilson *et al*., 2008).

In endemic areas, infection of cattle is common and usually inapparent. The viraemia in cattle commonly lasts several weeks facilitating acquisition of virus by insect vectors. Consequently, cattle are considered to be important reservoirs of virus (Barratt-Boyes and MacLachlan, 1995). During the viraemic phase, virus can be detected in the semen of a proportion of rams and bulls. Venereal and transplacental transfers of BTV infection are generally considered to be uncommon but the strain of BTV-8 circulating in northern Europe appears to be capable of vertical transmission in the ruminant population (Menzies *et al*., 2008; Wilson *et al*., 2008). Embryos collected from infected ewes may transmit infection to recipient ewes but this can be prevented by washing the embryos extensively (Singh *et al*., 1997).

Pathogenesis and pathology

After experimental infection, the virus replicates initially in regional lymph nodes. It is then carried in blood or lymph to other lymphoid tissues, lungs and spleen where further replication takes place. Virus localizes and multiplies in the endothelium of small blood vessels producing vascular damage with stasis, exudation and tissue hypoxia. The initiation and development of surface lesions in areas of tissue hypoxia relate to minor trauma and may be complicated by secondary bacterial infection. Lesions are particularly evident in the oral cavity, around the mouth and on the coronet of the hoof. In the bloodstream, the virus is highly cell-associated, particularly with erythrocytes. It has been suggested that this may protect virus from antibody and allow the virus to persist. Infectious virus can be detected for 35 to 60 days after infection. Sporadic cases of clinical disease in cattle are thought to involve type I hypersensitivity reactions with participation of IgE as a result of previous exposure to BTV or related orbiviruses.

Clinical signs

The clinical presentation, which is highly variable, ranges from subclinical infection to severe disease with high mortality. Severe disease is generally confined to Merinos and European mutton breeds. Nutritional status, exposure to sunlight and age also appear to influence the severity of lesions. The incubation period in sheep is up to 10 days. Affected animals are febrile and depressed with vascular congestion of the lips and muzzle. Oedema of the lips, face, eyelids and ears develops. Erosions and ulcers are evident on the oral mucosa. There is excessive salivation and a watery discharge that subsequently becomes mucopurulent and dries forming crusts around the nares. The tongue may be swollen and cyanotic. Lameness may result from coronitis and laminitis. Some animals develop torticollis. Abortion may occur and lambs may be weak or deformed at birth. Mortality rate may be up to 30% and, in some outbreaks, may be higher. Animals recovering from infection may lose part of their fleece some weeks later. Clinical cases in susceptible cattle are characterized by fever, stiffness, ulceration of the oral mucosa, ‘burnt muzzle’ and dermatitis. Cattle infected during pregnancy may abort or give birth to malformed calves.

Diagnosis

A presumptive diagnosis of BT may be based on clinical findings and post-mortem lesions. Diagnostic methods include identification of the virus or

demonstration of BTV-specific antibodies in non-vaccinated animals.

- Samples suitable for virus isolation include unclotted blood from febrile animals or fresh spleen and lymph node collected at post-mortem. Virus may be isolated by intravenous inoculation of embryonated eggs or in cell culture.
- A highly sensitive nested RT-PCR has been developed for detecting BTV nucleic acid in clinical samples (Aradaib *et al*., 2003). Molecular techniques can detect BTV nucleic acid in blood for at least 30 days beyond the period when virus can be isolated. Protocols for quantifying viral load (Shaw *et al*., 2007; Toussaint *et al*., 2007) and differentiating viral strains (Mertens *et al*., 2007) are available.
- Antigen detection ELISA systems have also been described (Stanislawek *et al*., 1996; Hamblin *et al*., 1998).
- Serological tests for the detection of antibodies to the BTV serogroup include CFT, indirect immunofluorescence and competitive ELISA. Neutralization assays are used for demonstrating type-specific antibodies. In animals from endemic regions, a rising antibody titre must be demonstrated using paired serum samples.

Control

As bluetongue is an OIE-listed disease, it is subject to international regulations controlling trade. The discovery of a number of serotypes of BTV in the Northern Territory of Australia resulted in disruption of trade in animals, semen and embryos although clinical disease was not present (Muller, 1995). Populations of insect vectors may be reduced by the use of larvicides at breeding sites. Insecticides applied to susceptible animals may temporarily halt feeding by vectors. Live attenuated vaccines have been used successfully for many years and provide protection against virulent virus of homologous serotype. Polyvalent vaccines are essential in regions where a number of serotypes are present. Attenuated vaccines may produce viraemia and may be teratogenic when used in ewes during the first half of gestation. They should not be used during periods of vector activity because of the risk of transferring vaccinal virus to pregnant ewes and the possibility of genetic reassortment with field virus and reversion to virulence (Osburn *et al*., 1996). Killed adjuvanted vaccines can induce protection but are more expensive to produce and require two inoculations. Recombinant virus-like particles, capable of inducing protective immunity, have

been produced in insect cells infected with recombinant baculoviruses expressing BTV proteins. However, vaccines produced by this method are not yet available commercially.

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Chapter 70

Birnaviridae

Birnaviruses are so named because their genomes contain two segments (A, B) of linear, double-stranded RNA. Genetic reassortment has been described. The icosahedral virions are about 60 nm in diameter (Fig. 70.1). Five polypeptides, designated VP1, VP2, VP3, VP4 and VP5, have been identified. The major capsid protein (VP2) contains epitopes which induce neutralizing antibodies. Replication occurs in the cytoplasm of host cells and involves a virion-associated RNA-dependent RNA polymerase. The family *Birnaviridae* contains four genera: *Avibirnavirus* and *Entomo-birnavirus*, which infect chickens and insects respectively, while members of *Aquabirnavirus* and *Blosnavirus* infect fish. Virions, which are non-enveloped, are stable over a wide pH range and at a temperature of 60°C for 1 hour. They are resistant to treatment with ether and chloroform.

Clinical infections

Two economically important diseases associated with birnaviruses are infectious bursal disease of chickens and infectious pancreatic necrosis of salmonids. These diseases occur worldwide and cause considerable losses in poultry units and in farmed salmon.

Infectious bursal disease

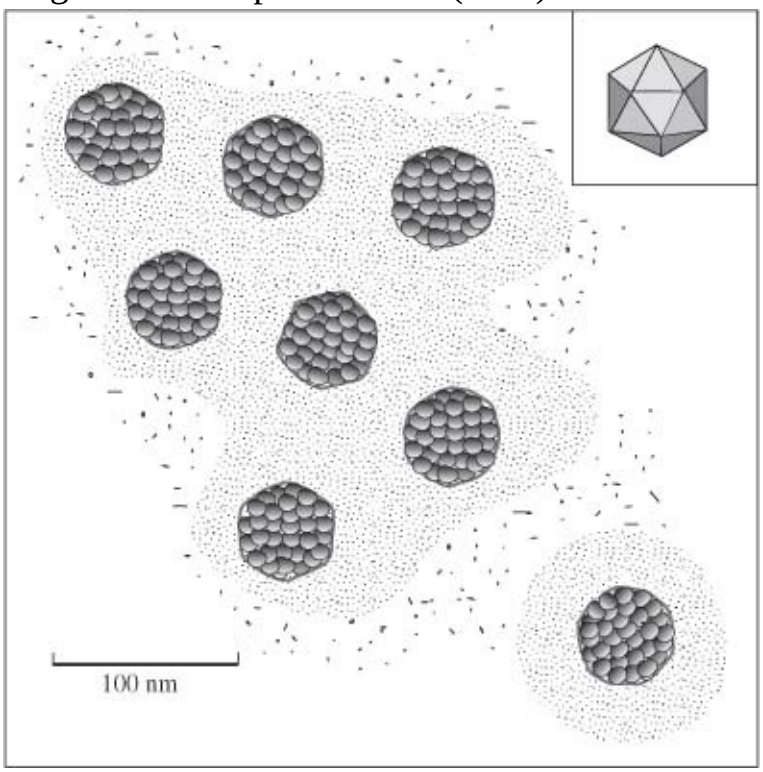
This condition is a highly contagious disease of young chickens which is caused by infectious bursal disease virus (IBDV). The causal agent was first isolated in Gumboro, Delaware, and the disease was originally known as Gumboro disease. Although turkeys and ducks are susceptible to infection, clinical disease occurs only in chickens. Based on neutralization tests, isolates of IBDV are assigned to two serotypes. There is considerable variation in the virulence and antigenicity of serotype 1 isolates. Both ‘very virulent’ (vv) strains and antigenic variants have emerged in vaccinated flocks. ‘Very virulent’ strains were first reported in

Europe and Asia in the late 1980s. These strains, although antigenically similar to classical serotype 1 strains, can cause disease even when maternally-derived antibody against the classical vaccine strains is present. Serotype 2 isolates are not associated with clinical disease. A variety of antigenic variants are recognized within each serotype. In the United States in recent years, variant serotype 1 isolates have been detected in flocks that had been vaccinated with classical strain vaccines. These antigenic variants, which are highly immunosuppressive in young chicks, cause rapid bursal atrophy.

Key points

- Non-enveloped, double-stranded RNA viruses with icosahedral symmetry
- Replicate in cytoplasm
- Stable in the environment
- The family contains two genera of veterinary importance:
  - –*Avibirnavirus* contains viruses which cause infectious bursal disease in chickens
  - –*Aquabirnavirus* contains viruses which cause infectious pancreatic necrosis in salmonids

Figure 70.1 Birnavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Epidemiology

Infection, which is usually acquired by the oral route, occurs when maternally-derived antibody levels are waning at 2 to 3 weeks of age. Virus is shed in the faeces for up to 2 weeks after infection and can remain infectious in the environment of a poultry house for several months. Spread to other poultry units can occur by fomites. Neither a carrier state nor vertical transmission has been demonstrated.

Pathogenesis

Within hours of ingestion, virus can be detected in macrophages and lymphoid cells in the caeca, duodenum and jejunum. Virus reaches the liver via the portal circulation and infects Kupffer cells. Infection spreads to the bursa of Fabricius where rapid replication results in a pronounced secondary viraemia and dissemination to other tissues. The main target cells are B lymphocytes and their precursors in the bursa. The major antigenic protein, VP2, has been shown to induce apoptosis in infected cells. Depletion of B lymphocytes in early life results in impaired humoral immune responses, lowered resistance to infectious diseases and ineffective responses to vaccines. In chickens older than 3 weeks of age, viral infection only marginally affects immune competence because many B lymphocytes are distributed throughout the tissues before bursal damage occurs.

Clinical signs

The severity of clinical signs is influenced by the virulence of the virus, the age of chicks at the time of infection, the breed of the chicks and the level of maternally-derived antibody. Chicks develop an acute form of the disease, usually between 3 and 6 weeks of age, following a short incubation period. Affected birds are depressed and inappetent and show evidence of diarrhoea and vent pecking. Morbidity ranges from 10% to 100% with a mortality rate up to 20% or, occasionally, higher. The course of the disease is short with surviving birds recovering in about 4 days. Many outbreaks are mild, detectable only by impaired weight gains. Although infections before 3 weeks of age are usually subclinical, severe depression of antibody responses may result. In general, the earlier in life infection occurs, the more pronounced the immunosuppression. Clinical signs in these birds are usually vague. Suboptimal growth, predisposition to secondary infections and poor response to vaccination may be encountered.

Diagnosis

- In acute disease, clinical signs and a swollen oedematous bursa at post-mortem are often sufficient for diagnosis. Confirmation and identification of subclinical infection require laboratory tests.
- Viral antigen can be detected in smears or frozen sections of the bursa using immunofluorescence. Macerated bursal tissue is suitable for detection of viral antigen by ELISA or by gel diffusion tests. Antigen capture ELISA with suitable monoclonal antibodies is capable of differentiating the very virulent IBDV phenotype from less pathogenic types.
- Specimens of bursa, spleen or faeces are suitable for virus isolation. Most strains grow on the chorioal-lantoic membrane of embryonated eggs. Classical and variant serotype 1 isolates can be differentiated using the virus neutralization assay.
- Reverse transcriptase-PCR can be used for the diagnosis of IBD, utilizing primers directed against the *VP2* gene or the *VP1* gene. Restriction enzyme analysis and nucleotide sequencing of PCR products are widely used for the characterization of IBDV strains, particularly for the identification of vvIBDV. Real -time RT - PCR protocols are available.
- Birds that have recovered from the acute disease develop high antibody titres as mature peripheral B lymphocytes are largely unaffected. Suitable serological assays include ELISA and virus neutralization.

Control

Depopulation, thorough cleaning and effective disinfection programmes are required following an outbreak of disease in a unit. Most commercial units rely on vaccination for control. Both modified live and inactivated serotype 1 vaccines are available. Live vaccines can be administered by aerosol or in drinking water. Birds selected for breeding are usually vaccinated at 4 to 10 weeks of age with a live vaccine and again close to laying, with an inactivated oil-adjuvanted vaccine, to ensure high levels of maternally-derived antibody in chicks. Vaccines used in parent stock should contain both classical and variant strains of IBDV. Chicks can be actively immunized after maternally-derived antibody levels decline at about 4 weeks of age. In high-risk flocks, vaccination may begin at 1 day of age to protect birds with little or no maternally-derived antibody, followed by booster inoculations at 2 and 3 weeks of age. Partially attenuated vaccines termed ‘intermediate’ and ‘intermediate plus’ (‘hot’) are

generally used in this way in broilers and commercial layer replacements as they are capable of overcoming low levels of maternally-derived antibody. Recent vaccine developments include *in vitro* expression of *VP2* gene and several recombinant vaccines using suitable vectors such as turkey herpesvirus, fowl adenovirus and fowlpox virus.

Further reading

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Chapter 71

Orthomyxoviridae

The family *Orthomyxoviridae* (Greek *orthos*, proper, and *myxa*, mucus) contains those viruses that cause influenza in humans and animals. Orthomyxoviruses are spherical or pleomorphic, enveloped viruses, 80 to 120 nm in diameter (Fig. 71.1), but long filamentous forms also occur. The envelope, which is derived from host cell membrane lipids, contains glycosylated and non-glycosylated viral proteins. Surface projections of glycoproteins form 'spikes' or peplomers which, in influenza A and B viruses, are of two types: a haemagglutinin (H) responsible for virus attachment and envelope fusion, and a neuraminidase (N) capable of cleaving viral receptors, degrading mucus and promoting both entry of virus into cells and release of virions from infected cells.

Influenza viruses haemagglutinate erythrocytes from a wide range of species. Antibodies to the H and N glycoproteins are mainly responsible for virus neutralization. The nucleocapsid has a helical symmetry, and the genome, which is composed of six to eight segments, consists of linear, negative-sense, single-stranded RNA. Replication occurs in cell nuclei with release of virions by budding from plasma membranes. Virions are labile in the environment and are sensitive to heat, changes in pH, lipid solvents, detergents, irradiation and oxidizing agents.

The family contains five genera, namely *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus* and *Isavirus*. Influenza B and C viruses are primarily pathogens of humans, although occasional infections of pigs with influenza C virus have been described and influenza B virus has been isolated from seals.

Key points

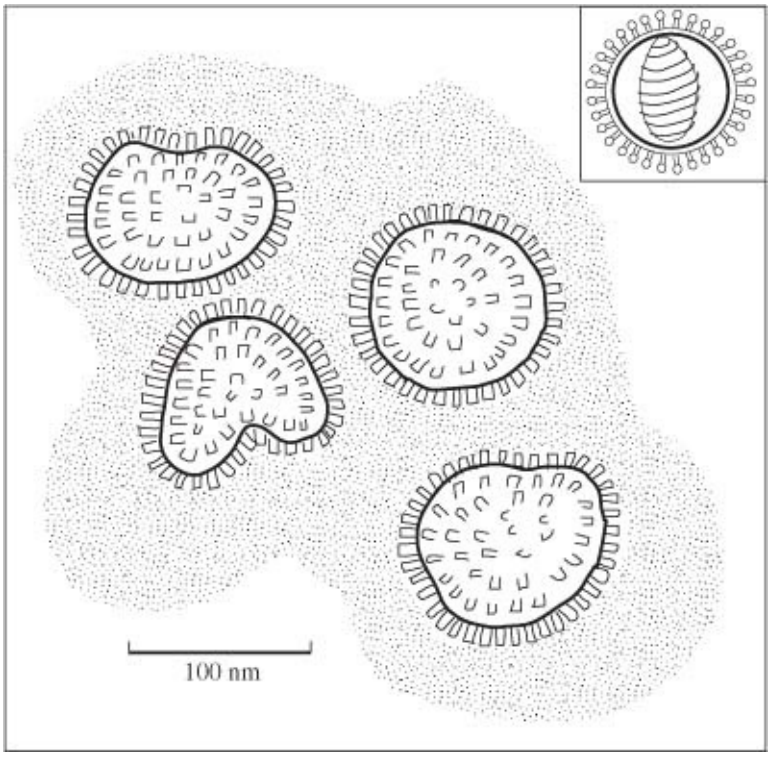
- Enveloped viruses with helical nucleocapsids and spherical or pleomorphic morphology
- Linear, negative-sense, single-stranded RNA
- Replication occurs in the nucleus
- Two important glycoproteins, one a haemagglutinin which binds to cell receptors and

the other with neuraminidase activity, are present in the envelope
- Genome is segmented, facilitating genetic reassortment
- Subtypes of influenza A virus are important human, avian, porcine and equine pathogens

Thogotovirus and Dhori virus are tick-borne arboviruses isolated from camels, cattle and humans in parts of Africa, Europe and Asia. Infectious salmon anaemia virus is an important cause of disease in Atlantic salmon. Influenza A virus, the most important member of the family, is a significant pathogen of birds and mammals.

Isolates of influenza A virus are grouped into subtypes on the basis of their H and N antigens. Currently, 16 H antigens and nine N antigens are recognized (Fouchier *et al*., 2005). New subtypes of influenza A virus emerge periodically as a result of point mutation and genetic reassortment. Point mutations give rise to antigenic drift in which variation occurs within a subtype. Genetic reassortment, a more complex process producing antigenic shift, results in the development of new subtypes. To assess the risk posed by the emergence of new variant viruses, a precise international standard classification of isolates has been adopted by the World Health Organization. This system is based on the influenza virus type, host, geographical origin, strain number, year of isolation and subtype. An example of this classification system, influenza virus A/equine/Prague/1/56 (H7N7), indicates that this virus was isolated from a horse in Prague during 1956. Antigenic subtypes of influenza A virus which cause disease in humans and animals are presented in Table 71.1.

Figure 71.1 Influenza A virus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Clinical infections

Influenza A viruses cause significant infections in humans, pigs, horses and birds. Antibodies to influenza A virus have been detected in cattle in association with respiratory disease, but their significance is unclear (Brown *et al*., 1998). Aquatic birds, particularly ducks which are reservoirs of influenza A virus, provide a genetic pool for the generation of the new subtypes capable of infecting mammals. Migratory waterfowl disseminate the virus across international borders. Although isolates of influenza A virus are usually species specific, there are well-documented instances of transfer between species. The viruses replicate in the intestinal tract of birds and the faecal-oral route is considered the main route of transmission. Human influenza pandemics have been attributed to the close association of concentrated human populations with domestic fowl and pigs. Genetic reassortment in these animal populations can lead to the emergence of virulent influenza virus subtypes which are capable of infecting humans, thereby initiating pandemics (Fig. 71.2). Influenza viruses bind to sialic acid–galactose disaccharides on the cell surface. Sialic acid can be linked at positions 3 or 6 to the backbone of galactose. In humans, α-2,6-linkages predominate whereas α-2,3-linkages are the most common molecular

arrangements in avian species. Avian influenza viruses bind preferentially to α-2,3-linkages and accordingly have a low affinity for human ciliated epithelium. Because pigs express both types of linkages, human and avian influenza subtypes can replicate readily in porcine cells and thus pigs are considered to be the species in which reassortment (‘mixing vessel’ hypothesis) is most likely to occur (Shortridge, 1997). Due to the fact that the genome of influenza A virus is segmented, mixed infections can give rise to genetic reassortment with the emergence of new subtypes. Such novel subtypes have been implicated in most major pandemics which have occurred to date. As there is limited immunity to the new subtypes in the human population, spread from country to country tends to occur rapidly. It is estimated that the pandemic of ‘Spanish flu’ in 1918 had a mortality rate of 2–20% and was responsible for more than 40 million human deaths worldwide. There is evidence to suggest that the cause of the ‘Spanish flu’ was an avian H1N1 influenza virus (Taubenberger *et al*., 2005) that became adapted to humans and swine. In contrast, both the 1957 H2N2 ‘Asian flu’ and the 1968 H3N2 ‘Hong Kong flu’ human pandemics were the result of reassortant viruses that contained both avian and human influenza virus genes. In 2009, a new pandemic strain of H1N1 influenza A began circulating in humans (Zimmer and Burke, 2009). It initially appeared in Mexico and rapidly spread to other parts of the world. Sequencing studies indicated similarities with the sequences (six gene segments) of North American triple-reassortant swine influenza isolates (H1N2 and H3N2), so called because they contain genes from avian, human and swine influenza viruses. In addition, two of the gene segments (neuraminidase and matrix protein) of this new virus were similar to European swine influenza A virus strains from 1992 (Babakir-Mina *et al*., 2009). Pigs were considered to be the likely source of this virus and were shown to be susceptible to infection but circulation has occurred primarily in the human population.

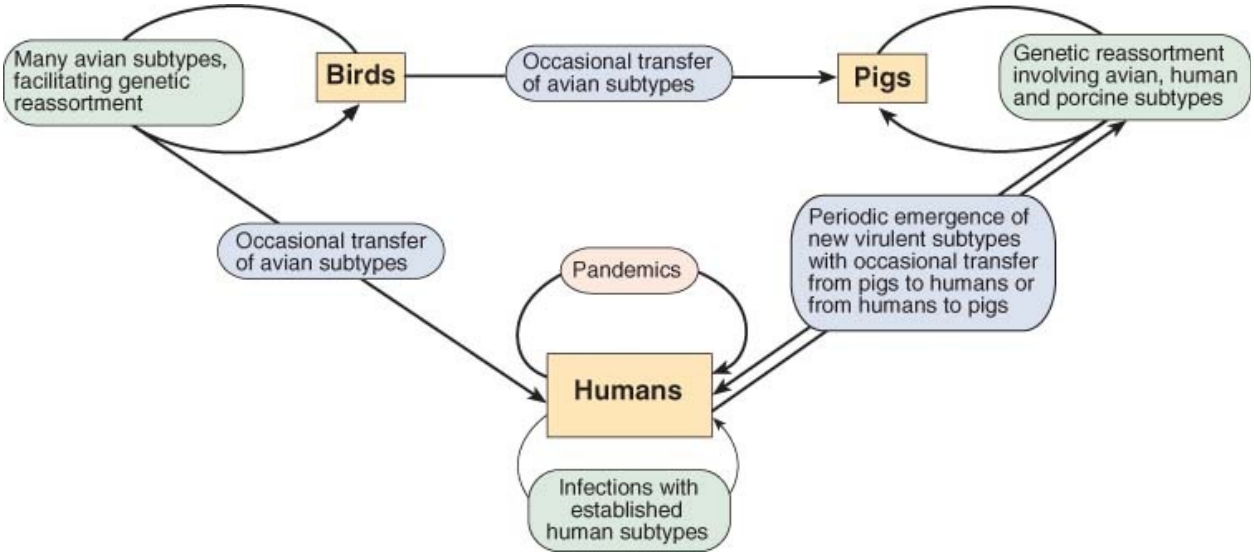
Table 71.1 Antigenic subtypes of influenza A virus isolated from humans and animals.

Hosts	Antigenic subtypes	Comments
Humans	H2N8 (1890)[a]	Human influenza pandemics occur at 10 to 40-year intervals and affect
	H3N8 (1900)	20% to 40% of the world population, causing significant mortality.
	H1N1 (1918)	Usually each new pandemic strain replaces the previous circulating virus.
	H2N2 (1957)	However, in 1977 the H1N1 strain re-emerged, probably due to accidental

	H3N2 (1967)	release from a laboratory source, and co-circulated with the H3N2 strain
	H1N1 (2009)	
Birds	Many antigenic subtypes represented by different combinations of haemagglutinin (H) and neuraminidase (N) peplomers have been recognized	Disease is usually associated with subtypes expressing H5 or H7. Wild birds, especially migrating ducks, act as carriers
Pigs	Predominantly H1N1, H1N2 and H3N2	Severity of disease is determined by the antigenic subtype
Horses	H7N7 and H3N8 (H3N8 has replaced	Wide geographical distribution, but absent from Australia (outbreak in
	H7N7 as the predominant subtype)	2007), New Zealand and Iceland

a, Year of recognition.

Figure 71.2 The circulation of subtypes of influenza A virus in bird and pig populations resulting in periodic emergence of virulent subtypes with the potential to cause pandemics in the human population.



Less serious outbreaks of human influenza, which are of relatively frequent occurrence, can be attributed to subtle antigenic changes arising from errors during replication of viral nucleic acid. If these subtypes exhibit antigenically distinct haemagglutinins, they are less affected by existing neutralizing antibodies and a proportion of the susceptible population may become infected. Such outbreaks occur abruptly, typically in the winter months in temperate regions, and have a mortality rate of about 0.1%.

Subtypes of influenza A virus, which are well established as pathogens in animal populations, have also been implicated in human infections. In 1976 an outbreak of respiratory disease involving a swine influenza H1N1 virus occurred in soldiers in Fort Dix, New Jersey. A large vaccination campaign was mounted with 40 million doses administered before the programme was halted. The virus did not spread outside the military training base. In 1997, following a large

epidemic of avian influenza in chickens, an H5N1 subtype was isolated from a fatal case in a young child in Hong Kong. This subtype had not previously been described outside of avian species. Human health fears prompted the destruction of 1.2 million birds. Fortunately, human-to-human transmission did not occur to any significant extent, although other human cases did occur as a result of contact with infected poultry. By the end of 1997, 18 human cases had been confirmed, six of which were fatal. In 2002, a highly pathogenic subtype of H5N1, capable of producing severe disease in domestic birds, reappeared in Hong Kong. Spread of this subtype, believed to have originated in southern China, marked the start of an unprecedented epizootic of avian influenza involving a large number of countries across south-east Asia over several years. Transmission has been principally related to the movement of poultry and poultry products although infection in parts of Africa and Europe may indicate a role for the spread of virus by migrating wild birds. Cases of human disease have been rare to date, but the case fatality rate has been high at 60% (Abdel-Ghafar *et al*, 2008). The virus has also infected tigers, leopards, domestic cats and dogs fed uncooked poultry meat. Concern has also been expressed regarding the possibility of transmission of low pathogenic avian influenza H9N2 isolates to humans (Lupiani and Reddy, 2009).

Avian influenza (fowl plague)

Many combinations of H and N antigens in influenza A virus are represented in isolates from avian species, particularly waterfowl (Raleigh *et al*., 2009). Influenza A virus subtypes are distributed worldwide and are frequently recovered from clinically normal birds. Outbreaks of severe clinical disease, caused by H5 and H7 subtypes, occur periodically in chickens and turkeys. In these species, acute infection is referred to as fowl plague or highly pathogenic avian influenza (HPAI) and is categorized as a listed disease by the OIE. All H5 and H7 isolates are notifiable. Phylogenetic studies have shown close similarities between wild and domestic bird isolates and indicate that clades and lineages of isolates are associated with geographical and temporal factors.

Epidemiology

Infection is maintained in wild bird populations. Waterfowl are considered to be responsible for introducing the virus to domestic birds. Although ducks, particularly juveniles, regularly become infected with influenza A virus, they

rarely show signs of illness. Following replication in the intestinal tract, virus is shed in faeces. Secondary spread can result from the movement of personnel and contaminated equipment between poultry farms, and live-bird markets may contribute to the spread of infection in some countries.

Pathogenesis

Spread of influenza virus in tissues is dependent on the type of proteases present in a given tissue and the structure of the viral haemagglutinin molecule. The production of infectious virions requires post-translational cleavage of the viral precursor haemagglutinin (HA0) molecule into a disulphide-linked HA1–HA2 dimer to attain its full biological potential. In the majority of influenza A virus subtypes, haemag-glutinin is cleaved by particular host proteases such as trypsin-like enzymes found only in the epithelial cells of the respiratory and digestive tracts. The presence at the cleavage site of multiple basic amino acids, arginine or lysine, renders haemagglutinins of virulent subtypes susceptible to cleavage by intracellular host proteases present in many tissues, thereby facilitating the development of generalized infection. It has been suggested that HPAI isolates arise by mutation from low virulence isolates probably after the transfer of virus from the natural wild bird host to poultry. Such change, which is unpredictable, may occur soon after introduction or following several months of circulation of low pathogenic avian influenza (LPAI) virus in poultry.

Clinical signs

The incubation period, which is variable, is up to 3 days in naturally-infected individual birds and 14 days in the entire flock. The incubation period is influenced by virus and route of infection. Clinically, the disease may be inapparent, mild or, in some instances, severe with high mortality. Factors such as overcrowding, poor ventilation and concurrent infections may predispose to the development of severe disease. Highly virulent subtypes cause explosive outbreaks of disease with high mortality. Clinical signs are more apparent in birds which survive for a few days. Respiratory distress, diarrhoea, oedema in the cranial region, cyanosis, sinusitis and lacrimation are features of the clinical presentation. Infection of laying birds results in a dramatic drop in egg production.

Diagnosis

The severe form of the disease may be difficult to distinguish from velogenic, viscerotropic Newcastle disease or from fowl cholera. Mild forms of the disease resemble other respiratory conditions in birds.

- Laboratory confirmation, which involves virus isolation and characterization, is essential. Suitable specimens for laboratory examination include tracheal and cloacal swabs, faeces and pooled samples of organs.
- Tissue suspensions are inoculated into 9 to 11-day-old embryonated eggs. Allantoic fluid, harvested after incubation for 4 to 7 days, is tested for haemagglutinating activity.
- The presence of influenza A virus can be confirmed by immunodiffusion using a suspension of chorioallantoic membrane from eggs inoculated with material from an outbreak and positive antiserum to the nucleocapsid or matrix antigens common to all influenza A viruses.
- Antisera with broad specificity may be used in haemagglutination inhibition (HI) or immunodiffusion tests to confirm that an isolate is influenza A virus. Definitive subtyping is performed by haemagglutination inhibition (HI) and neura-minidase inhibition (NI) in reference laboratories using monospecific antisera prepared against the 16 haemagglutinins and nine neuraminidase determinants.
- All highly virulent avian influenza A subtypes identified possess either H5 or H7 antigens. However, numerous low virulence H5 and H7 isolates have been recorded. To assess pathogenicity, ten chickens 4 to 8 weeks of age should be inoculated intravenously. Isolates that cause more than 75% mortality within 8 days or have an intravenous pathogenicity index (IVPI) of greater than 1.2 are considered highly pathogenic.
- Genomic sequencing can be used to determine the amino acid composition at the cleavage site of the haemagglutinin molecule. H5 and H7 isolates with an IVPI of less than 1.2 should be sequenced to determine if they have multiple basic amino acids at the cleavage site of the precursor haemagglutinin molecule as these are associated with pathogenicity (Alexander, 2008).
- RT-PCR techniques (Senne *et al*., 1996; Starick *et al*., 2000; Munch *et al*, 2001) and real-time RT-PCR (Spackman *et al*., 2002) are commonly employed for the rapid detection and subtype identification of virus in clinical samples, particularly for the rapid identification of subsequent outbreaks once the primary infected premises has been detected and the

virus isolate characterized. Primers for conserved sequences of the matrix protein gene are useful for screening samples from a range of different species for all subtypes of the virus (Fouchier *et al*., 2000). Primers and protocols are also available for the rapid and specific detection of H5 and H7 subtypes of the virus (Slomka *et al*., 2007).

- Commercial antigen immunoassays are available for the detection of influenza A viruses in poultry (Slemons and Brugh, 1998; Cattoli *et al*., 2004). The tests are rapid and generally detect any influenza A virus, typically based on a monoclonal antibody against the nucleoprotein. Because these assays may lack sensitivity, their use should be confined to flock testing.
- Serological testing for antibodies to influenza virus can be carried out using an agar gel immunodiffusion test or haemagglutination inhibition test or by competitive ELISA (Shafer *et al*., 1998). A neurami-nidase inhibition test has been developed as part of a DIVA (differentiating infected from vaccinated animals) strategy (Capua *et al*., 2003).

Control

Outbreaks of avian influenza in domestic species are notifiable to national regulatory authorities. In countries free of the disease, outbreaks are controlled by slaughter of affected flocks, imposition of movement restrictions and implementation of rigorous disinfection procedures. Imported birds are quarantined. In high-risk areas along the migration routes of waterfowl, poultry should be housed in bird-proof buildings.

Vaccination is usually prohibited in those countries implementing a slaughter policy because of international trade restrictions and possible difficulties in establishing freedom from infection. Some countries accept the presence of mildly pathogenic subtypes because of the expense of implementing control measures. In such countries, inactivated oil emulsion vaccines are available commercially and are used, particularly in turkeys, to protect against subtypes of low virulence. Recombinant haemagglutinin protein vaccines and recombinant fowlpox virus vector vaccines containing a haemagglutinin gene insert have been developed (Swayne *et al*., 1997; Crawford *et al*., 1999).

Vaccines that are effective against a particular virulent influenza A subtype may not be effective against new emerging subtypes. Because of the risk of reversion to virulence, live vaccines against influenza A virus are not used.

However, clinical trials in humans with an attenuated, cold- adapted, reassortant influenza virus vaccine have produced good results (Couch, 2000) and may lead to the development of similar vaccines for poultry.

Swine influenza

This highly contagious disease of pigs occurs worldwide. Swine influenza was first described in 1918, its occurrence coinciding with a major pandemic of human influenza. The most common subtypes currently circulating in pigs are H1N1, H1N2 and H3N2. Frequently two or more subtypes circulate in a pig population. In Europe during 1979, H1N1 isolates, clearly distinguishable from classical H1N1 subtypes and with haemagglutinins structurally similar to avian haemagglutinins, were identified. These H1N1 subtypes, which are more virulent than classical H1N1 isolates, now predominate in Europe. The H3N2 virus is a triple reassortant containing haemagglutinin and neuraminidase proteins from a human influenza virus and internal proteins from porcine, avian and human viruses, while the H1N2 subtype is a triple reassortant comprised of an HA of human origin, an NA of swine H3N2 origin and internal proteins of avian-like swine H1N1 viruses. A novel H3N1 subtype has recently been described in pigs in Korea, Italy and the USA. It has been suggested that pigs may act as a ‘mixing vessel’ with transfer of virulent reassortant subtypes from pigs to humans resulting in pandemics in the human population (Fig. 71.2).

Epidemiology

An outbreak of swine influenza is usually associated with the recent introduction of pigs into a herd. Virus, shed in high concentrations in the nasal secretions of infected pigs, spreads rapidly within a herd. The principal route of transmission is by direct contact. Airborne spread between farms may occur under suitable weather conditions in areas with high pig populations. Outbreaks of disease usually occur when environmental temperatures are low. Between outbreaks, it is probable that virus circulates in herds without evidence of clinical disease and that maintenance of infection on farms for many months is related to the continual availability of susceptible animals. Pigs housed in multiple age groups are at greater risk of acquiring infection with swine influenza virus than those of uniform age (Poljak *et al*., 2008).

Pathogenesis and pathology

Infection is limited to the respiratory tract; the lungs are the major target organs. Following infection, virus multiplies in nasal, tracheal and bronchial epithelium. Spread of infection throughout the respiratory tract may result in necrosis, extensive pneumonic change and consolidation. Experimental inoculation of some swine influenza virus isolates demonstrated very low virulence and resulted in minor histological changes without gross post-mortem lesions (Ferrari *et al*., 2010). Lesions are often limited to the apical and cardiac lobes. The acute phase of the disease persists for more than 72 hours after which virus replication declines.

Clinical signs

Onset of the disease in a herd is often abrupt, many pigs becoming clinically ill simultaneously. The incubation period is up to 4 days. The severity of the illness ranges from subclinical to acute and is strongly influenced by the strain of the infecting virus. Secondary bacterial infections frequently complicate the course of the disease and delay recovery. Acute disease is characterized by huddling in groups, paroxysmal coughing, dyspnoea and fever, while some pigs may have a discharge from the eyes and nose. Most pigs recover within 6 days. Although morbidity is high, mortality is usually low except in very young pigs or when intercurrent infection is present. The economic impact of the disease is mainly attributable to loss of weight, while in fully susceptible herds abortion can occur in affected sows (Karasin *et al*., 2002).

Diagnosis

- Samples suitable for virus isolation include nasal mucus and lung tissue collected from acute cases early in the disease. As the virus is labile, transport media should be used for rapid transfer of specimens to the laboratory. Isolation is performed in embryonated eggs or cell culture. After incubation for 72 hours, haemagglutinating activity is demonstrable in the allantoic fluid.
- Demonstration of a rise in antibody levels in paired serum samples using haemagglutination–inhibition test or ELISA procedures is indicative of infection.
- Viral antigen can be detected using immunofluorescence or ELISA.
- Viral nucleic acid can be detected with RT-PCR (Fouchier *et al*., 2000) and real time RT-PCR (Richt *et al*., 2004).

Control

Good husbandry, including the elimination of stress factors, may help to minimize losses from swine influenza. Depopulation is the only means of eliminating infection, and an all-in/all-out management system is the best measure to prevent the introduction of infection. Inactivated vaccines are available commercially. Vaccination can be beneficial provided that the subtypes of virus incorporated in vaccines include those involved in the outbreaks (Thacker and Janke, 2008).

Equine influenza

This economically important, acute respiratory disease of horses has occurred worldwide with the exception of a small number of countries such as New Zealand and Iceland. Equine influenza occurred in Australia in 2007 for the first time. Two immunologically distinct subtypes of influenza A virus are described in horses. The first virus, isolated from horses in 1956, was designated A/equine/Prague/1/56 (H7N7) or influenza A/equine 1. In 1963, a second subtype was isolated in the USA and designated A/equine/Miami/2/63 (H3N8) or influenza A/equine 2. Infection or vaccination with one subtype does not induce protection against infection with the other subtype. Although the last outbreak of disease attributed to influenza A/ equine 1 occurred in 1979, there is serological evidence that this subtype continues to circulate in the horse population.

Antigenic drift accounts for several variants of influenza A/equine 2 with two antigenically and genetically distinct lineages identified in Europe and the Americas (Oxburgh *et al*., 1998). However, considerable crossover of viruses, of both lineages, from the two continents has been recorded. Both North American and European lineages of H3N8 have been detected in horses in China. In contrast, an H3N8 subtype isolated from horses in China in 1989 was more closely related to avian strains than to the H3N8 subtype circulating in horses in other countries. Outbreaks of influenza in greyhounds have been associated with isolates closely related to H3N8 equine influenza virus (Crawford *et al*., 2005).

Epidemiology

Outbreaks are associated with movement and assembly of horses for shows, sales, racing or training. The initial source of infection is often a partially

immune horse shedding virus without showing clinical signs. Equine influenza is highly contagious and spreads rapidly among susceptible horses. Large quantities of virus are shed in aerosols by the frequent coughing of affected animals. Infection has been reported to spread over 1–2 km via windborne aerosol (Davis *et al*., 2009). Indirect transmission through contamination of clothing, equipment and vehicles can also occur.

Pathogenesis

The virus replicates in the epithelium of the respiratory tract resulting in destruction of ciliated epithelium within 4 to 6 days and hypersecretion from submucosal glands. This destruction of ciliated epithelial cells is a result of apoptosis triggered by infection of cells by equine influenza virus.

Clinical signs

The incubation period is usually 1 to 3 days with a range of 18 hours to 5 days, the length of incubation being inversely related to the infecting dose (Newton and Mumford, 2004). The disease is characterized by pyrexia, nasal discharge, dry cough, anorexia and depression. It is frequently associated with secondary bacterial infections (Newton *et al*., 2006). Ocular discharge, limb oedema and stiffness may also be present. Age and previous exposure or vaccination status may influence the severity of the clinical signs and the likelihood of secondary bacterial infection with the development of respiratory complications. Exercise exacerbates the clinical signs (Gross *et al*., 1998). Animals with mild infections usually recover within 3 weeks. In severe cases, several months may be required for convalescence. Excretion of virus has been reported to last from 7 to 10 days in fully susceptible horses.

Diagnosis

Although clinical signs may be suggestive of equine influenza, laboratory confirmation is required.

- Nasopharyngeal swabs collected during the acute phase of the infection are suitable for isolation of the virus in embryonated eggs or in cell culture. New isolates should be closely monitored for antigenic drift.
- A commercial diagnostic kit, developed for the detection of the nucleoprotein of human influenza A virus, can be used for the diagnosis of equine influenza (Chambers *et al*., 1994).

- Viral nucleic acid can be demonstrated using RT-PCR (Donofrio *et al*., 1994; Oxburgh and Hagstrom, 1999; Fouchier *et al*., 2000) and real-time RT-PCR (Quinlivan *et al*., 2005; Lu *et al*., 2009).
- Serological diagnosis of equine influenza is possible. Haemagglutination inhibition or single radial haemolysis tests on paired serum samples can be used for diagnosis. Serum used in the HI test must be pretreated in order to remove non-specific inhibitors.

Treatment and control

Supportive therapy and rest are indicated for affected horses. The antiviral drugs amantadine and rimantadine have been shown to be effective for inhibiting replication of influenza A virus *in vitro*. Several inactivated vaccines are commercially available which are effective in reducing the severity of clinical signs and shedding of virus (Paillot *et al*.,2010). However, immunity is usually short-lived and booster injections are required in accordance with the manufacturer's instructions. The incorporation of polymer adjuvants or Quil-A-based immuno-stimulating complexes (ISCOMs) into vaccine preparations extends the duration of protective levels of immunity. Commercial recombinant canarypoxvirus vaccines expressing the haemagglutinin gene of A/equine 2 have been tested and used during the successful control and eradication programme for equine influenza in Australia in 2007 (Garner *et al*., 2010). Serological assays capable of differentiating infected from vaccinated animals (DIVA) are applicable with this vaccine. Protective immunity generated by natural exposure is related both to a mucosal IgA immune response and to humoral IgGa and IgGb responses, a pattern of protective immunity not generated by conventional vaccines (Nelson *et al*., 1998). Vaccinated horses generally exhibit milder clinical signs and shed virus for shorter periods than unvaccinated animals. Vaccine manufacturers must update vaccinal strains regularly and vaccines should include antigenic material representative of the influenza A virus subtypes prevalent in the horse population. Current commercial vaccines contain strains of both lineages of H3N8 as well as H7N7, and have been shown to give broad cross-protection when circulating antibodies are sufficiently high (Park *et al*., 2004).

The rate of mutation is likely to be increased by vaccination (Newton and Mumford, 2004). For this reason, it is considered necessary to update the strains of equine influenza used in vaccine production every 3 to 5 years (Anon.–EU–

EI, 1998). In addition to vaccination, control of equine influenza requires isolation of affected horses and cleaning, disinfection and isolation of infected premises. Animal movement should cease until contaminated premises have been cleaned and disinfected.

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Chapter 72

Paramyxoviridae

Paramyxoviruses and orthomyxoviruses were formerly grouped together as the ‘myxoviruses’ (Greek *myxa*, mucus), a name which describes their affinity for mucous membranes. Paramyxoviruses are pleomorphic, 150 nm or more in diameter and enveloped (Fig. 72.1). They contain a single molecule of negativesense, single-stranded RNA. Two types of glycoprotein ‘spikes’ or peplomers are present in the envelope: an attachment protein and a fusion protein (F). The attachment protein may be either a haemagglutinin-neuraminidase protein (HN) or a protein without neuraminidase activity (G). The attachment proteins allow the virus to bind to cell surface receptors and the fusion protein causes the virus envelope to fuse with the host cell membrane. Both types of peplomers can induce production of virus-neutralizing antibodies. There is also an envelope-associated non-glycosylated membrane protein (M). Paramyxoviruses may exhibit haemagglutinating, haemolytic and neuraminidase activities. The nucleocapsid, which has helical symmetry, is 13 to 18 nm in diameter and has a characteristic herringbone appearance. Replication occurs in the cell cytoplasm. Virions are released by budding from the plasma membrane at sites containing virus envelope proteins. The labile virions are sensitive to heat, desiccation, lipid solvents, non-ionic detergents and disinfectants.

Families containing enveloped viruses with genomes consisting of a single molecule of negative sense, single-stranded RNA, namely *Paramyxoviridae*, *Rhabdoviridae, Filoviridae* and *Bornaviridae*, have been assigned to the order *Mononegavirales*. The family is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. Recently, classification of the *Paramyxoviridae* has been changed to include three new genera, *Metapneumovirus, Henipavirus* and *Avulavirus* and, in addition, the genus *Paramyxovirus* has been renamed *Respirovirus* (Fig. 72.2). Although paramyxoviruses are genetically stable and do not exhibit recombination, antigenic variation may occur through mutation.

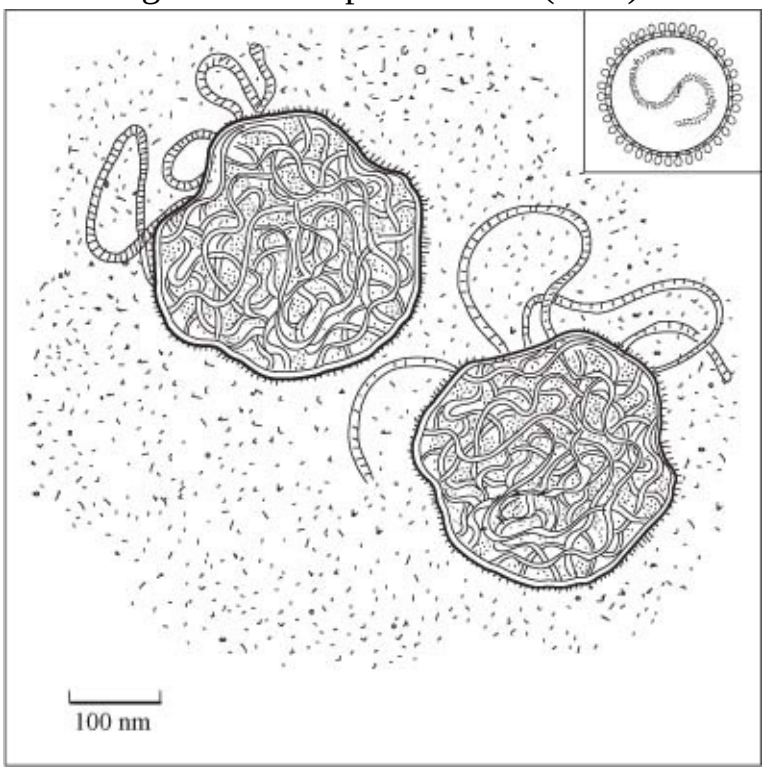
Key points

- Large pleomorphic enveloped viruses
- Negative-sense, single-stranded RNA
- Helically symmetrical nucleocapsid
- Replicate in the cytoplasm
- Subfamilies *Paramyxovirinae* and *Pneumovirinae* divided into seven genera, each containing viruses of veterinary importance
- Cause rinderpest, peste des petits ruminants, canine distemper, Newcastle disease and a range of respiratory diseases in domestic animals

Clinical infections

Paramyxoviruses, which have a narrow host range, infect mainly mammals and birds (Table 72.1). Following transmission through close contact or by aerosols, replication occurs primarily in the respiratory tract. Infection is generally cytolytic but persistent infections are described *in vitro*. Formation of syncytia and intracytoplasmic, acidophilic inclusions are features of infection with these viruses. Serious diseases caused by paramyxoviruses in animals include rinderpest, peste des petits ruminants, canine distemper and Newcastle disease, and in humans measles and mumps.

Figure 72.1 Paramyxovirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Rinderpest

This acute disease, which occurs primarily in ruminants and is also referred to as cattle plague, has been recognized for centuries as a major cause of mortality in cattle and domestic buffalo. Originally an Asian disease, devastating outbreaks in Europe resulted in the foundation of the first veterinary school in Lyon in 1761. Following its introduction into the Horn of Africa a devastating outbreak followed throughout sub-Saharan Africa during the last decade of the nineteenth century. Rinderpest was considered endemic in the Somali pastoral ecosystem which straddles the borders of Kenya, Ethiopia and Somalia. However, the virus has not been confirmed in this area since 2001. The virus is considered to have been eradicated from its traditional homeland of central Asia and also from Africa. It is classed as a listed disease by the World Organization for Animal Health, also known as Office International des Epizooties (OIE).

Epidemiology

Although only one serotype of this morbillivirus is recognized, strains differ in both host range and virulence. Individual host species exhibit differences in susceptibility to virus strains. Domestic cattle, buffalo and several wildlife species including giraffe, warthog, Cape buffalo and eland are highly susceptible to infection. Gazelles and small domestic ruminants are less susceptible. Asiatic breeds of domestic pig develop disease whereas infection in European breeds is sub-clinical. Transmission, which occurs through aerosols, usually requires close contact as the virus is labile and remains viable in the environment for short periods only. Virus shedding in all secretions and excretions begins a few days before clinical signs develop. In endemic areas, the disease is usually mild and is restricted to young cattle in which maternally-derived immunity has declined. As there is no carrier state, maintenance of infection requires continuous transmission to susceptible animals. Transmissibility and virulence of the virus are directly related. During epidemics, selection of virulent, highly transmissible virus occurs, with shedding of large quantities of virus from infected animals. The absence of highly susceptible hosts in endemic areas results in the selection of milder strains of virus. Epidemics usually occur following movement of susceptible animals into an endemic area or the introduction of infected animals into susceptible populations. All ages of animals are affected in epidemics. Morbidity may reach 90% and mortality can approach 100%.

Pathogenesis

After inhalation of the virus, multiplication occurs in the pharyngeal and mandibular lymph nodes. Viraemia develops within 3 days, resulting in spread to other lymphoid tissues and to the mucosae of the respiratory and digestive tracts. Leukopenia and immunosuppression follow necrosis in lymphoid tissues. Virus shedding, which continues throughout the acute phase of the disease, subsides a few days after body temperature returns to normal.

Clinical signs

After an incubation period of 3 to 9 days, infected animals develop a fever and become anorexic and depressed. Mucosal erosions in the mouth and nasal passages become evident within 5 days. Profuse salivation is accompanied by an oculonasal discharge. About 3 days after the appearance of the mucosal ulcers, fever regresses and a profuse diarrhoea develops. The dark fluid faeces often contain mucus, necrotic debris and blood. Dehydration and wasting soon become evident. Severely affected animals may collapse and die within 12 days of the onset of clinical signs. In surviving animals, convalescence lasts several weeks. Secondary infections and activation of latent protozoal infections are frequent complications. Pregnant animals may abort during the convalescent period.

Figure 72.2 A classification of paramyxoviruses with emphasis on those of veterinary importance. Viruses in red cause OIE-Iisted diseases.

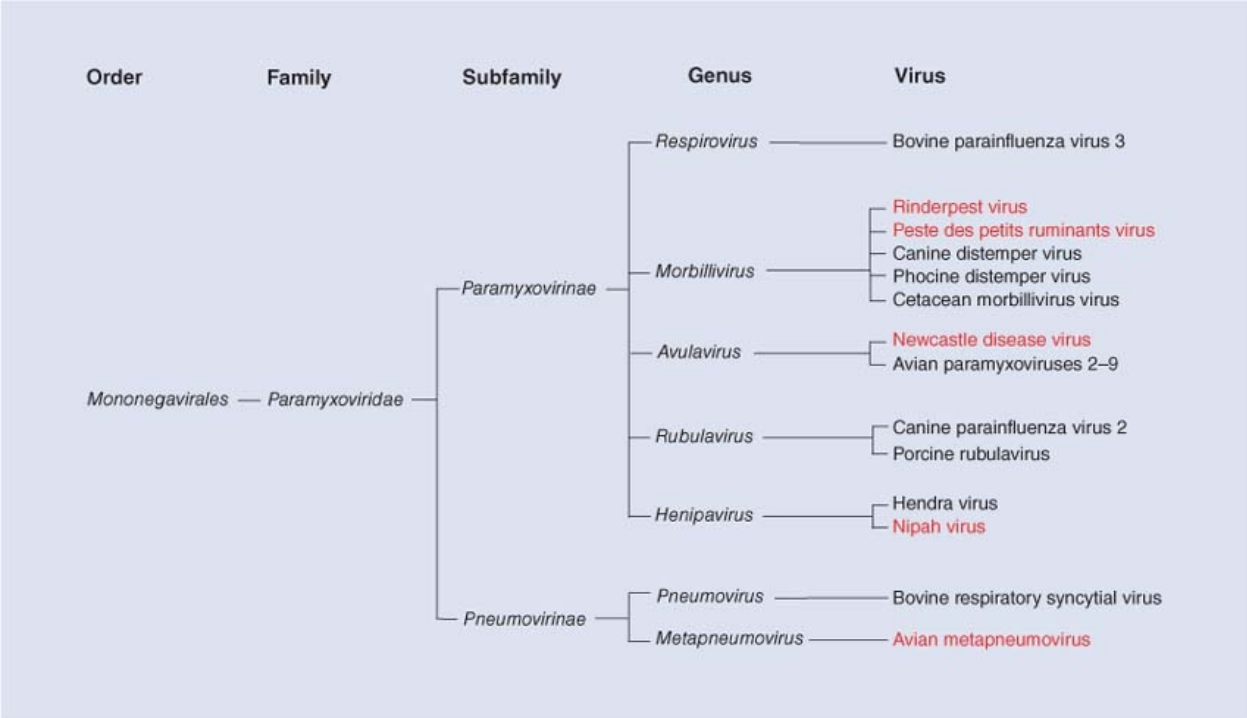


Table 72.1 Paramyxoviruses of veterinary importance.

Genus	Virus	Comments
Morbillivirus	Rinderpest virus	Causes highly contagious disease in domestic and wild
		ruminants characterized by high morbidity and high mortality
	Peste des petits ruminants virus	Causes severe disease in small ruminants, particularly sheep
		and goats, resembling rinderpest, with high morbidity and
		high mortality rates
	Canine distemper virus	Causes acute disease in dogs and wild carnivores;
		characterized by multisystemic involvement and variable
		mortality
Avulavirus	Newcastle disease virus (avian	Causes Newcastle disease in domestic and wild birds. Isolates
	paramyxovirus 1)	vary in virulence: velogenic, mesogenic and lentogenic strains.
		Generalized infection characterized by respiratory, intestinal
		and nervous signs
Rubulavirus	Porcine rubulavirus	Causes blue eye disease; described only in Mexico
	Canine parainfluenza virus 2	Causes inapparent or mild respiratory disease in dogs;
		sometimes associated with kennel cough; related to or
		possibly a subtype of simian virus 5 (SV5)
Respirovirus	Bovine parainfluenza virus 3	Causes subclinical or mild respiratory disease in cattle and
		sheep. Sometimes associated with shipping fever in cattle.
		Predisposes to secondary bacterial infection particularly with
		Mannheimia haemolytica
Pneumovirus	Bovine respiratory syncytial virus	Common subclinical infection in adult cattle. Associated with
		respiratory disease outbreaks of varying severity in young
		cattle

Metapneumovirus	Avian metapneumovirus (turkey	Causes severe upper respiratory tract infection in turkeys with
	rhinotracheitis virus)	coryza and swollen sinuses. In chickens, the disease is
		referred to as swollen head syndrome

Diagnosis

Clinical and pathological findings may be sufficient for diagnosis in endemic areas. They may also be adequate in individual animals in outbreaks which have been confirmed by laboratory tests. Lesions are found throughout the gastrointestinal tract with characteristic congestion and haemorrhage along the longitudinal folds of the large intestine, referred to as ‘zebra striping’. In regions where rinderpest is uncommon or absent, laboratory confirmation is required to differentiate it from bovine viral diarrhoea, infectious bovine rhinotracheitis, malignant catarrhal fever and foot-and-mouth disease. When investigating an outbreak, specimens for laboratory examination should be collected from several febrile animals which have not developed diarrhoea.

- Specimens suitable for virus isolation include white cells from the buffy coat of heparinized blood samples, lymph node and spleen.
- The presence of rinderpest virus, which produces cytopathic effects in cell cultures, can be confirmed by immunofluorescence.
- Agar gel immunodiffusion or a counter immunoe-lectrophoresis test is used as a rapid antigen detection test. Specimens suitable for these procedures include ocular discharge and mesenteric lymph nodes. A pen-side test, based on a rapid chromatographic strip test, may be useful for field personnel investigating outbreaks of suspected rinderpest (Bruning *et al*., 1999).
- A reverse transcriptase polymerase chain reaction method, which can detect rinderpest virus and differentiate it from the virus of peste des petits ruminants, has been developed (Forsyth and Barrett, 1995).
- A competitive ELISA for detecting serum antibodies to rinderpest virus is the test recommended by OIE for international trade.
- Post mortem enteric lesions are characteristic but not pathognomonic. Congestion of the folds of the colonic mucosa often produces a zebra-stripe pattern.
- Syncytia may form in stratified squamous epithelium of the upper alimentary tract and in crypts of the small intestine.

Control

The Global Rinderpest Eradication Programme of the Food and Agricultural Organization (FAO) was begun in 1994 to achieve worldwide eradication of rinderpest. It was declared a success in 2010, making this only the second virus (after smallpox virus) to be eradicated. Factors which made this achievable included the availability of a vaccine developed by Walter Plowright that induces lasting immunity, reliable diagnostic tests and the absence of carrier animals and wildlife reservoirs.

In countries free of rinderpest, control is based on restriction of animal movement, quarantine of imported animals and slaughter of infected animals. In endemic areas, control is achieved by vaccination of domestic cattle and buffaloes with a modified live tissue-culture-based vaccine that induces immunity lasting at least 5 years. This stable freeze-dried vaccine is thermolabile following reconstitution. Recombinant vaccinia and capripoxvirus vaccines expressing either haemagglutinin protein or fusion protein of rinderpest virus have high heat stability and have been used to protect cattle (Inui *et al*, 1995; Ngichabe *et al*, 1997). Control of animal movement is the single most important measure for preventing disease transmission.

Peste des petits ruminants

This condition, also referred to as goat plague, is an acute contagious disease of small ruminants, particularly goats. It is caused by the morbillivirus, peste des petits ruminants virus (PPRV), which is closely related to other members of the genus. Peste des petits ruminants (PPR), which occurs in sub-Saharan Africa north of the equator, the Middle East, India and Pakistan, is an OIE-listed disease.

Epidemiology

Close contact is required for transmission of this labile virus which occurs by aerosols. The introduction of infection into a flock is invariably associated with movement of animals. Although a carrier state is not known to occur, subclinical infection and the onset of viral shedding before overt clinical signs facilitate spread of infection. In West Africa, epidemics tend to occur during the rainy season, when flocks are gathered together in preparation for sales. Infection rates are similar in sheep and goats but the disease is generally more severe in goats.

Pathogenesis

The pathogenesis of PPR is similar to that of rinderpest. Mucosal erosions and profuse diarrhoea are features of the condition. During the acute phase of the disease, virus is shed in all secretions and excretions.

Clinical signs

The incubation period is about 4 days. The disease is particularly severe in young animals. Affected goats exhibit fever, dry muzzle and a serous nasal discharge which becomes mucopurulent. Erosions on the mucous membrane of the buccal cavity are accompanied by marked salivation. Ulcers develop in the mucosae of the alimentary, respiratory and urinary tracts. Conjunctivitis with ocular discharge is a feature of the disease. A profuse diarrhoea, which results in dehydration, develops within days of infection. Signs of tracheitis and pneumonia are common. There is a severe leukopenia which facilitates secondary bacterial infection. Pulmonary infections caused by *Pasteurella* species are common in the later stages of the disease. Pregnant animals may abort. Mortality rates in severe outbreaks often exceed 70% and acutely affected goats may die within 10 days of exposure to the virus.

In sheep, infection with PPRV, which tends to be less severe than in goats, is characterized by fever, nasal catarrh, mucosal erosions and intermittent diarrhoea. Affected animals usually recover after 10 to 14 days.

Diagnosis

Specimens for laboratory examination should be taken from animals in the acute phase of the disease. Suitable specimens include nasal and ocular swabs, unclotted blood and scrapings of buccal and rectal mucosae. Samples of lung, spleen and lymph node from animals slaughtered early in the course of the disease are also suitable. Laboratory confirmation is based primarily on virus isolation in tissue culture and on antigen detection. Rapid antigen detection methods include ELISA, counter immunoelectrophoresis and agar gel immunodiffusion. Specific primers for use in RT-PCR are available. Antibodies can be detected by virus neutralization or by competitive ELISA.

Control

Slaughter policies apply in countries free from PPR. Quarantine and vaccination

are used in regions where the disease is endemic. Modified live rinderpest vaccine, which induces adequate protection against PPRV, has been used in sheep and goats for many years. A modified live PPRV vaccine has also been developed.

Canine distemper

This highly contagious disease of dogs and other carnivores has a worldwide distribution. Canine distemper virus (CDV), a pantropic morbillivirus, produces a generalized infection involving many organ systems.

Epidemiology

The wide host range of CDV includes members of the families *Canidae, Ailuridae, Hyaenidae, Mustelidae, Procyonidae, Ursidae, Viverridae* and *Felidae*. Outbreaks of disease have been documented in several wildlife species including foxes, skunks, racoons, black-footed ferrets and lions (Appel and Summers, 1995; Roelke-Parker *et al*., 1996). The virus is relatively labile, requiring transmission by direct contact or by aerosols. In urban dog populations, the virus is maintained by infection of susceptible animals. Infection spreads rapidly among young dogs, usually between 3 and 6 months of age, when maternally-derived immunity declines. The number of dogs in populations in rural areas is often too low to maintain continuous infection with the result that, irrespective of age, unvaccinated dogs are susceptible and significant outbreaks of the disease can occur.

Pathogenesis

The virus, which replicates in the upper respiratory tract, spreads to the tonsils and bronchial lymph nodes. A cell-associated viraemia follows with spread to other lymphoreticular tissues. Viral replication produces lymphocytolysis and leukopenia resulting in immunosuppression which facilitates a secondary viraemia to develop. The extent of spread to tissues and organs is determined by the rapidity and effectiveness of the immune response. In the absence of a sufficiently vigorous response, dissemination and replication of CDV occurs in the respiratory, gastrointestinal, urinary and central nervous systems. Spread to the skin may also occur.

Virus infects both neurons and glial cells within the CNS and may persist there for very long periods. Old dog encephalitis is apparently associated with

prolonged persistence of the virus in the brain, possibly as a result of non-cytolytic spread from cell to cell without budding from the cell membrane, thus evading immune detection (Stettler *et al*., 1997). This mechanism appears to be analogous to that causing subacute sclerosing panencephalitis of children which is associated with persistent infection with defective measles virus. The presence of viral antigen in these conditions stimulates a low-grade prolonged inflammatory response eventually leading to the development of neurological signs.

Clinical signs

The incubation period is usually about 1 week but may extend to 4 weeks or more when nervous signs appear without prior evidence of infection. The severity and duration of illness are variable and are influenced by the virulence of the infecting virus, the age and immune status of the infected animal and the rapidity of its immune response to infection. The pyrexic response to infection is biphasic although the initial elevation of temperature may not be noticed. During the second period of pyrexia, oculonasal discharge, pharyngitis and tonsillar enlargement become evident. Coughing, vomiting and diarrhoea are often consequences of secondary infections. A skin rash and pustules may be present on the abdomen. Some affected dogs have hyperkeratosis of the nose and footpads, referred to as ‘hardpad’. Acute disease, which may last for a few weeks, is followed either by recovery and life-long immunity or by the development of neurological signs and, eventually, death. Common neurological signs include paresis, myoclonus and seizures. The convulsions may begin as petit mal ‘chewing gum’ fits where the animal salivates and makes chewing movements with the jaws before convulsions become more frequent and severe, resulting in grand mal epileptiform seizures. A grave prognosis is indicated in animals displaying neurological disturbance. Residual neurological deficits are common in dogs that survive. Old dog encephalitis, characterized by motor and behavioural deterioration, is invariably fatal.

Diagnosis

A febrile, catarrhal illness with neurological sequelae in young dogs is highly suggestive of canine distemper.

- Viral antigen may be demonstrated by immunofluorescence in conjunctival or vaginal impression smears or in smears of cells from the buffy coat.

- Cryostat sections of lymph nodes, urinary bladder and cerebellum are also suitable for the demonstration of viral antigen.
- Eosinophilic inclusions can be demonstrated in nervous and epithelial tissues.
- Sensitive molecular methods for the detection of CDV RNA in clinical samples include one-step, nested and real-time RT-PCR (Frisk *et al*., 1999; Kim *et al*., 2001; Shin *et al*, 2004; Elia *et al*., 2006). Retrospective diagnosis using a semi-nested RT-PCR on formalin-fixed, paraffin-embedded tissue is also possible (Stanton *et al*., 2002). Six major genetic lineages, America-1 and-2, Asia-1 and-2, European and Arctic, have been identified, based on sequencing of the H gene (Martella *et al*., 2007). Commercial vaccines typically contain strains from the America-1 lineage.
- Serological demonstration of either IgM antibodies or a fourfold rise in antibody titre between acute and convalescent sera may be determined by virus neutralization, ELISA or indirect immunofluorescence. Antibody may be detected in cerebrospinal fluid.
- Virus isolation may prove difficult. Urinary bladder and brain are suitable post-mortem specimens for virus isolation. Cells from the buffy coat of heparinized blood are also suitable.

Control

Modified live vaccines, which are available commercially, provide adequate protection when administered to pups after maternally-derived antibody has declined to negligible levels, usually after 12 weeks of age. Most CDV vaccines are produced from egg-adapted or avian cell culture-adapted virus (Onderstepoort strain) or from canine cell culture-adapted virus (Rockborn strain). Because post-vaccinal encephalitis has been reported occasionally following the use of canine cell culture-adapted strains, the avian cell culture-adapted strain is considered to be safer. Heterotypic measles virus vaccines have been used in young pups to induce protection in the presence of moderate levels of maternally-derived antibody. A recombinant canarypox virus expressing CDV envelope glycoproteins has been shown to be an effective vaccine (Larson and Schultz, 2006). Following successful vaccination, dogs remain immune for several years and booster vaccinations are recommended every 3 years (Gore *et al*., 2005). Because of the labile nature of the virus, control can be achieved after an outbreak of disease in kennels by strict isolation and disinfection.

Infections caused by other morbilliviruses

During the late 1980s, serious outbreaks of a disease, with clinical and pathological features similar to canine distemper, were recorded in the seal populations, particularly harbour seals, in the Baltic and the North Sea. The interest generated by these disease outbreaks of viral infections in marine mammals led to the recognition of new morbilliviruses including phocine distemper virus and cetacean distemper virus. Serological evidence of morbillivirus infection has been recorded in several cetacean species.

Hendra and Nipah viruses

During an outbreak of severe respiratory disease in horses in Australia during 1994, Hendra virus, originally considered to be an equine morbillivirus, was isolated. Two humans in contact with infected horses were also affected. Fourteen horses and their trainer died. Sporadic cases with acute onset, fever accompanied by either respiratory or neurological signs and high mortality rates are reported periodically. A related virus, Nipah virus, was isolated in Malaysia during 1999 following outbreaks of disease in pigs and humans working in affected pig units. The disease, which caused a febrile encephalitis, resulted in more than 100 deaths in humans. The natural hosts of Hendra and Nipah viruses are considered to be *Pteropus* species of bats (flying foxes). As a consequence of the discovery of these two viruses, a new genus, *Henipavirus*, has been created within the subfamily *Paramyxovirinae*.

Newcastle disease

A large number of avian paramyxovirus (APMV) isolates has been recorded worldwide from a range of domestic and wild birds. Nine species of antigenically distinct APMV are currently recognized in the genus *Avulavirus*. New isolates are assigned to a species on the basis of antigenic relatedness in haemagglutination inhibition tests. Although infections with most avian paramyxovirses are associated with mild or inapparent disease, infections with APMV-2 and APMV-3 cause respiratory disease in turkeys.

The most important avian paramyxovirus is Newcastle disease virus (NDV), also designated avian paramyxovirus 1 (APMV-1), which causes Newcastle disease. This disease occurs in poultry worldwide. Newcastle disease was first described in 1926 when severe outbreaks were reported in Newcastle, England,

and in Java. Other major outbreaks of the disease occurred in the Middle East during the late 1960s and in the 1970s when pigeons were the species primarily affected.

Epidemiology

A wide range of avian species including chickens, turkeys, pigeons, pheasants, ducks and geese is susceptible. Infection with NDV is probably endemic in wild birds especially waterfowl (Takakuwa *et al*., 1998). Strains of NDV differ in their virulence. On the basis of virulence and tissue tropism in poultry, isolates are categorized into five groups or pathotypes:

- Viscerotropic velogenic isolates causing severe fatal disease characterized by haemorrhagic intestinal lesions (Doyle's form)
- Neurotropic velogenic isolates causing acute disease characterized by nervous and respiratory signs with high mortality (Beach's form)
- Mesogenic isolates causing mild disease with mortality confined to young birds (Beaudette's form)
- Lentogenic isolates causing mild or inapparent respiratory infection (Hitchner's form)
- Asymptomatic enteric isolates associated with sub-clinical intestinal infection by lentogenic strains.

These clinical forms overlap and, in addition to the pathogenicity of the NDV isolate, factors such as the host species, dose, age of bird and environmental conditions influence the severity of clinical signs. It appears that virulent strains may arise from progenitor viruses of low virulence following passage in chickens (Shengqing *et al*., 2002). Virus is shed in all excretions and secretions. Transmission usually occurs by aerosols or by ingestion of contaminated feed or water. The relative stability of the virus permits mechanical transfer of infective material through the movement of personnel and equipment. Virus, which can survive in carcasses for some weeks, is present in all organs of acutely affected birds and in eggs.

Captive and wild birds can contribute to the spread of infection. Pigeons are susceptible to all strains of NDV and may play a role in the transmission of Newcastle disease. Mesogenic isolates, which can be distinguished from other NDV isolates using monoclonal antibodies, were obtained from racing pigeons in Europe during the early 1980s. These isolates, often referred to as 'pigeon' paramyxovirus 1, are associated with clinical disease in pigeons resembling the

neurotropic form of Newcastle disease. Outbreaks of Newcastle disease in poultry in the United Kingdom during 1984 were linked to feed contaminated by infected feral pigeons.

Pathogenesis

Viral replication, which occurs initially in the epithelia of the respiratory and intestinal tracts, is followed by haematogenous spread to the spleen and bone marrow. Secondary viraemia results in infection of other organs including lungs, intestine and CNS. The extent of spread within the body relates to strain virulence which is determined by the amino acid sequence of the F glycoprotein. The fusion (F) glycoprotein of NDV is synthesized in an infected cell as a precursor molecule (F_0) which is cleaved by host cell proteases to F_1 and F_2 subunits. If cleavage fails to occur, non-infectious particles are produced. The F_0 molecules of virulent strains of NDV possess basic amino acids at critical positions which facilitate cleavage by proteases in a wide range of host tissues. In contrast, the replication of lentogenic strains is confined to the respiratory and intestinal epithelia where suitable trypsin-like proteases are produced.

Clinical signs

The incubation period is usually about 5 days. Respiratory, gastrointestinal and nervous signs occur in chickens. The particular clinical presentation relates to the virulence of the virus strain, its tissue tropism and the age and immune status of the host. Highly virulent strains may produce sudden high mortality in a flock in the absence of premonitory clinical signs. The mortality rate in fully susceptible flocks may be close to 100%. When present, signs in these flocks include listlessness, weakness and a decrease in egg production. Viscerotropic strains tend to produce respiratory signs such as gasping and rales, oedema of the head and neck and greenish diarrhoea. Birds that survive the acute phase may develop neurological signs. Infection with neurotropic velogenic strains results in respiratory disease followed by nervous signs such as wing paralysis, leg paralysis, torticollis and muscle spasms. Mesogenic strains usually cause respiratory disease. Lentogenic strains do not produce disease in adult birds but may produce respiratory signs in young birds. Pathogenicity of NDV isolates relates not only to their virulence but also to host susceptibility. Infection in turkeys, which usually involves the respiratory and central nervous systems, is less severe than that in chickens. Pigeons infected with ' pigeon' paramyxovirus

1 present with neurological signs and diarrhoea, and mortality in affected birds may approach 10%. Clinical infections have also been described in cormorants and geese.

Humans may develop a transitory conjunctivitis if exposed to high concentrations of NDV.

Diagnosis

A presumptive clinical diagnosis may be made when the characteristic signs and lesions associated with virulent strains are present. Laboratory confirmation by isolation and identification of the virus is necessary.

- Tracheal and cloacal swabs from live birds are suitable for virus isolation. Suitable post-mortem specimens for laboratory examination include faeces, intestinal contents and portions of trachea, intestine, spleen, brain and lung. Samples may be stored at 4°C for up to 4 days.
- Virus isolation is carried out in embryonated eggs from specific pathogen-free (SPF) flocks, usually by inoculation into the allantoic cavity. After incubation, allantoic fluid is tested for haemagglutination activity.
- Haemagglutination-inhibition test using specific antiserum confirms the presence of NDV.
- Virulence of NDV isolates is assessed using *in vivo* tests including the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index in SPF chicks. The pathogenicity index is the mean score per bird per observation over an 8-or 10- day observation period. The mean death time (MDT) using embryonated eggs has been employed to classify isolates as velogenic (embryonic death (ED) in less than 60 hours), mesogenic (ED between 60 and 90 hours) and lentogenic (ED after more than 90 hours). The current OIE definition for reporting an outbreak of ND is infection of birds by avian paramyxovirus serotype 1 with either an ICPI ≥0.7 in day-old chicks or with multiple (at least three) basic amino acids at the C-terminus of the F_2 protein and phenylalanine at residue 117 (N-terminus of the F_1 protein).
- Molecular techniques are increasingly being used for the detection of NDV in clinical specimens (Gohm *et al*., 2000; Creelan *et al*, 2002). Primers are usually selected to cover the cleavage site of the F_0 protein gene, thus providing information on the virulence of the virus detected (Cavanagh, 2001). Real-time RT-PCR has also been used successfully in ND outbreaks

(Wise *et al*., 2004).

- Demonstration of antibody to NDV is of diagnostic value only in unvaccinated flocks. The haemagglutination-inhibition test is the most widely used assay. Commercial ELISA kits are available.
- Demonstration of viral antigen in tracheal sections or impression smears using immunofluorescence is a less sensitive technique than virus isolation.

Control

General control measures include locating poultry farms several kilometres apart, bird-proofing of houses and feed stores, controlled access to farms, thorough cleaning and disinfection of vehicles and equipment and restriction of movement between poultry farms. National control policies for Newcastle disease differ from country to country and range from compulsory vaccination to slaughter of infected flocks. A combination of vaccination and slaughter policies is frequently employed. Vaccination is particularly important for birds in breeder flocks. Lentogenic or mesogenic strains of NDV propagated in eggs or tissue culture are used in live vaccines. They are administered as a spray, in drinking water or by intranasal or intraconjunctival instillation. The presence of maternally-derived antibodies interferes with the efficacy of live vaccines. In order to avoid this undesirable effect, vaccination should be delayed until 2 to 4 weeks of age when most birds will be susceptible, or alternatively live vaccine can be administered to day-old chicks by conjunctival instillation or by a coarse spray with large droplets in order to ‘seed’ a flock with vaccinal virus. This method, which may result in respiratory disease in fully susceptible birds, establishes active infection in some birds that persists until maternally-derived immunity has waned sufficiently in the rest of the birds to allow infection to develop. Revaccination is normally carried out 3 to 4 weeks later. A schedule of vaccination employing both live and inactivated vaccines gives good results. Vaccinated birds, although protected from clinical disease, can be infected with wild-type virus and become shedders. Several recombinant vaccines employing different vector viruses have been developed.

Blue eye disease in pigs

This condition, caused by porcine rubulavirus, was first observed in pigs during 1980 in Mexico. Blue eye disease is characterized by neurological signs and corneal opacity in young pigs and reproductive failure in adults. Morbidity and

mortality are highest in young pigs. Diagnosis is based on clinical signs, histopathological changes and serological testing of paired serum samples. Suitable tests for the detection of antibodies include haemagglutination-inhibition, ELISA and virus neutralization. Methods aimed at preventing the introduction of the infection include strict isolation procedures combined with serological testing of replacement animals. Inactivated vaccines have been produced. The disease has not been reported outside Mexico. However, another rubulavirus, Menangle virus, was isolated from stillborn piglets during an outbreak of reproductive disease at a large Australian commercial piggery in 1997.

Infection caused by bovine parainfluenza virus 3

Infection with bovine parainfluenza virus 3 (BPIV-3), which occurs worldwide, is often subclinical. Transmission, which occurs by aerosols and direct contact, is facilitated by overcrowding in poorly ventilated conditions. Although uncomplicated infections are frequently subclinical, mild respiratory disease may be seen. The virus is commonly isolated from animals during outbreaks of serious respiratory disease such as enzootic calf pneumonia and shipping fever, conditions in which other respiratory viruses and bacteria are often involved. Various stress factors such as transportation or adverse environmental conditions may contribute to the severity of the disease.

The virus infects ciliated epithelium of the respiratory tract, alveolar epithelium and macrophages. Infection causes destruction of ciliated epithelium resulting in interference with the mucociliary clearance mechanism. In addition, phagocytosis and intracellular destruction of bacteria by alveolar macrophages are depressed, predisposing to secondary bacterial infection in the lungs. Most uncomplicated infections with BPIV-3 are mild and are characterized by fever, nasal discharge and coughing. The majority of affected animals recover within a few days. The virus can be isolated in suitable bovine cell lines from nasal swabs or lung tissue. Samples should be taken from several animals in the early stages of the disease and transferred immediately to the laboratory in viral transport medium. Direct immunofluorescence for detecting viral antigen can be carried out on samples of nasal mucus or on cryostat sections of lung. Haemagglutination-inhibition tests, virus neutralization, ELISA and indirect immunofluorescence are commonly used to demonstrate a four-fold rise in antibody titre between acute and convalescent sera.

Both inactivated and modified live BPIV-3 vaccines are available, often combined with other respiratory viruses. Modified live vaccines are designed either for intranasal administration or for intramuscular injection. Immunity tends to be short-lived and reinfection may occur after some months.

Infection caused by bovine respiratory syncytial virus

Pulmonary disease, caused by bovine respiratory syncytial virus (BRSV), is recorded in beef and dairy calves worldwide. Infection has been recorded in sheep and goats with isolates designated as ovine respiratory syncytial virus and caprine respiratory syncytial virus, respectively. However, these isolates may represent strains of a ruminant respiratory syncytial virus rather than separate species. The virus is so named because of the characteristic syncytia which it induces in infected cells *in vivo* and *in vitro*.

Epidemiology

Infection in cattle is common. Moderate to severe respiratory signs often develop in infected calves. Infection in adult animals is usually mild or subclinical but severe disease may occasionally occur (Ellis *et al*., 1996; Elvander, 1996). Persistent infection in individual animals is considered to be responsible for the maintenance of infection in herds. Transmission occurs through aerosols or through direct contact with infected animals. Most clinical cases are recorded during autumn and winter months. Transportation, overcrowding or adverse weather conditions can precipitate outbreaks of the disease. Concurrent infection with bovine viral diarrhoea virus results in more severe clinical signs than those encountered in infection with each individual virus (Pollreiz *et al*., 1997).

Pathogenesis

The virus replicates primarily in the ciliated epithelium of the respiratory system. Destruction of bronchiolar epithelium results in necrotizing bronchiolitis. Multinucleate cells are occasionally formed by fusion of infected type 2 pneumocytes. Infection results in the induction of pro-inflammatory chemokines and cytokines and it has been suggested that much of the pathological changes are due to the host’s response (Valarcher and Taylor, 2007). Bovine respiratory syncytial virus is considered to be immunosuppressive. This effect, along with the accumulation of cellular debris and exudate in pulmonary airways, facilitates bacterial proliferation.

Clinical signs

Affected animals are typically between 3 and 9 months old. The incubation period is 2 to 5 days. Clinical signs, which range from mild to severe, include fever, nasal and lacrimal discharge, coughing and polypnoea. As the disease progresses, open-mouth and abdominal breathing may be present. The course of the disease is usually up to 2 weeks. A biphasic pattern is commonly observed in outbreaks among beef calves. Mild respiratory disease is followed by apparent recovery and, within a few days, dyspnoea and pulmonary emphysema develop. Mortality in these outbreaks may reach 20%.

Diagnosis

Clinical signs and pathological findings may permit a presumptive diagnosis. Laboratory confirmation is necessary for definitive diagnosis.

- Suitable specimens for laboratory examination include nasal swabs, bronchoalveolar lavage fluid, lung tissue and paired serum samples. Specimens should be collected from several animals in the affected group.
- As the virus is thermolabile, specimens must be transferred promptly to the laboratory in a suitable transport medium.
- Virus isolation is not attempted routinely as it is difficult and requires several blind passages in cell culture.
- Commercial ELISA kits are available for the detection of viral antigen. Immunofluorescence is a rapid and useful technique. Viral antigen can be detected more readily in specimens from the lower respiratory tract than in nasal swabs.
- Polymerase chain reaction procedures have been described for the detection of specific viral nucleic acid (Larsen *et al*., 1999; Valarcher *et al*., 1999; Willoughby *et al*., 2008).
- Suitable serological tests for demonstrating a rising antibody titre include virus neutralization and ELISA. Serum samples should be taken early in the course of the disease as antibody levels tend to rise rapidly.

Control

Suitable control measures include reducing stress factors, maintaining good hygiene in calf pens, rearing calves away from older age groups and implementing a closed herd policy. Prior vaccination with formalin-inactivated

virus may exacerbate BRSV disease (Antonis *et al*., 2003). Modified live vaccines may be administered parenterally or intranasally. Although vaccination tends to reduce the likelihood of clinical disease in exposed animals, the duration of protection is short, and frequent boosters may be required. Vaccines developed for the control of BRSV infection include an intranasal recombinant herpes-1 vaccine expressing the attachment (G) glycoprotein (Taylor *et al*., 1998), a vaccinia recombinant virus (Antonis *et al*., 2003), a DNA vaccine encoding the fusion gene (Taylor *et al*., 2005) and a subunit fusion glycoprotein vaccine expressed in recombinant baculovirus-infected insect cells (Sharma *et al*., 1996).

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Chapter 73

Rhabdoviridae

Members of the family *Rhabdoviridae* (Greek *rhabdos*, rod) have characteristic rod shapes (Fig. 73.1). This family, along with the families *Paramyxoviridae, Bornaviridae* and *Filoviridae*, belongs to the order *Mononegavirales* (Fig. 73.2). Viruses in this order possess a linear, non-segmented, single-stranded RNA genome of negative polarity encased in a ribonucleo-protein complex. Rhabdoviruses of vertebrates are bullet-shaped or cone-shaped while those infecting plants are generally bacilliform. This large family contains viruses of vertebrates, invertebrates and plants. The family *Rhabdoviridae* comprises six genera: *Vesiculovirus, Lyssavirus, Ephemerovirus, Cytorhabdo-virus, Novir habdovirus* and *Nucleorhabdovirus*. Moreover, a large number of rhabdoviruses have yet to be assigned to a genus. *Vesiculovirus, Lyssavirus* and *Ephemerovirus* genera contain viruses that infect vertebrates. Infectious haematopoietic necrosis virus and related rhabdoviruses of fish are included in the genus *Novirhabdovirus*.

Rhabdoviruses usually contain five major proteins: a large RNA-dependent RNA polymerase (L), a surface glycoprotein (G), a nucleoprotein (N), a protein component of the viral polymerase (P) and a matrix protein (M). The G protein forms the surface peplomers which interact with host cell receptors, facilitating endocytosis of the virion. In addition, the G protein induces virus-neutralizing antibodies and cell-mediated immunity. Replication occurs in the cytoplasm (with the exception of nucleorhabdoviruses). Newly synthesized nucleocapsids acquire envelopes from the plasma membrane as virions bud from the cell. Virions (100 to 430nm × 45 to 100 nm) are stable in the pH range 5 to 10. They are rapidly inactivated by heating at 56°C, by treatment with lipid solvents and by exposure to UV light.

Key points

- Enveloped RNA viruses with helical symmetry and rod-shaped morphology
- Rabies virus and related lyssaviruses

–present in saliva; transmitted by biting carnivores and bats

–cause encephalitis in mammals which is invariably fatal

- Vesicular stomatitis viruses

  –transmitted by direct contact and environmental contamination or by arthropod vectors

  –cause febrile disease with vesicular lesions especially in cattle, horses and pigs

- Bovine ephemeral fever virus

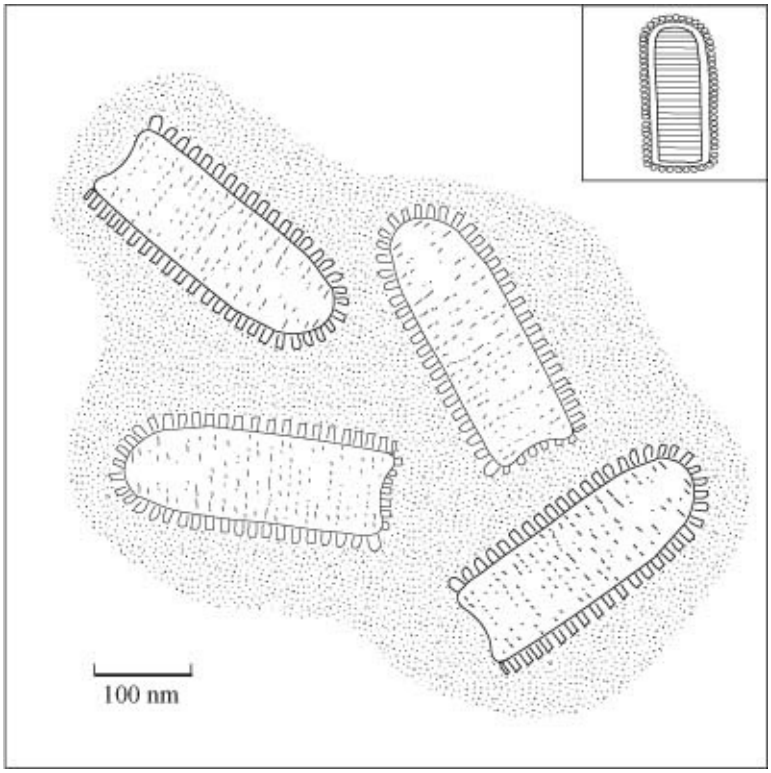
  – transmitted by biting arthropods

  – causes transient febrile illness with ill-defined clinical signs

Clinical infections

Rhabdoviruses of veterinary importance are presented in Tables 73.1 and 73.2. They can be transmitted by bites of mammals, arthropod vectors or direct contact. Infection may also be acquired through environmental contamination.

Figure 73.1 Rhabdovirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



The best known and most important member of the *Rhabdoviridae* is rabies virus, a *Lyssavirus* (Greek *lyssa*, rage or fury). A number of distinct *Lyssavirus*

genotypes produce clinical signs indistinguishable from rabies (Table 73.1). Four new lyssaviruses have been isolated from bat species: Aravan virus, Khujand virus, Irkut virus and West Caucasian bat virus. More than 25 viruses isolated from animals have been classified in the genus *Vesiculovirus* The most important vesiculoviruses which infect domestic animals are the vesicular stomatitis Indiana virus and the vesicular stomatitis New Jersey virus (Table 73.2). Bovine ephemeral fever virus, of significance in some countries, is the type species of the genus *Ephemerovirus*. Some fish diseases, such as infectious haematopoietic necrosis, viral haemorrhagic septicaemia and spring viraemia of carp are also caused by rhabdoviruses.

Rabies

This viral infection, which affects the central nervous system of most mammals including humans, is invariably fatal. However, mammalian species vary widely in their susceptibility. Most clinical cases are due to infection with rabies virus (genotype 1). A number of other neurotropic lyssaviruses, closely related to the rabies virus, produce clinical signs indistinguishable from rabies (Table 73.1). Genetic sequencing and antigenic studies have been used to categorize lyssaviruses into seven genotypes and four serotypes (Smith, 1996; Gould *et al*., 1998). Each genotype is assigned separate species status. Classical rabies caused by genotype 1 lyssavirus is endemic on continental land masses with the exception of Australia and Antarctica. Many island countries are also free of the disease.

Epidemiology

Several species-adapted strains of rabies virus have been described. Strains affecting a particular species are transmitted more readily to members of that species than to other animal species. In a given geographical region, rabies is usually maintained and transmitted by particular mammalian reservoir hosts. Two epidemiologically important infectious cycles are recognized, urban rabies in dogs and sylvatic rabies in wildlife. More than 95% of human cases are the result of bites from rabid dogs. Racoons, skunks, foxes and bats are important reservoirs of rabies virus in North America (Krebs *et al*., 1998). In continental Europe, the principal reservoir is the red fox. The vampire bat is an important reservoir of the virus in Central and South America and in the Caribbean islands. In developed countries, the control of stray dogs and the use of vaccination programmes have reduced the importance of urban rabies and have focused

attention on wildlife reservoirs.

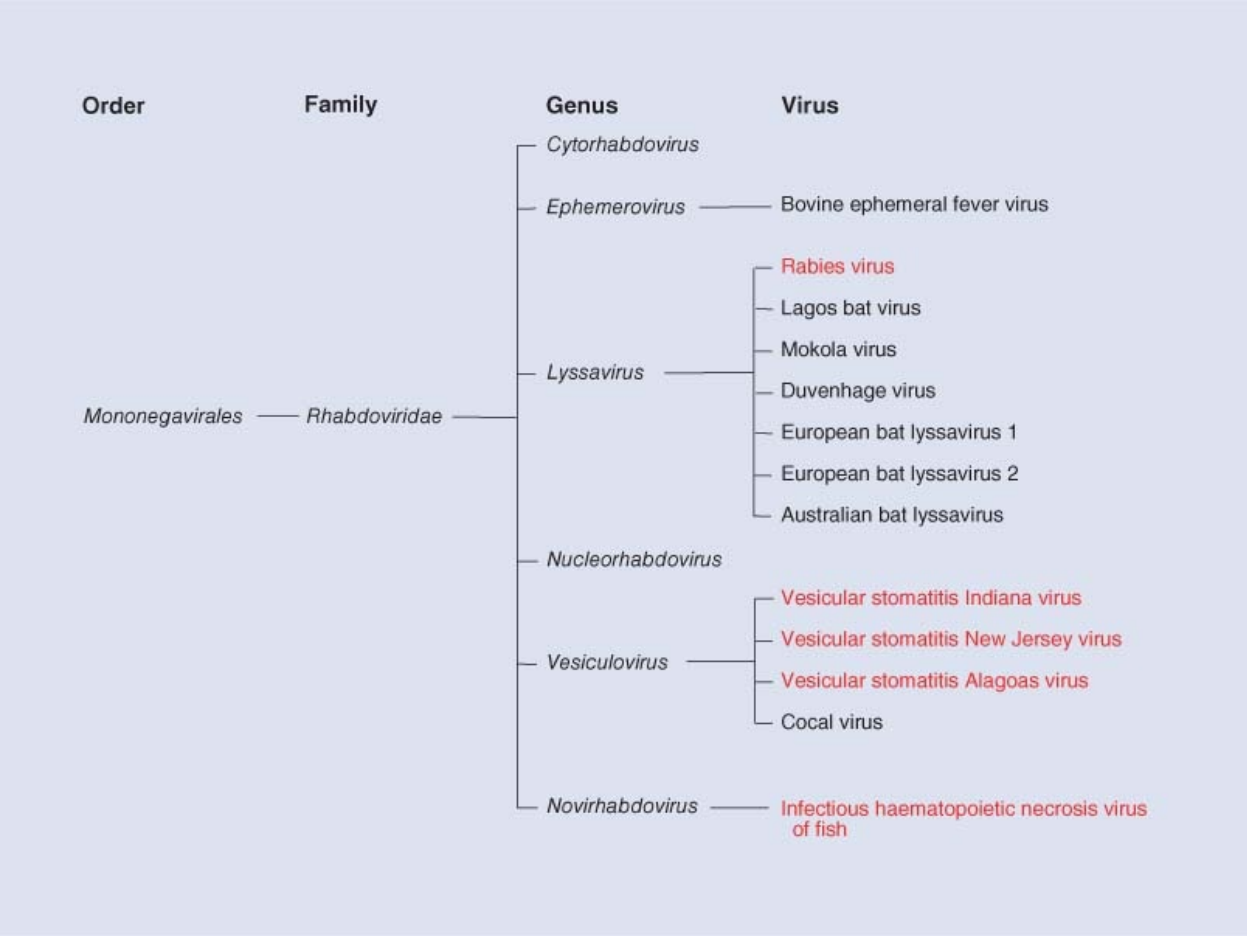
Species susceptibility to rabies virus is important epidemiologically. Domestic animals and humans are considered to be moderately susceptible to the virus, whereas foxes, wolves, coyotes and jackals are considered to be highly susceptible. Although virus may be transmitted through scratching and licking, transmission usually occurs through bites. Infected animals may excrete virus in their saliva for some time before the onset of clinical signs.

Pathogenesis

Following introduction into the tissues, virus enters peripheral nerve endings. There may be limited replication locally in myocytes or other tissue cells. The virus is transported to the central nervous system by retrograde axoplasmic flow and becomes widely disseminated in nervous tissue by intra-axonal spread. Clinical signs develop following neuronal damage caused by viral replication. Virus spreads centrifu-gally within nerve cell processes and is released at axon terminals where it infects many non-nervous tissues including the salivary glands. The presence of virus in saliva, especially in carnivores, is an important factor in rabies transmission.

Although rabies viral antigens are highly immuno-genic, immune detection is delayed because intracellular transport prevents contact with the cells of the immune system in the early stages of infection.

Figure 73.2 A classification of rhabdoviruses with emphasis on those of veterinary importance. Viruses in red cause OIE-listed diseases.



Clinical signs

The incubation period, which is highly variable and can be of many months' duration, is influenced by various factors including host species, virus strain, the quantity of virus in the inoculum and the site of introduction of the virus. Large amounts of virus introduced into deep bite wounds in the head region are usually associated with short incubation periods. The clinical course in domestic carnivores, which usually lasts for days, may encompass prodromal, furious (excitative) and dumb (paralytic) phases. In certain rabid animals, some of these phases may not be observed. In the prodromal phase, affected animals are often confused and disorientated; wild animals may lose their natural fear of humans. The furious phase is characterized by an increase in aggressiveness and hyperexcitability, and there is a tendency to bite at inanimate objects and at other animals. Affected animals may roam over long distances. The furious form is observed more often in cats than in dogs. Foxes rarely exhibit this form of the disease. In dumb rabies, muscle weakness, difficulty in swallowing, profuse

salivation and dropping of the jaw are the usual features. These cli nical signs may be mistaken for those caused by a foreign body in the mouth or throat. The term hydrophobia, a synonym for rabies in humans, relates to the ina bility to swallow water because of pharyngeal paralysis.

Diagnosis

Ante mortem diagnostic tests for rabies are not generally used except in humans where saliva is examined by PCR. In endemic areas, suspect domestic carnivores which have bitten humans should be isolated and observed for up to 7 days. The brains of animals that develop clinical signs should be examined for the presence of virus. Rabies virus is particularly abundant in Ammon's Horn of the hippocampus, cerebrum, cerebellum and medulla. Rapid laboratory confirmation is essential for the implementation of appropriate treatment of human patients.

- The preferred method of diagnosis is the direct fluorescent antibody test (FAT) on acetone-fixed brain tissue smears. The FAT provides a rapid and specific diagnosis but may yield false-negative results with autolysed brain specimens. The conjugated antisera used for diagnosis are usually most sensitive for detecting rabies virus (serotype 1), but will detect other serotypes if a potent conjugate directed against conserved antigenic sites on the nucleocapsid proteins is used.
- Non-suppurative encephalitis characterized by perivascular lymphoid cuffing and intracytoplasmic inclusions (Negri bodies) may be demonstrable histologically.
- Rabies virus can be cultured in neuroblastoma cells or in baby hamster kidney cells. Culture of the virus is of value when results of the FAT are uncertain. Rabies virus, which is non-cytopathic, can be detected in tissue culture using conjugated antisera.
- Unweaned mice, inoculated intracerebrally with brain tissue from suspect rabies cases, should be observed over several days for the development of disease. The FAT is used to confirm the presence of rabies virus in infected mice.
- Reverse transcriptase polymerase chain reaction (RT-PCR) has been used to detect viral RNA in brain samples. This test can distinguish rabies virus (genotype 1) from rabies-related lyssaviruses. The sensitivity of RT-PCR can be enhanced by combining the technique with ELISA which aids detection of amplified product (Whitby *et al*., 1997).

- Serological tests, although rarely used to confirm a diagnosis due to late seroconversion, are commonly used for trade and for animal movement. Internationally recognized virus neutralization tests include the rapid fluorescent focus inhibition test (RFFIT) and the fluorescent antibody virus neutralization test (FAVN). Commercially available indirect ELISAs, using rabies glycoprotein as antigen, are useful screening tests to determine if vaccinated cats and dogs have seroconverted.

Table 73.1 Lyssaviruses which cause classical rabies and rabies-like disease.

Virus	Phylogroup	Genotype	Serotype	Geographical distribution	Comments
Rabies virus	1	1	1	Apart from Australia and Antartica, rabies virus (genotype 1) occurs on all continents. Many island countries are free of the disease	Causes fatal encephalitis in many mammalian species. Transmitted by wildlife species including foxes, racoons and bats; domestic carnivores also involved in transmission. Rabies is a major zoonotic disease with more than 50,000 human fatalities worldwide each year
Lagos bat virus	2	2	2	Africa	Isolated initially from fruit bats; also isolated from domestic animals with encephalitis
Mokola virus	2	3	3	Africa	Isolated initially from shrews; also isolated from domestic animals. Human infection reported
Duvenhage virus	1	4	4	Africa	Originally isolated from a human bitten by an insectivorous bat; additional cases reported in humans. Not reported in domestic animals
European bat lyssavirus 1	1	5	–	Europe	Identified with increasing frequency in insectivorous bats. Human infection reported
European bat lyssavirus 2	1	6	–	Europe	Isolated initially from a human with symptoms of rabies; present in insectivorous bats. Additional human cases reported; not reported in domestic animals
Australian bat lyssavirus	1	7	–	Australia	Identified in fruit bats and in insectivorous bats; human infection reported

Table 73.2 Viruses of veterinary significance in the genera *Vesiculovirus* and *Ephemerovirus*.

Genus, virus	Hosts	Comments
Vesiculovirus		
Vesicular stomatitis Indiana virus	Cattle, horses, pigs, humans	Causes febrile disease with vesicular lesions; resembles foot-and-mouth disease clinically. Occurs in North and South America
Vesicular stomatitis New Jersey virus	Cattle, horses, pigs, humans	Causes febrile disease with vesicular lesions; infection more severe than that caused by the Indiana virus. Occurs in North and South America
Vesicular stomatitis Alagoas virus (Brazil virus)	Horses, mules, cattle, humans	Originally isolated from mules in Brazil
Cocal virus (Argentina virus)	Horses	Isolated initially from mites in Trinidad; occurs in South America
Ephemerovirus		

Bovine ephemeral fever virus	Cattle	Causes febrile illness of short duration; occurs in Africa, Asia and Australia

Control

Most countries that are free of rabies rely on rigorous quarantine measures to prevent the introduction of disease. Movement of vaccinated domestic carnivores is permitted between some countries provided that strict identification and testing procedures are in place. In countries where rabies is endemic, control methods are aimed mainly at reservoir species. Urban rabies can be effectively controlled by vaccination and restriction of dog and cat movement and by the elimination of stray animals. Control of sylvatic rabies requires special measures. Regional depopulation of reservoir species, which has rarely been successful, is ecologically unacceptable. Vaccination of red foxes with live oral vaccines, delivered in baits, has eliminated sylvatic rabies from several regions of western Europe. A number of modified live rabies virus vaccines have been used including SAD B19, SAG-1 and SAG-2. However, as a result of concerns about the safety of live vaccines, a vaccinia–rabies virus glycoprotein (VRG) recombinant vaccine was developed and has proved effective for vaccinating foxes (Pastoret and Brochier, 1999), coyotes (Fearneyhough *et al*., 1998) and racoons (Hanlon *et al*., 1998). The rapid increase in racoon rabies in the USA has proved difficult to control through vaccination (Smith, 1996), and several challenges remain for successful eradication in North America including development of practical vaccination methods for bats (Rupprecht *et al*., 2004).

Commercial vaccines available for the immunization of domestic carnivores by parenteral inoculation contain inactivated virus (genotype 1) and are potent and safe. These inactivated rabies vaccines are considered to be effective against strains of genotype 1 virus. However, their ability to induce a protective immune response against other lyssavirus genotypes is variable and cross-protection against individual lyssavi-ruses such as Mokola virus may not develop. A commercial recombinant canarypox virus vaccine is available for cats. Promising developments include oral vaccination using either a recombinant canine type-2 adenovirus expressing the rabies virus glycoprotein (Tordo *et al*., 2008) or a double glycoprotein rabies virus (RV) variant as a live-attenuated vaccine (Faber *et al*., 2009).

Vesicular stomatitis

This febrile disease affects mainly horses, cattle and pigs. Other susceptible

species include camelids, several wildlife species and humans. Vesicular stomatitis is clinically similar to foot-and-mouth disease and is a disease listed by the Office International des Epizooties. A number of closely related, antigenically distinct members of the genus *Vesiculovirus*, of which the type species is vesicular stomatitis Indiana virus, can cause the disease. Most outbreaks are associated with vesicular stomatitis Indiana virus or with vesicular stomatitis New Jersey virus, which is more virulent. Cocal virus and vesicular stomatitis Alagoas virus, also referred to as subtypes 2 and 3 of vesicular stomatitis Indiana virus respectively, have been isolated from outbreaks of disease in horses and cattle in South America (Table 73.2).

Epidemiology

Infection is endemic in Central America and in regions of South America and the USA. Outbreaks of the disease occur every 2 to 3 years in tropical and subtropical regions, with clinical cases most common at the end of the rainy season and early in the dry season. Rapid spread from endemic areas to other regions may occur during some summer seasons. Disease outbreaks in temperate regions, which occur every 5 to 10 years, usually cease abruptly with the onset of winter.

Although the mode of transmission is incompletely understood, direct contact and insect vectors have been implicated. Virus is shed in saliva and can contaminate water and feed troughs. The involvement of insect vectors is inferred from the seasonal occurrence of cases and from the pattern of spread with clustering of cases along river valleys and in irrigated areas. Virus has been isolated from many insect species including blackflies, mosquitoes, sandflies and house-flies. Viral replication in blackflies has been demonstrated experimentally. It is unclear how biting insects acquire the virus from domestic animals as a viraemic phase has not been demonstrated in these species.

Pathogenesis

Virus probably enters the body through abrasions on the skin or mucous membranes or following an insect bite. Vesicles, which develop at the site of infection, may coalesce. Spread may occur locally by extension from primary lesions. Although secondary lesions at distant sites may develop, it is unclear how transfer of the virus occurs and if these lesions result from viraemia or following environmental contamination (Clarke *et al*., 1996).

Clinical signs

The incubation period is up to 5 days. Subclinical infection is common: around 10 to 15% of animals in infected herds show clinical signs. Affected animals, which are usually more than 1 year old, become febrile. Vesicles develop on the tongue and on oral mucous membranes, often accompanied by profuse salivation. Secondary lesions may occur on the coronary band and teats. Lameness is often a prominent feature of the disease in pigs. Mastitis may develop in cows with severe teat lesions. In the absence of secondary infection, lesions generally heal within 2 weeks.

The economic impact of the disease relates to production losses, culling and other disease control measures (Hayek *et al*., 1998). Following infection, animals develop high levels of neutralizing antibodies but the duration of protection is variable. Cross-protection between vesicular stomatitis Indiana virus and vesicular stomatitis New Jersey virus is limited.

Diagnosis

Because of similarities between vesicular stomatitis, foot-and-mouth disease and swine vesicular disease, prompt laboratory confirmation is required. If horses present with vesicular lesions, infection with vesicular stomatitis virus should be considered.

- Suitable specimens for isolation of virus or for the detection of viral antigen include epithelium from lesions and vesicular fluid.
- Viral antigen can be detected by CFT or ELISA.
- Virus may be isolated in suitable cell lines, in embryonated eggs or in suckling mice by intracerebral inoculation. The virus is cytopathic. The fluorescent antibody test, ELISA, CFT or the virus neutralization test are suitable procedures for identification of isolates.
- Electron microscopy can be used to identify virus in specimens or tissue culture.
- Protocols for RT-PCR for the diagnosis of vesicular stomatitis have been published (Rasmussen *et al*., 2005).
- Antibody levels in recovered animals may be assayed by liquid-phase blocking ELISA, CFT, the virus neutralization test, competitive ELISA or IgM-specific capture ELISA. Because levels of complement-fixing and IgM antibodies persist for only short periods, assays based on procedures

involving these antibodies can be used to confirm recent infections in endemic areas.

Treatment and control

- Specific treatment is not available. Measures aimed at minimizing secondary infections may be beneficial.
- Suspected cases should be notified to the relevant authorities. Movement restrictions and a 30-day quarantine period following the last clinical case are recommended for infected premises. International trade restrictions are generally instituted following an outbreak.
- Insect-proofing of buildings and avoidance of habitats associated with insect vectors reduce the likelihood of infection.
- Although both inactivated and attenuated vaccines have been used, they are not commercially available.
- Vesicular stomatitis is a zoonotic disease with clinical signs that resemble influenza.

Bovine ephemeral fever

This arthropod-borne viral disease of cattle and water buffalo occurs in tropical and subtropical regions of Africa, Asia and Australia. The virus causes subclinical infection in many other ruminant species including Cape buffalo, wildebeest, waterbuck and deer.

Epidemiology

Epidemiological evidence suggests that insects, particularly mosquitoes and possibly certain midge species, are involved in transmission of the virus. In tropical areas where bovine ephemeral fever is endemic, subclinical infections are common. Outbreaks often follow periods of rainfall. In more temperate regions, epidemics occur during summer months and tend to decline with the onset of winter. Transmission does not occur by direct contact or by fomites. Persistence of virus in animals following recovery from acute illness has not been documented. It is probable that virus persistence occurs in arthropod vectors.

Pathogenesis

Blood-sucking insects acquire the virus when feeding on animals during the brief viraemic stage of the disease. The virus, which multiplies in the insect vector, is shed in its saliva and is transmitted to a new host during feeding. The virus primarily affects the endothelium of small blood vessels and gives rise to a major systemic inflammatory response. Many of the pathological changes observed in infected animals are attributed to host response rather than to direct viral damage.

Clinical signs

The incubation period is typically 3 to 5 days. The severity of the clinical signs is influenced by the immune status of infected animals and the virulence of the virus strain. The disease tends to be severe in well-fed animals and high-yielding dairy cows. A biphasic high fever is commonly observed. Affected animals become depressed, anorexic, lame and constipated. Milk production drops dramatically. Muscle stiffness and ruminal stasis may develop. Pregnant animals may abort. Recumbency may be accompanied by salivation and ocular and nasal discharge. Muscular fibrillation and paresis frequently occur, reflecting the accompanying hypocalcaemia. The disease is of short duration and affected animals usually recover after a few days. Most recovered animals develop a solid immunity.

Diagnosis

Diagnosis of bovine ephemeral fever is usually based on clinical signs. Virus neutralization tests or ELISA should be carried out on paired serum samples to detect a rise in virus-specific antibody. Other serological tests such as immunofluorescence are less useful as interpretation is complicated by cross-reacting antibodies induced by infections with related non-pathogenic ephemeroviruses such as Kimberley virus. Neutrophilia, increased plasma fibrinogen and decreased plasma calcium levels are commonly present. Isolation of bovine ephemeral fever virus is difficult. A real time RT-PCR protocol for detection of viral RNA has been described (Stram *et al*., 2005).

Treatment

Affected animals should be rested. Anti-inflammatory drugs such as phenylbutazone, flunixin meglumine and ketoprofen have proved useful for treatment (Fenwick and Daniel, 1996). Intravenous or subcutaneous

administration of calcium borogluconate is recommended. Oral drenching should be avoided during the acute phase of the illness because swallowing may be impaired.

Control

Vector control is usually impractical in endemic areas. Control is based on the use of vaccines, both inactivated and attenuated. Trials have been carried out using a subunit vaccine based on the envelope glycoprotein (Uren *et al*., 1994). A recombinant virus-vectored glycoprotein vaccine has been developed.

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Chapter 74

Bornaviridae

The family *Bornaviridae*, which belongs to the order *Mononegavirales*, contains a single genus, *Bornavirus*. The sole member of the genus is Borna disease virus (BDV). This enveloped virus, which has only recently been demonstrated by electron microscopy, is spherical with a diameter of about 90 nm (Fig. 74.1). The envelope surrounds an inner core, 50 to 60 nm in diameter. The genome consists of a single molecule of negative-sense, single-stranded RNA. In contrast to other non-segmented, negative-sense RNA animal viruses, replication occurs in the nucleus of host cells with budding at the cell surface. The virus is highly neurotropic and highly cell-associated, spreading by cell-to-cell contact in a non-cytolytic manner. This labile virus is sensitive to heat, lipid solvents and low pH values.

Clinical infections

Borna disease virus is associated with a severe T cellmediated meningoencephalitis in horses and sheep in central Europe. For many years efforts have been made to confirm a link between the virus and staggering disease in cats. Recently a number of reports have suggested that proventricular dilatation disease, a fatal disorder of parrots, is caused by an avian bornavirus (Honkavuori *et al*., 2008; Kistler *et al*., 2008).

Borna disease

This fatal neurological disease of horses is named after Borna, the town in Saxony where in 1885 many horses died in an epidemic of the disease. The disease occurs sporadically in Germany, Switzerland and other parts of Europe. It has also been reported in Japan. Seroepidemiological studies, however, indicate a wide geographical distribution. Antibodies reacting with BDV have been found in the sera of human patients with psychiatric disorders and a possible link between infection with the virus and human neurological disorders

has been proposed (Boucher *et al*., 1999).

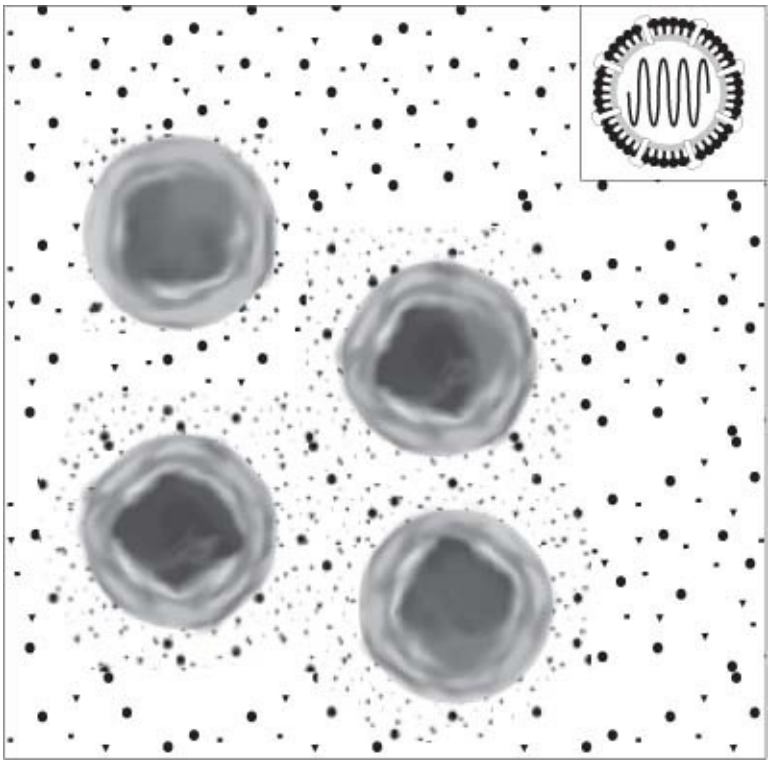
Key points

- Labile, enveloped, spherical virus with a particular affinity for nervous tissues
- Genome consists of negative-sense, single-stranded RNA
- Replicates in nuclei of neural cells
- The single genus *Bornavirus* contains one species
- Causes Borna disease, an infection of the CNS, fatal in some domestic species, particularly horses

Epidemiology

Neurological disease attributed to BDV has been described in horses, sheep and cats. Serological evidence of infection has been recorded in several other species including cattle, rabbits, parrots and ostriches. The virus, which is thought to be present in nasal, salivary and conjunctival secretions, may be transmitted through ingestion or, more likely, through inhalation. Most cases of Borna disease occur in spring and early summer; prevalence varies from year to year. It has been suggested that rodents and wild birds may act as reservoir hosts (Berg *et al*., 2001; Hilbe *et al*., 2006). Persistent infections can be established experimentally in rats.

Figure 74.1 Bornavirus particles as they appear in an electron micrograph and diagrammatic representation (inset).



Pathogenesis and pathology

Following oronasal infection, the virus gains entry to the CNS by intra-axonal spread either through the olfactory nerve or through nerves supplying the oropharyngeal and intestinal regions. Spread within the CNS and into the peripheral nerves also occurs within axons. Viral antigens expressed on the surface of infected neurons induce a cell-mediated response involving cytotoxic $CD8^+$ and resulting in destruction of the infected cells. A non-suppurative encephalitis with lymphocytic perivascular cuffing is largely confined to grey matter. Neuronal degeneration is prominent and intranuclear inclusions (Joest-Degen bodies) may be present, particularly in the hippocampus. Antibody levels in infected animals, which are relatively low and non-protective, are not linked to the pathogenesis of the disease. Despite the persistence of infection, clinical signs do not develop in immunodeficient animals.

Clinical signs

Borna disease has been described mainly in young horses. The incubation period, which is highly variable, ranges from weeks to several months. Factors which may influence the severity of clinical signs include the age and immunological status of the infected animal and the strain of infecting virus. On

farms where infection in horses is present, clinical disease is usually confined to individual animals. Clinical signs include fever, somnolence and evidence of neurological disturbance. Ataxia, pharyngeal paralysis and hyperaesthesia may be present. The course of the disease is up to 3 weeks and mortality rates may approach 100%. Surviving horses have permanent CNS damage and may exhibit recurrent episodes of neurological disturbance. ‘Staggering disease’ in cats has been associated with BDV infection (Lundgren *et al*., 1995). In defined geographical regions, sheep flocks with large numbers of seropositive animals have been reported.

Diagnosis

Borna disease may vaguely resemble other neurological conditions in the horse. However, the distribution of lesions in the CNS differs from that in other equine encephalomyelitides and, if eosinophilic intranuclear inclusions (Joest-Degen bodies) are present, they may be confirmatory. Viral antigen can be demonstrated in the brain by immunohistochemical methods. Demonstration of antibodies in serum or in cerebrospinal fluid by indirect immunofluorescence, immunoblotting or ELISA may aid diagnosis. Reverse transcriptase-PCR and real-time RT-PCR for the demonstration of BDV-RNA are valuable diagnostic tools (Legay *et al*., 2000; Wensman *et al*., 2007).

Control

Although BDV does not appear to be readily transmitted by infected horses, seropositive animals should be isolated. Standard hygienic measures should be applied to suspect animals. The disease is notifiable in Germany.

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Chapter 75

Bunyaviridae

The name of this family is derived from Bunyamwera, the place in Uganda where the type species Bunyamwera virus was first isolated. The family *Bunyaviridae* contains more than 300 viruses. Virions (80 to 120 nm in diameter) are spherical and enveloped. Glycoprotein peplomers project from the surface of the envelope which encloses three circular, helical, nucleocapsid segments (Fig. 75.1). The viruses are sensitive to heat, acid pH levels, lipid solvents, detergents and disinfectants. The genera in the family are *Orthobunyavirus, Phlebovirus, Nairovirus, Hantavirus* and *Tospovirus*. Based on antigenic relatedness, viruses within each genus are placed in serogroups. The genome consists of three single-stranded, negative or ambisense RNA segments designated small (S), medium (M) and large (L). Genetic reassortment occurs between closely related viruses. There are four structural proteins: a nucleocapsid (N) protein, a large (L) transcriptase protein and two glycoproteins (Gn and Gc). Haemagglutinating and neutralizing antigenic determinants are present on one or both of the envelope glycoproteins. Non-structural (NS) proteins may be expressed by the S segment (designated NSs) and by the M segment (designated NSm). Replication takes place in the cytoplasm of host cells. In the final stages of assembly, virions acquire envelopes by budding into the Golgi network. They are then transported through the cytoplasm in secretory vesicles and released by exocytosis at the cell surface.

Viruses in the genera *Orthobunyavirus, Phlebovirus, Nairovirus* and *Hantavirus* infect vertebrates; those in the genus *Tospovirus* infect plants.

Key points

- Medium-sized, enveloped, single-stranded RNA viruses
- Replicate in the cytoplasm
- Labile in the environment
- More than 300 viruses in the family, the majority are arthropod-borne
- The family is composed of five genera:
  – *Orthobunyavirus* contains viruses which cause congenital defects in cattle and sheep

– *Phlebovirus* contains the virus which causes Rift Valley fever

– *Nairovirus* contains the virus which causes Nairobi sheep disease

– *Hantavirus* contains many viruses which cause haemorrhagic fever in humans; rodents act as reservoirs

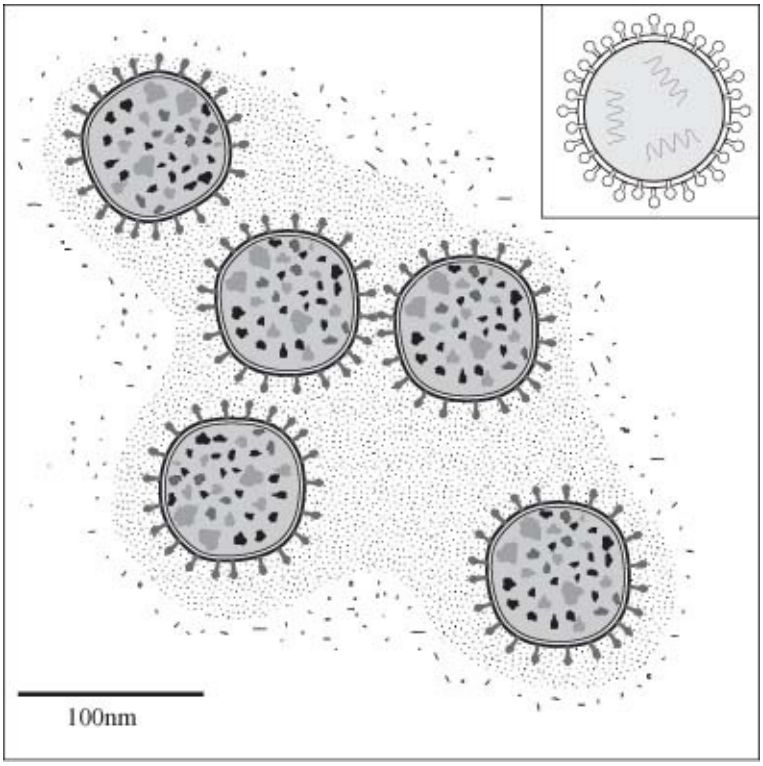
– *Tospovirus* contains viruses of plants

Clinical infections

With the exception of viruses in the genus *Hantavirus*, bunyaviruses are arthropod-borne. In nature, these arboviruses are maintained in complex transmission cycles involving replication in both arthropod vectors and vertebrate hosts. Infection of mammalian cells often results in cytolysis while infection of invertebrate cells is non-cytolytic and persistent. Mosquitoes are the most important vectors. Ticks, sandflies and midges may act as vectors for some bunyaviruses. Arthropod vectors acquire virus from vertebrate hosts during viraemic periods. Each bunyavirus species replicates in a limited number of vertebrate and invertebrate hosts.

Hantaviruses, which are primarily human pathogens, are maintained in nature by non-cytolytic, persistent infections in rodents, which shed virus in urine, faeces and saliva. Transmission between rodent hosts can occur by aerosols and biting. Individual hantaviruses are associated with particular rodent species. Many bunyaviruses infect humans and frequently cause serious diseases including California encephalitis, haemorrhagic fever with renal syndrome, hantavirus pulmonary syndrome and Crimean-Congo haemorrhagic fever. Such human infections are generally considered to be incidental and do not usually result in disease transmission.

Figure 75.1 Bunyavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Three important ruminant diseases, Rift Valley fever, Nairobi sheep disease and Akabane disease are caused by bunyaviruses (Table 75.1).

Rift valley fever

This peracute or acute disease of domestic ruminants in Africa is characterized by abortion and also by high mortality rates in newborn animals. Although a wide range of ruminant species are susceptible to infection, Rift Valley fever occurs primarily in sheep, cattle and goats. Inapparent infection occurs in camels but abortion rates may be as high as in cattle. Indigenous African ruminants are less susceptible to infection than imported species. Rift Valley fever is also an important zoonotic disease.

Table 75.1 Bunyaviruses of veterinary importance.

Genus	Virus	Hosts	Comments
Phlebovirus	Rift Valley fever virus	Sheep, cattle, goats	Causes high mortality rates in neonatal animals and abortion in pregnant animals. Endemic in southern and eastern Africa, transmitted by mosquitoes. Important zoonotic disease
Nairovirus	Nairobi sheep disease virus	Sheep, goats	Causes severe, often fatal disease in susceptible animals. Present in central and eastern Africa. Transmitted by ticks
Hantavirus	Hantaan virus, Puumala virus, Dobrava virus, Sin Nombre virus, Andes virus	Rodents	Rodent species are the major reservoir. Cause of haemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome in humans, usually transmitted by aerosol. Specific antibodies have been demonstrated in cats, dogs, cattle, pigs and deer but significance is unclear
Orthobunyavirus	Akabane virus, Tinaroo virus, Aino virus, Peaton virus, Douglas virus	Cattle, sheep	Viruses belonging to several serogroups within the genus, associated with congenital defects and abortion. Transmitted by mosquitoes and midges. Widely distributed geographically in tropical and subtropical regions of the Old World
	Cache Valley virus	Sheep	Belongs to the Bunyamwera serogroup; transmitted by mosquitoes. Occasionally associated with congenital defects in sheep flocks in North America

Epidemiology

Outbreaks of the disease tend to occur unpredictably in eastern and southern Africa at intervals of 5 or more years and are associated with abnormally heavy rains and a dramatic rise in vector populations. In exceptional years, large-scale epidemics have occurred in West Africa and in the Nile valley and delta. In recent years, outbreaks have occurred in Saudi Arabia and Yemen. Transovarial transmission of Rift Valley fever virus (RVFV) occurs in *Aedes* species. During inter-epidemic periods, the virus is maintained in the eggs of floodwater species of mosquito laid in undrained shallow depressions at the edges of temporary pools. Eggs of these species must remain dry for a period before they hatch following re-immersion in water. During epidemics, RVFV replicates in both wild and domestic ruminants and can be transmitted by many species of mosquitoes. Humans are frequently infected during these epidemics. Infected ruminants develop marked viraemia for up to 5 days after infection. During this period, blood and tissues of affected animals are infectious. Direct and indirect transmission can occur through aerosols, contact with infected placentae or aborted foetuses, fomites or mechanical transfer on the mouthparts of flies. Abattoir workers and veterinarians are particularly at risk of acquiring infection.

Pathogenesis and pathology

Following infection and local replication, viraemia leads to invasion of the liver

and other major organs. Cell necrosis is widespread, particularly in the liver. In pregnant animals the virus crosses the placenta and the extensive cytolysis results in foetal death.

Clinical signs

In mature sheep and goats, clinical signs include fever, weakness, regurgitation of ingesta, foetid diarrhoea, blood-tinged mucopurulent nasal discharge and, occasionally, icterus. Pregnant ewes may abort. Abortion rates approaching 100% are not uncommon. Mortality rates in adult sheep may be up to 60%. In lambs, the incubation period is up to 36 hours. Affected animals are pyrexic, listless and disinclined to move; they may show signs of abdominal pain. Affected lambs rarely survive more than 36 hours after the onset of clinical signs. The mortality rate in lambs less than 1 week old approaches 90%. In cattle, mortality rates are usually below 10% and abortion rates range from 15% to 40%.

Human infections with RVFV are often inapparent or may present as a moderate to severe influenza-like illness. The haemorrhagic and encephalitic forms of the disease, which occur in a small number of patients, can be fatal.

Diagnosis

- The histopathological lesions, particularly in the livers of lambs, are considered pathognomonic. Demonstration of viral antigen in fixed tissues by immunohistochemical methods is confirmatory.
- Virus can be isolated in suitable cell cultures, susceptible laboratory animals or embryonated eggs. Specimens for laboratory examinations include blood from viraemic animals, foetal organs and post-mortem specimens of liver, spleen and brain. Because the virus can infect laboratory personnel, suspect specimens should be handled only in properly equipped laboratories.
- Rapid confirmatory tests include detection of viral antigen in serum by ELISA or in impression smears by immunofluorescence. Viral RNA can be readily detected in serum and tissues, as well as in mosquitoes, using RT-PCR (Sall *et al*., 2001; Bird *et al*., 2007).
- Demonstration of IgM antibodies by ELISA in a serum sample or of seroconversion in paired serum samples by virus neutralization, ELISA or haemagglutination inhibition may be used for confirmation of the disease.

Control

Although vector control and environmental management can assist in limiting the spread of Rift Valley fever, such measures are often not feasible. Modified live vaccine containing Smithburn's attenuated strain of RVFV is widely used in endemic regions and during outbreaks. This vaccine is unsafe in pregnant animals because it may cause congenital defects or abortion. A mutagen-attenuated vaccine, developed for use in humans, is effective but may produce teratogenic effects depending on the stage of pregnancy (Morrill *et al*., 1997; Hunter *et al*, 2002). Another candidate vaccine is the attenuated clone 13, a small plaque variant which has a large deletion in the small RNA segment that codes for non-structural proteins (Muller *et al*., 1995). Inactivated vaccines prepared from highly immunogenic, virulent strains of RVFV are suitable for use in pregnant animals and may be used in Rift Valley fever-free countries bordering endemic regions.

Nairobi sheep disease

This severe, tick-borne, viral infection of sheep and goats occurs in central and eastern Africa. The causal agent, Nairobi sheep disease virus (NSDV), is closely related to Ganjam virus of sheep and goats in India. The virus is currently classified as a strain of Dugbe virus. Although humans are susceptible to NSDV, infection appears to be uncommon.

Epidemiology

The brown ear tick (*Rhipicephalus appendiculatus*) is the principal vector of the virus. Transovarial and transstadial infection occurs with transmission by all stages of the tick. In endemic areas, lambs and kids exposed to infection while protected by maternally-derived antibody develop active immunity. Outbreaks of disease arise from movement of susceptible animals into endemic areas or from the introduction of infected ticks into Nairobi sheep disease-free areas.

Pathogenesis

Following inoculation by an infected tick, regional lymph nodes draining the site become enlarged and oedematous. The virus has a predilection for vascular endothelium and replicates to high titres in liver, spleen, lungs and other organs.

Clinical signs

The incubation period is up to 6 days. There is marked pyrexia and depression followed, within 48 hours, by foetid dysentery. A mucopurulent nasal discharge and conjunctivitis may be observed. Pregnant animals often abort. The mortality rate ranges from 30% to 90%; death may occur up to 11 days after the onset of clinical signs. The disease is more severe in native breeds of sheep than in Merinos. Clinical signs are milder in goats than in sheep.

Diagnosis

A history of a high mortality rate in a flock recently introduced into an endemic area may point to Nairobi sheep disease. Specimens suitable for virus isolation in cell culture include blood, mesenteric lymph nodes and spleen from febrile or dead animals. Direct immunofluorescence is useful for identification of the virus in tissue culture cells. Viral antigen can be detected directly in tissue specimens by AGID. The indirect immunofluorescent test is recommended for the detection of antibodies to NSDV.

Control

Dipping is used to control the tick vector, and animals at risk should be vaccinated. Modified live and inactivated vaccines have been used experimentally but the limited demand has not justified commercial production.

Bunyaviruses and congenital defects in domestic ruminants

Orthobunyaviruses, which cause congenital defects, such as arthrogryposis and hydranencephaly, and abortion in cattle, sheep and goats include Akabane virus (Akabane serogroup), Aino virus (Shuni virus serogroup), Peaton virus (Shamonda virus serogroup), Douglas virus (Sathuperi virus serogroup) and Tinaroo virus (Akabane virus serogroup). Cache Valley virus (Bunyamwera serogroup) has been linked to neonatal deaths and malformed lambs in parts of the United States. The most important and virulent of these viruses is Akabane virus.

Akabane disease

Serological studies indicate a widespread distribution of this virus in tropical and subtropical regions in the Middle East, Asia, Australia and Africa. Sporadic epidemics, associated with developmental defects, have been described in Japan, Australia, Israel and parts of Africa. The virus is transmitted by midges and mosquitoes. Disease outbreaks appear to coincide with movements of the vectors or following the introduction of susceptible animals into endemic areas. Encephalomyelitis and polymyositis develop in foetuses infected with Akabane virus. The extent and degree of the pathological changes that occur following infection can be related to the stage of gestation at which infection occurred. The most severe damage, resulting in neurological defects, is evident in calves born to cows infected at 12 to 16 weeks of gestation. Clinical signs are generally not observed in the dam (Lee *et al*., 2002). Diagnosis is based on gross pathological findings in the foetal CNS and the detection of specific neutralizing antibody in the sera of aborted calves or of newborn animals prior to suckling. Molecular techniques for the detection and differentiation of Akabane virus from other orthobunyaviruses have been described (Akashi *et al*., 1999; Stram *et al*., 2004). Vector control and vaccination are the methods used to prevent outbreaks of the disease. An inactivated vaccine is available in Japan and Australia. A live vaccine is commercially available in Japan. In order to minimize the occurrence of congenital defects in cattle and sheep, breeding stock should be introduced into endemic areas in advance of the breeding season.

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Chapter 76

Picornaviridae

Picornaviruses (Spanish *pico,* very small), which are icosahedral and non-enveloped, contain a molecule of single-stranded RNA. Virions are 30 nm in diameter (Fig. 76.1). The capsid is composed of 60 identical subunits, each containing four major proteins, VP1, VP2, VP3 and VP4. The VP4 protein is located on the inner surface of the capsid. Viral replication occurs in the cytoplasm in membrane-associated complexes and infection is usually cytolytic. Picornaviruses are resistant to ether, chloroform and non-ionic detergents. Individual genera differ in their thermal lability and pH stability. A substantial reorganization of the family has occurred in recent years. The family originally comprised eight genera: *Enterovirus, Cardiovirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus* and *Teschovirus,* Four new genera have recently been added: *Tremovirus, Sapelovirus, Senecavirus* and *Avihepatovirus*. The genus *Rhinovirus* was removed following the re-classification of human rhinovirus A and human rhinovirus B as enteroviruses. Many of the porcine isolates, referred to as porcine enteroviruses (PEV) 1-13, have been placed in the genus *Teschovirus*. Several isolates previously referred to as avian enteroviruses have been renamed and reassigned to another genus (*Sapelovirus* and *Avihepatovirus*) or left unassigned, while avian nephritis virus 1 and 2 have been renamed chicken astrovirus and placed in the family *Astroviridae*.

Viruses of veterinary importance in the family *Picornaviridae* are presented in Fig. 76.2. Aphthoviruses are unstable at pH values below 6.5 and rhinoviruses are unstable below pH 5.0. Other picornaviruses are generally stable at acid pH values. Some viruses in the genera *Hepatovirus* and *Parechovirus,* including hepatitis A virus, are important human pathogens. Poliovirus (human enterovirus C), which causes serious neurological disease in humans, and the human rhinoviruses, which are associated with the common cold in humans, are enteroviruses.

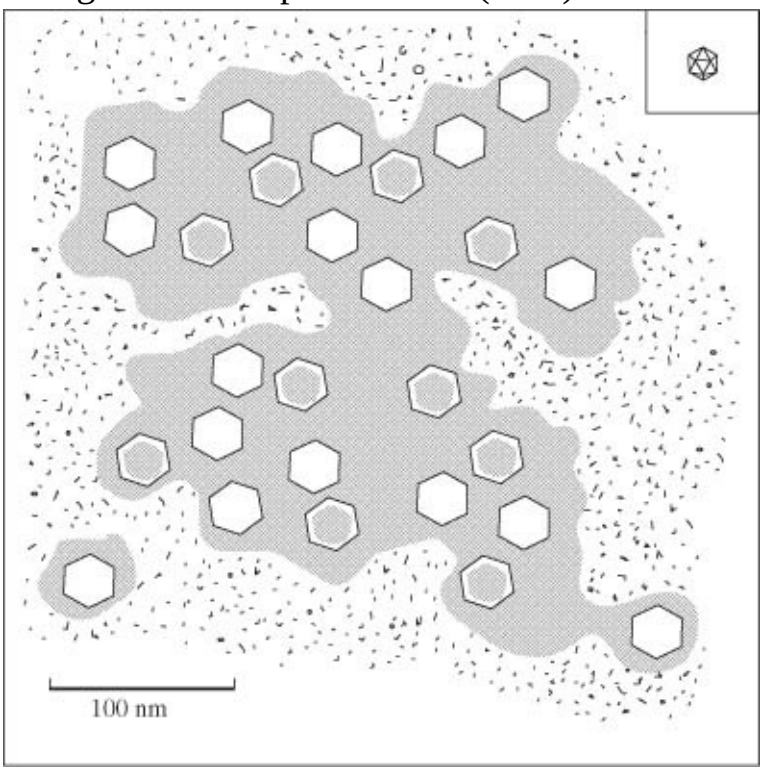
Key points

- Non-enveloped, positive-sense, single-stranded RNA viruses with icosahedral symmetry
- Replicate in cytoplasm
- Resistant to many organic solvents; individual members differ in their susceptibility to pH change
- A number of genera: *Aphthovirus, Enterovirus, Teschovirus, Erbovirus, Cardiovirus* and *Tremovirus* contain viruses of veterinary significance
- Foot-and-mouth disease virus belongs to the genus *Aphthovirus*
- Enteroviruses cause swine vesicular disease
- Teschoviruses cause Teschen/Talfan disease, reproductive problems and enteritis in pigs
- Cardioviruses cause encephalomyocarditis in young pigs
- Avian encephalomyelitis virus causes encephalomyelitis in chickens

Clinical infections

With the exception of foot-and-mouth disease virus and encephalomyocarditis virus, picornaviruses typically infect a single or a limited number of host species.

Figure 76.1 Picornavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Transmission usually occurs by the faecal–oral route but may also occur by

fomites or by aerosols. Some picornaviruses, notably foot-and-mouth disease virus (Bergmann *et al*., 1996; Mezencio *et al*., 1999) and swine vesicular disease virus (Lin *et al*., 1998), can produce persistent infections. Antigenic variation, which may contribute to the development of persistent infection (Woodbury, 1995), has been attributed to a number of molecular mechanisms including genetic recombination. Mixed infections with different serotypes of foot-and-mouth disease virus are known to occur in individual animals, particularly in African Cape buffaloes. Porcine teschovirus (PTV) infections, which are widespread in pig populations, are typically subclinical. However, a number of clinical presentations, including polioencephalomyelitis (PTV-1, PTV-2, PTV-3, PTV-5), reproductive disorders (PTV-1, PTV-3, PTV-6), diarrhoea (PTV-1, PTV-2, PTV-3, PTV-5), pneumonia (PTV-1, PTV-2, PTV-3) and pericarditis/myocarditis (PTV-2, PTV-3), have been associated with certain serotypes. Bovine enteroviruses have been isolated from both normal cattle and animals with enteric, respiratory and reproductive disease. Duck hepatitis A virus (formerly duck hepatitis virus type 1) is an important cause of hepatitis in ducklings; three genotypes of the virus have been described.

Swine vesicular disease

This mild vesicular disease of pigs occurs sporadically in parts of Europe and Asia; it is endemic in southern Italy. Because it is indistinguishable clinically from foot-and-mouth disease, accurate laboratory diagnosis of swine vesicular disease (SVD) is essential and this is the main reason it is still classified as a listed OIE disease. Swine vesicular disease virus (SVDV), an enterovirus, is closely related to human coxsackievirus B5, a subtype of human enterovirus B. The pig is the natural host for the virus. Laboratory workers handling contaminated material can become infected.

Epidemiology

In the presence of organic matter, SVDV is stable for long periods in the environment and transmission can occur directly or indirectly. Spread of disease from farm to farm is dependent on the movement of infected pigs or contaminated vehicles or fomites. Tissues of infected pigs, which contain large quantities of virus, remain infective despite the low pH values associated with *rigor mortis*. The virus exhibits prolonged survival in refrigerated pork.

Pathogenesis

Following entry through damaged skin or by ingestion, the virus replicates locally and subsequently spreads via the lymphatics to the bloodstream. During viraemia of short duration, many organs and tissues become infected. Virus shedding, which is greatest during the first week following infection, begins before clinical signs become evident. The faeces and tissue material from infected pigs may contain viable virus for months (Lin *et al*., 1998).

Clinical signs

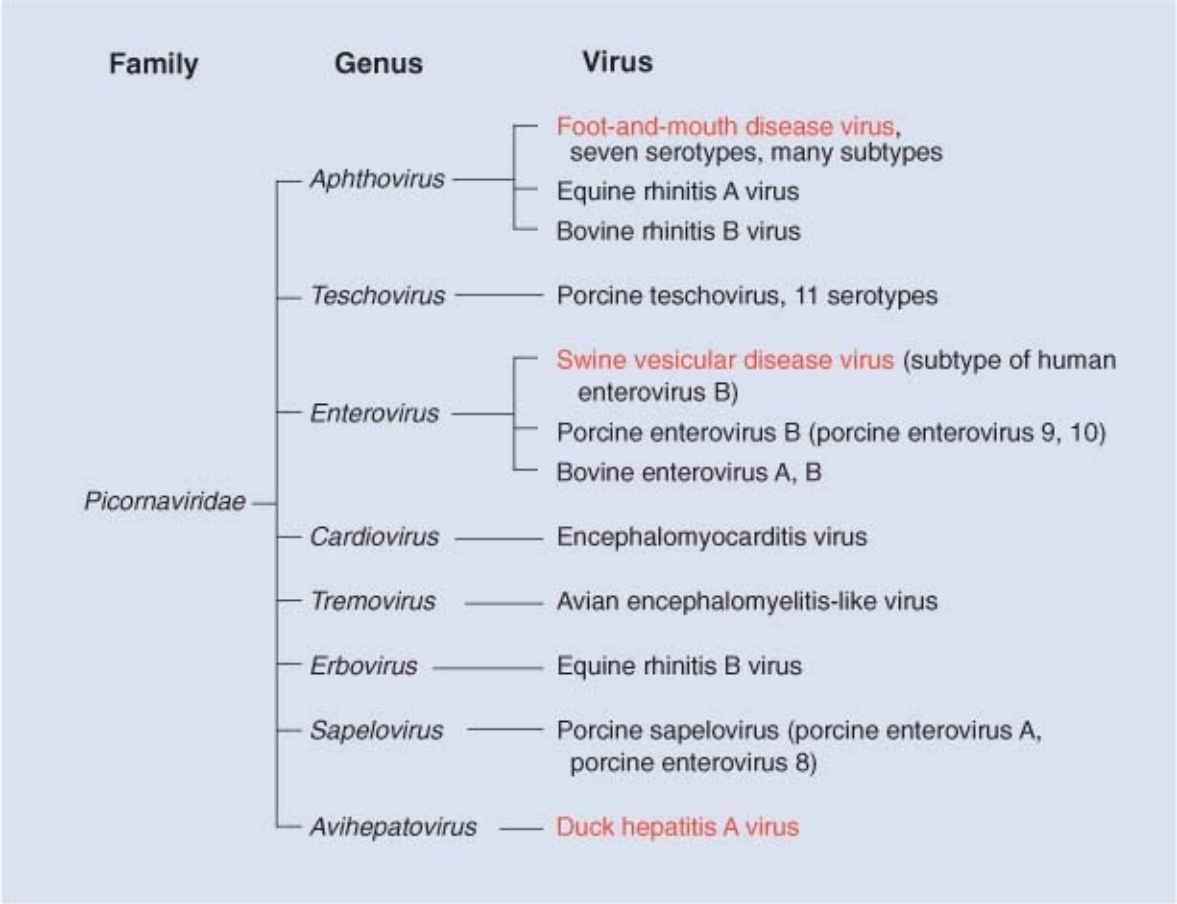
The incubation period is up to 7 days. Infection is usually characterized by a mild febrile illness. Strains of the virus vary in virulence. Subclinical disease is common. Transient fever is followed by the development of vesicular lesions on the feet, particularly on the coronary bands. Less commonly, vesicles appear on the lips, tongue and snout. Lameness, dullness and inappetence may be present. Lameness is exacerbated by penning on concrete floors. Affected animals remain in good condition and the lesions heal within a few weeks.

Diagnosis

Laboratory tests are essential for differentiating SVD from other important vesicular diseases of pigs.

- An ELISA is available for the rapid detection of viral antigen in vesicular fluid or in epithelial tissues.

Figure 76.2 Viruses of veterinary importance in eight genera of the family *Picornaviridae*. Viruses in red cause OIE-listed diseases.



- Samples should be inoculated on to monolayers of susceptible cells. The virus produces cytopathic effects.
- A number of RT-PCR protocols for the detection of SVD in clinical samples, using primers to highly conserved regions of genes coding for major structural proteins, have been described (Lin *et al*., 1997 ; Nunez *et al*., 1998). In addition, real time RT-PCR assays have been used successfully (Reid *et al*., 2004).
- Several serological procedures are suitable for herd screening for antibodies to SVDV. Virus neutralization and ELISA are the tests most frequently used. Virus neutralization, although the standard test, requires tissue culture procedures and takes longer to complete than ELISA. A limitation of serological testing is that it may occasionally yield false-positive results in some individual animals, referred to as ‘singleton reactors’.

Control

Effective vaccines against SVD have been prepared. Commercial vaccines are

not available because vaccination is not considered a suitable control measure in countries free of important vesicular diseases. In most countries, SVD is a notifiable disease and eradication policies are enforced with restrictions on the importation of pigs and pork. Control measures, applied following an outbreak of SVD, include thorough cleaning and disinfection of premises, control of pig movement and boiling of waste food fed to pigs.

Teschen/Talfan disease

This condition, formerly referred to as porcine enteroviral encephalomyelitis, was first described in Teschen, Czechoslovakia, in 1929 and subsequently caused significant losses in several European countries. The clinical presentation varies in accordance with the virulence of the infecting strain of porcine enterovirus (PEV). Severe clinical disease is now rare and is largely confined to eastern Europe and Madagascar.

Epidemiology

Thirteen serotypes of porcine enteroviruses were recognized formerly. Comparative analysis of genome sequences of porcine enteroviruses has resulted in PEV 1 to 7 and PEV 11 to 13 being assigned to a newly-created genus, *Teschovirus*, which comprises a single species and eleven serotypes, porcine teschovirus (PTV) 1 to 11. Porcine teschovirus serotypes associated with polioencephalomyelitis include PTV-1, PTV-2, PTV-3 and PTV-5. The most important neurotropic strains belong to PTV serotype 1 (PEV1), which includes both the highly virulent isolates associated with Teschen disease and the less virulent but widely distributed strains that cause endemic posterior paresis (Talfan disease). In common with swine vesicular disease, transmission occurs by the faecal–oral route either directly or indirectly. Clinical disease is most severe in young pigs in herds which have not been previously exposed to infection. In herds that are endemically infected, sporadic clinical disease tends to occur after mixing of weaned pigs when maternally-derived immunity has declined.

Pathogenesis

Following ingestion, virus replicates in the tonsils, intestines and associated lymph nodes. Viraemia and invasion of the central nervous system may follow, particularly when virulent strains are involved. Faecal excretion of virus may

continue for several weeks.

Clinical signs

Fever, depression and listlessness may be followed about 1 week after infection by neurological signs. Weakness and incoordination progress to paraplegia and paralysis. Pigs with posterior paralysis may adopt a dog-sitting posture. Severely affected animals exhibit nystagmus, opisthotonos, convulsions and coma. Mortality rate in these animals is high. Mildly affected pigs usually recover.

Diagnosis

- A mild to severe non-suppurative encephalomyelitis is demonstrable histologically. The presence of viral antigen may be detectable by immunohisto-chemical staining.
- Virus can be isolated in porcine kidney cell lines from specimens of brain and spinal cord. The virus produces a cytopathic effect.
- It is possible to use RT-PCR for the rapid detection of the viral RNA (Palmquist *et al*., 2002), while specific primer sets have been designed to differentiate the different species of porcine picornaviruses (Zell *et al*., 2000). A real-time assay has also been described (Krumbholz *et al*., 2003).
- Virus neutralization and ELISA are the most frequently used serological methods for demonstrating antibodies to PTV. Because antibodies to PTV strains are common in pig populations, it is necessary to demonstrate a fourfold rise in titre between acute and convalescent sera for disease confirmation.

Control

Both inactivated and modified live vaccines are effective and have been used for the control of Teschen disease, a disease which is notifiable in many countries. Outbreaks can be controlled by slaughter, strict sanitary measures and ring vaccination.

Reproductive disorders caused by porcine picornaviruses

Porcine teschovirus serotypes PTV1, PTV3 and PTV6 as well as porcine sapelovirus (porcine enterovirus A, PEV8) are associated with the SMEDI

syndrome (stillbirths, mummification, embryonic death and infertility) in pigs. Although these viruses are widely distributed in commercial pig herds, they are pathogenic only for embryos and foetuses. Clinical disease follows infection of naive, pregnant animals. Cross-protection between serotypes does not occur. Transmission is by the faecal-oral route. Infection of the alimentary tract is followed by viraemia and transplacental spread to developing foetuses. The clinical effects of infection depend on the stage of gestation. Infection during early to mid-gestation results in embryonic death and mummification, whereas infection during the later stages of pregnancy may result in stillbirths or the birth of live piglets. A susceptible sow may, therefore, give birth to mummified, stillborn and live piglets, reflecting the stage of foetal development at the time of infection. The clinical presentation is indistinguishable from porcine parvovirus infection, a more common cause of the SMEDI syndrome. Laboratory confirmation requires either isolation of the virus from lung tissues of stillborn piglets or demonstration of antibody in the serum of stillborn or newborn piglets prior to ingestion of colostrum. Mummified foetuses carried to term usually do not contain live virus but viral antigen may be detectable by immunofluorescence. Commercial vaccines are not available. Gilts should be exposed to porcine tescho-viruses by contact with older sows, faeces or mummified foetuses prior to breeding.

Avian encephalomyelitis

This viral disease of young birds has been recorded in domestic fowl, pheasants, quail and turkeys. Avian encephalomyelitis (AE) is of considerable economic importance in chickens. Although avian encephalomyelitis virus (AEV) was formerly considered to be an enterovirus, it has been shown to be most closely related to hepatitis A virus and tentatively assigned to the genus *Hepatovirus* (Todd *et al*., 1999). A new genus, *Tremovirus*, has been created. Horizontal and vertical transmission occurs. The virus produces enteric infection and is shed in the faeces. A proportion of the eggs of infected hens are infected. Chicks infected *in ovo* hatch normally but shed virus and infect other chicks in the incubator shortly after hatching. Infection of the intestinal tract is followed by viraemia and a competent immune response is required to prevent infection of the central nervous system. Clinical signs, which usually become evident within 2 weeks, include ataxia and fine tremors of the head and neck. Progressive paralysis leads to death due to inanition or following trampling. Non-suppurative

encephalomyelitis and lymphocytic accumulations in viscera, particularly the pancreas, are characteristic. Demonstration of viral antigen in tissues by immunofluorescence or by virus isolation in embryonated hens' eggs from brain or pancreas is confirmatory. Viral RNA in tissues can be detected using RT-PCR (Xie *et al*., 2005). Serological testing of paired sera may be of diagnostic value. Control is achieved by vaccination of breeding flocks with a modified live vaccine to ensure the presence of maternally-derived antibodies in chicks.

Foot-and-mouth disease

This highly contagious disease of even-toed ungulates is characterized by fever and the formation of vesicles on epithelial surfaces. Foot-and-mouth disease (FMD) is a listed disease of major importance internationally on account of its rapid spread and the dramatic economic losses that it causes in susceptible animals. Isolates of foot-and-mouth disease virus (FMDV) are grouped into seven major serotypes with differing geographical distributions (Table 76.1). Infection with one serotype does not confer immunity against other serotypes. A large number of subtypes is recognized within each serotype. Nucleotide sequencing of the *VP1* (1D) gene, which encodes for a capsid protein, is used for comparing an isolate from an outbreak with other isolates of the same serotype in order to determine possible sources of infection. Study of VP1-based phylogenies has revealed that different genotypes within the O and SAT types evolve in discrete geographical regions and such variants are known as topotypes. The dramatic spread of the pan-Asian lineage (topotype) of type O virus in recent years, which resulted in a very extensive and expensive outbreak in the United Kingdom in 2001, has been of particular concern in European countries.

Table 76.1 The geographical distribution of foot-and-mouth disease virus serotypes. Australia, New Zealand, North and Central America, the Caribbean countries and the countries of western Europe are currently free from the disease.[a]

Foot-and-mouth disease virus serotypes	Geographical distribution
O, A and C	South America
O, A and C	Eastern European countries
O, A, C, SAT1, SAT2 and SAT3	Africa
O, A, C and Asia1	Asia

a, large outbreak in UK during 2001.

Epidemiology

Cattle, sheep, goats, pigs and domesticated buffalo are susceptible to FMD. Several wildlife species including African buffalo, elephants, hedgehogs, deer and antelopes are also susceptible. Large numbers of virus particles are shed in the secretions and excretions of infected animals. Virus shedding begins during the incubation period, about 24 hours before the appearance of clinical signs. Transmission can occur by direct contact, by aerosols, by mechanical carriage by humans or vehicles, on fomites and through animal products such as meat, offal, milk, semen or embryos. Because of their large respiratory volume and the low dose of virus required to establish infection, cattle are highly susceptible and are often the first species in which clinical signs are evident. Infected groups of animals, particularly pigs, shed large quantities of virus in aerosols (Donaldson *et al*., 2001). Under favourable conditions of low temperature, high humidity and moderate winds, virus in aerosols may spread up to 10 km over land. Turbulence is generally less marked over water than over land. In 1981, virus was carried a distance of more than 200 km from France to the south coast of England.

The virus, which is moderately resistant to environmental factors, is sensitive to acid and alkaline conditions outside the range pH of 6.0 to 9.0. Virus can remain infective on soil for 3 days in summer and for up to 28 days in the winter. Following death of the host, lactic acid production in muscle inactivates the virus but it may survive in offal and in bone marrow. Foot- and- mouth virus can persist in the pharyngeal region of carrier animals which have recovered from FMD. It can also persist in vaccinated animals infected with a subtype different from the vaccinal subtype. Infection may persist for up to 3 years in cattle, for many months in sheep and for up to 5 years in the African Cape buffalo. Transmission of infection from persistently-infected African Cape buffalo to domestic cattle has been clearly demonstrated, whereas there is only circumstantial evidence to indicate the occasional transmission of infection from persistently-infected cattle. It is unclear if virus can persist in pigs (Bergmann *et al*., 1996; Mezencio *et al*., 1999; Zhang and Bashiruddin, 2009).

Pathogenesis

Although infection usually occurs through inhalation, the most efficient method of infection, virus can also gain entry to tissues through ingestion, insemination and inoculation, and through contact with abraded skin. Pigs are more resistant than ruminants to the virus and infection tends to occur through the feeding of

contaminated, untreated swill. Primary viral replication, after inhalation, takes place in the mucosal and lymphatic tissues of the pharynx. Viraemia follows primary multiplication with further viral replication in lymph nodes, mammary glands and other organs as well as the epithelial cells of the mouth, muzzle, teats, interdigital skin and coronary band. In these areas of stratified squamous epithelium, vesicle formation results from swelling and rupture of keratinocytes in the stratum spinosum.

Clinical signs

The incubation period ranges from 2 to 14 days, but is generally shorter than a week. Infected cattle develop fever and inappetence and show a drop in milk production. Profuse salivation, with characteristic drooling and smacking of lips, accompanies the formation of oral vesicles which rupture, leaving raw, painful ulcers. Ruptured vesicles in the interdigital cleft and on the coronary band lead to lameness. Vesicles may also appear on the skin of the teats and udders of lactating cows. Although the ulcers tend to heal rapidly, there may be secondary bacterial infection which exacerbates and prolongs the inflammatory process. Infected animals lose condition. Mature animals seldom die. Calves may die from acute myocarditis. Although the virus does not cross the placenta, abortion probably relates to the pyrexial response.

In pigs, foot lesions are severe and the hooves may slough. Marked lameness is the most prominent sign in this species. The disease in sheep, goats and wild ruminants is generally mild, presenting as fever accompanied by lameness which spreads rapidly through groups of animals.

Human infection, usually mild, has been described on rare occasions in laboratory personnel working with the virus and in individuals handling infected animals.

Table 76.2 Susceptibility of farm animals to viruses which cause vesicular diseases.

Virus	Species			
	Cattle	Sheep, goats	Pigs	Horses
Foot-and-mouth disease virus	Susceptible	Susceptible	Susceptible	Resistant
Swine vesicular disease virus	Resistant	Resistant	Susceptible	Resistant
Vesicular exanthema of swine virus	Resistant	Resistant	Susceptible	Resistant
Vesicular stomatitis virus	Susceptible	Resistant	Susceptible	Susceptible

Diagnosis

Foot-and-mouth disease clinically resembles other vesicular diseases of domestic animals including vesicular stomatitis in cattle and pigs, swine vesicular disease and vesicular exanthema in pigs (Table 76.2). Consequently, FMD requires laboratory confirmation. Laboratory procedures involving FMDV must be carried out in purpose-built laboratories suitable for containment of Group 4 pathogens.

- A confirmatory diagnosis is based on the isolation of FMDV from samples of tissue or vesicular fluid. Epithelium collected from an unruptured or recently ruptured vesicle is ideal for laboratory processing.
- Viral antigen demonstration may be carried out by ELISA or CFT.
- In persistent or subclinical infections, samples of oesophageal/pharyngeal fluid can be obtained with a probang (sputum) cup and examined by virus isolation or RT- PCR.
- Virus isolation is carried out in special cell lines such as primary bovine thyroid or kidney cells.
- The preferred test for virus detection is the RT-PCR which is sensitive and specific and has been adapted for the amplification of genome fragments of FMDV (Reid *et al*., 2000). Real-time protocols are available and considered to be as sensitive as virus isolation (Alexandersen *et al*., 2002) with potential for use in the field (King *et al*., 2008). Specific primers, which can distinguish the seven serotypes, are available.
- Demonstration of specific antibody by virus neutralization or ELISA can be

used to confirm a diagnosis in unvaccinated animals. In endemic areas, interpretation of antibody titres may prove difficult. The use of assays (De Diego *et al*., 1997; Bergmann *et al*., 2000) such as ELISA or immunoblotting, capable of detecting antibodies to non-structural proteins (NSPs), has proved useful for differentiating animals currently or previously infected from animals vaccinated with purified or recombinant protein preparations, which do not contain NSPs (Mackay *et al*., 1997 ; Sorensen *et al*., 1998 ; Paton *et al*., 2006). These assays are probably most appropriate for herd use rather than for individual animals because some vaccinated animals that subsequently become infected produce little antibody to NSPs (Mackay, 1998).

Control

In countries that are free from FMD, it is notifiable. Affected and in-contact animals are slaughtered. Following an outbreak, movement restrictions are applied and infected premises must be thoroughly cleaned and disinfected. Mild acids such as citric acid and acetic acid and alkalis such as sodium carbonate are effective disinfectants. Reserves of inactivated virus are maintained in several countries to provide an adequate supply of vaccine at short notice in the event of a major outbreak of the disease. Although ring vaccination around an affected premises may help to limit the spread of the disease, it may also allow the development of the carrier state in animals subsequently exposed to the virus.

In countries where FMD is endemic, efforts are generally directed at protecting high-yielding dairy cattle by a combination of vaccination and control of animal movement. Vaccines for FMD incorporating adjuvant are derived from tissue-culture-propagated virus which has been chemically inactivated. They are usually multivalent, containing three or more virus strains. Protection against antigenically-similar strains of virus is satisfactory and lasts for up to 6 months. Research is continuing into the development of improved vaccines based principally on peptide synthesis or recombinant DNA technology (Doel, 1996; Grubman, 2005).

Infections caused by equine rhinitis viruses

Genomic and other studies have shown that equine rhinovirus 1 is closely related to foot-and-mouth disease virus. It has been renamed equine rhinitis A virus and has been placed in the genus *Aphthovirus*. Equine rhinitis B virus (equine

rhinovirus 2, 3) comprises three serotypes and has been placed in the genus *Erbovirus*. Infection with equine rhinitis viruses appears to be widespread and most horses are exposed early in life. Although equine rhinitis A virus and equine rhinitis B virus have been associated with acute respiratory disease (Carman *et al*., 1997; Klaey *et al*., 1998; Dynon *et al*., 2007), they are generally considered to be minor respiratory pathogens. They may contribute to the development of disease following surgery or strenuous exercise or when present in mixed infections with bacteria or other viruses. Viraemia and prolonged shedding of virus in urine occurs in infection with equine rhinitis A virus.

Infection with encephalomyocarditis virus

Rodents are considered to be the natural hosts of encephalomyocarditis virus (EMCV). However, this cardiovirus has a wide host range including humans, monkeys and pigs. Infection in pigs is usually sub-clinical but sporadic deaths and minor outbreaks of disease have been described. Rats and mice, the principal reservoirs, excrete virus in faeces and urine. The virus is stable in the environment. Pigs acquire infection by ingesting contaminated feed. Pig-to-pig transmission may also occur (Koenen *et al*., 1999). Following ingestion, viraemia develops within a few days. Subsequently, high titres of virus can be demonstrated in the myocardium, spleen and mesenteric lymph nodes. Transplacental infection may occur. Virus isolates associated with myocardial disease are apparently distinct from those responsible for reproductive disease (Koenen *et al*., 1999).

Outbreaks of disease are generally restricted to one particular age group. The severity of the disease relates to the strain of virus and the age of the infected pigs, with severe disease usually confined to the first weeks of life. Piglets may die suddenly as a result of heart failure. Hydrothorax, hydropericardium and ascites may be demonstrated at post-mortem examination. Areas of myocardial necrosis with associated lymphoid infiltration may be demonstrable histologically. Changes in the CNS are minimal. Reproductive failure in sows is characterized by mummified foetuses and stillbirths. Laboratory confirmation relies on virus isolation and identification. The rapid detection of EMCV RNA in clinical specimens by RT-PCR has been described (Vanderhallen and Koenen, 1997). Virus neutralization, ELISA and haemagglutination tests can be used to detect specific antibodies. Control of rodents is important in reducing the likelihood of infection. An inactivated vaccine is available commercially in the

USA.

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Chapter 77

Caliciviridae

Caliciviruses (Latin *calix*, cup) have cup-shaped depressions on the surface of virions which are demonstrable by electron microscopy. The virions, 27 to 40 nm in diameter, are icosahedral and non-enveloped (Fig. 77.1). The genome consists of a single molecule of linear, positive-sense, single-stranded RNA. Replication takes place in the cytoplasm of infected cells, and virions are released by cell lysis. Many caliciviruses have not yet been cultured. The virions are resistant to ether, chloroform and mild detergents. They are relatively resistant to heat but are sensitive to acid pH values.

Caliciviruses, which are closely related to picorna-viruses, were formerly grouped within the *Picorna-viridae*. Currently, the family *Caliciviridae* is divided into four genera: *Vesivirus, Lagovirus* and two genera of human caliciviruses referred to as *Norovirus* and *Sapovirus*. A fifth genus, '*Nebovirus*', has been proposed with type species Newbury-1 virus (bovine enteric calicivirus) isolated from the faeces of a calf. The genus *Vesivirus* contains vesicular exanthema of swine virus, the type virus of the family, San Miguel sea lion virus and feline calicivirus. The *Lagovirus* genus contains two viruses of lagomorphs, rabbit haemorrhagic disease virus and European brown hare syndrome virus. The human caliciviruses, Norwalk virus and Sapporo virus, cause gastroenteritis. Due to the lack of clarity of surface detail and their fuzzy appearance when viewed by electron microscopy, noroviruses are also referred to as small, round, structured viruses. Bovine (Newbury-2 virus) and porcine noroviruses have been described (Scipioni *et al*., 2008). Hepatitis E virus of humans, formerly classified in the *Caliciviridae*, has been placed in a newly created family, *Hepeviridae*.

Key points

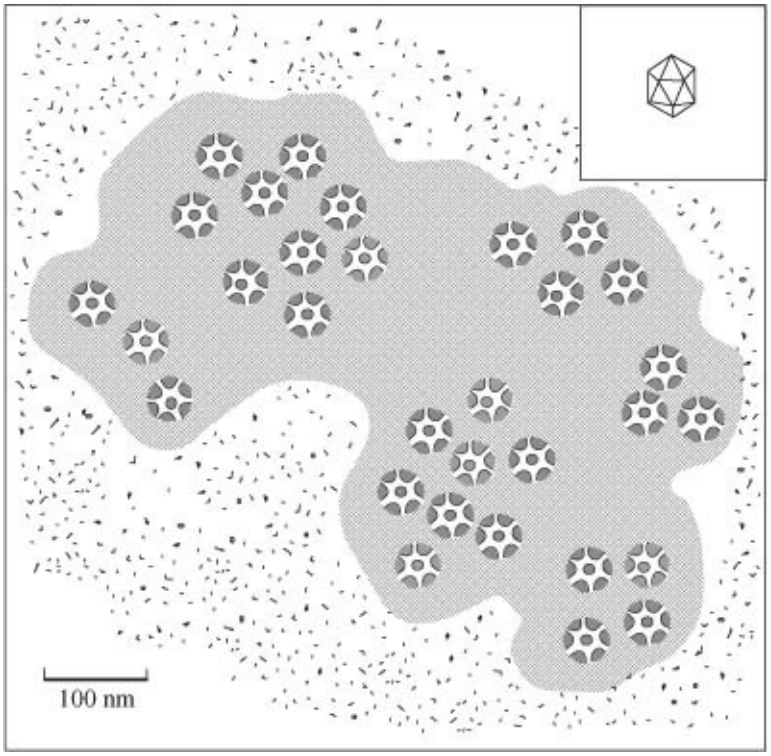
- Small, non-enveloped, single-stranded RNA viruses with icosahedral symmetry
- Replicate in the cytoplasm
- Stable in the environment

- Four genera:
  – *Vesivirus* contains viruses which cause vesicular exanthema of swine and feline calicivirus infection
  – *Lagovirus* contains viruses which cause rabbit haemorrhagic disease and European brown hare syndrome
  – *Norovirus* and *Sapovirus* contain human caliciviruses which cause gastroenteritis

Clinical infections

Caliciviruses have been recovered from many species including humans, cats, pigs, marine mammals, rabbits, hares, cattle, dogs, reptiles, amphibians and insects. They are associated with a wide range of conditions including respiratory disease, vesicular lesions, necrotizing hepatitis and gastroenteritis (Table 77.1). Infections with caliciviruses, which are frequently persistent, may be inapparent, mild or acute. Transmission occurs directly or indirectly without vector involvement. However, mechanical transmission of rabbit haemorrhagic disease virus by mosquitoes and fleas has been described.

Figure 77.1 Calicivirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Vesicular exanthema of swine

First reported in southern California in 1932, vesicular exanthema of swine (VES), an acute, highly contagious disease, became widespread throughout the USA during the 1950s. An eradication campaign included rigorous implementation of laws relating to the cooking of waste food. The last outbreak of VES was recorded in 1956 and the country was declared free of the disease in 1959. A reservoir of the virus exists in marine mammals. In 1972, the San Miguel sea lion virus (SMSV) was isolated from California sea lions, which had developed vesicles on their flippers. Premature parturition occurred in infected animals. Subsequently, SMSV has been isolated from a number of other marine mammals and from the opal eye fish. Strains of SMSV produce VES when inoculated into pigs and it is thought that the original outbreak of VES arose through the feeding of uncooked swill containing meat from infected marine mammals. These vesicular viruses show antigenic heterogeneity. There are 13 serotypes of vesicular exanthema of swine virus and 17 serotypes of SMSV. San Miguel sea lion virus is currently classified as a strain of vesicular exanthema of swine virus.

The incubation period of VES is up to 72 hours and the course of the disease is approximately 2 weeks. Vesicles appear on the tongue, lips, snout, interdigital skin and coronary bands. Affected pigs are febrile and acutely lame. Although morbidity is high, mortality is low. The disease is clinically indistinguishable from foot-and-mouth disease, vesicular stomatitis and swine vesicular disease. Because of its similarity to foot-and-mouth disease, vesicular exanthema of swine is an important disease. Weight loss in fattening pigs and mortality in neonatal pigs are economically important consequences of the infection.

Table 77.1 Caliciviruses of veterinary importance.

Virus	Hosts	Comments
Vesicular exanthema of swine virus (13 serotypes)	Pigs	Acute, contagious, vesicular disease, clinically similar to foot-and-mouth disease. Occurred in the USA before 1956. May have arisen from feeding sea lion and seal meat contaminated with San Miguel sea lion virus
San Miguel sea lion virus (17 serotypes)	Marine mammals, opal eye fish	Associated with cutaneous vesicles and premature parturition in pinnipeds; when inoculated into pigs, causes vesicular exanthema
Feline calicivirus	Domestic and wild cats	Important cause of upper respiratory tract infection in cats worldwide. Virulent systemic disease (VSD) described in some outbreaks
Rabbit haemorrhagic disease virus	European rabbits	Acute fatal disease in European rabbits over 2 months of age
European brown hare syndrome virus	European brown hares	Related to rabbit haemorrhagic disease virus. Causes hepatic necrosis and widespread haemorrhages with high mortality
Canine calicivirus	Dogs	Occasionally associated with diarrhoea

Vesicular fluid and the overlying flap of epithelium are rich in virus. Isolates can be identified by RT-PCR (Reid *et al*., 2007), ELISA, CFT, immunoelectron microscopy and virus isolation in pig kidney cell lines.

Feline calicivirus infection

Infections caused by feline calicivirus (FCV) account for about 40% of upper respiratory tract inflammatory disease in cats worldwide. All species of *Felidae* are considered to be susceptible but natural disease tends to be confined to domestic cats and to cheetahs in captivity. Most FCV isolates belong to a single genotype, although a second genotype has been described in Japan (Sato *et al*, 2002). There is a high degree of antigenic heterogeneity among FCV isolates. Sequence analysis studies have shown that individual isolates of FCV exist as quasispecies, which evolve and exhibit antigenic drift. Significant alterations in the antigenic profiles of sequential virus isolates from carrier cats are thought to be influenced by immune selection and may play an important part in viral persistence (Radford *et al*., 1998).

Epidemiology

Although cats of all ages are susceptible to infection with FCV, acute disease occurs most commonly in kittens as maternally-derived antibody wanes between 2 and 3 months of age. Infected cats excrete large amounts of virus in oronasal secretions. Many cats shed virus in oropharyngeal secretions for at least 30 days after recovery from acute infection or following subclinical infection while protected by maternally-derived antibody or by vaccination. A minority remain persistently infected and shed virus continuously for months and, occasionally, for years. Infection is maintained in a given cat population by these carrier animals and the prevalence of infection is highest where large groups of cats live together. It is thought that this persistence arises as a result of a combination of both viral and host factors. Sequential studies of isolates from endemically-infected colonies of cats indicate that a number of mechanisms operate to ensure the long-term survival of the virus in the host population. Progressive evolution of virus within persistently-infected individuals through mutation accumulation gives rise to changes in the immunodominant region of the capsid protein, allowing the virus to escape the host immune response. In addition, recombination between different viral strains has been described (Coyne *et al*., 2006b). As a result, the majority of cats within an infected colony undergo

sequential reinfection with viral variants of the same strain or with distinct, co-circulating strains (Coyne *et al*., 2007). Although infection usually follows contact with acutely-infected or carrier cats, indirect transmission can also occur with virus capable of persisting in the environment for up to 1 month on dry surfaces at room temperature.

Pathogenesis

Virus replication occurs primarily in the oropharynx with rapid spread throughout the upper respiratory tract and to the conjunctivae. A transient viraemia occurs with spread of virus to many other tissues. Infections range from subclinical to severe, reflecting differences in strain virulence. Virulent strains of FCV can cause interstitial pneumonia in young kittens. The virus has been recovered from the joints of lame cats. In recent years, virulent systemic disease (VSD-FCV) has been described, associated with certain virulent strains of the virus and characterized by vasculitis, multi-organ involvement and high mortality (Hurley *et al*, 2004; Coyne *et al*., 2006a).

Clinical signs

The incubation period is up to 5 days. Clinical signs, which are usually confined to the upper respiratory tract and the conjunctivae, are often less severe than those caused by feline herpesvirus 1 infection. Fever, oculonasal discharge and conjunctivitis are accompanied by the development of characteristic vesicles on the tongue and oral mucosa. These vesicles rupture leaving shallow ulcers. Morbidity may be high but mortality is usually low. Stiffness and shifting lameness, which usually resolve within a few days, are sometimes seen during the acute phase of FCV infection or following inoculation with FCV vaccine. An association between infection with FCV and chronic gingivitis and stomatitis has been suggested, when infection with feline immunodeficiency virus is also present.

In cats affected by VSD-FCV, the upper respiratory tract disease is accompanied by pyrexia, cutaneous oedema, ulcerative dermatitis and jaundice. Adult cats are often more severely affected than kittens and vaccination does not seem to be protective. Mortality rates may be as high as 50%. Outbreaks of VSD-FCV are usually associated with the introduction of cats from large rescue shelters into another colony and tend to be of short duration.

Diagnosis

- Upper respiratory tract signs along with ulcers on the oral mucosa are suggestive of infection with FCV. Differentiation from feline herpesvirus 1 infection requires laboratory testing.
- Feline calicivirus can be isolated in feline cell lines from oropharyngeal swabs or from lung tissue. The detection of FCV may not be aetiologically significant due to the large numbers of carrier and shedding animals in cat populations.
- Viral RNA can be detected in clinical specimens using RT-PCR (Sykes *et al*., 1998) and real-time RT-PCR (Wilhelm and Truyen, 2006) protocols. The design of the primers used must take account of the variability of the viral genome.
- On account of the high seroprevalence in cat populations, demonstration of a rising antibody titre in paired serum samples is required for laboratory confirmation of infection.

Control

Vaccination and management practices aimed at reducing exposure to the virus are the main methods of control. Inactivated vaccines for parenteral administration and modified live vaccines for either parenteral or intranasal administration are available. DNA vaccines and virus-vectored vaccines have been developed but are not yet commercially available. Although vaccination protects effectively against acute upper respiratory tract disease, it does not prevent subclinical infection or the development of a carrier state. Vaccines are based on a limited number of FCV isolates, which cross-react with a broad spectrum of field isolates. An increasing number of recent field isolates are not neutralized by vaccine-induced antisera *in vitro* (Lauritzen *et al*., 1997) and this has led to the incorporation of new vaccine strains (Addie *et al*., 2008). Live vaccines for administration by injection may cause clinical signs if administered by other routes. Intranasal vaccines are useful where a rapid onset of protection is needed, such as when there is a high risk of a disease outbreak, but they may give rise to mild clinical signs such as sneezing. Booster vaccinations at yearly intervals are recommended for cats in high risk circumstances, while triennial intervals may be sufficient for cats in low risk categories (European Advisory Board on Cat Diseases, 2007). Management practices aimed at reducing viral spread and load in large catteries include vaccination, use of quarantine facilities,

grouping of new arrivals, good hygiene practices, use of pens that prevent direct contact and avoidance of overcrowding. Additional measures in endemically-infected breeding colonies include reduction in stocking density, early vaccination and early weaning of kittens followed by rearing in isolation.

Rabbit haemorrhagic disease

This is a highly contagious, acute and often fatal disease of European rabbits (*Oryctolagus cuniculus*). Rabbits under 2 months of age are not susceptible. A single serotype of the virus (RHDV) and two major subtypes are recognized: RHDV and the antigenic variant RHDVa. Rabbit haemorrhagic disease (RHD) was first reported in China during 1984 and has since been encountered in many parts of the world. This virus is considered to be a mutant form of a non-pathogenic virus, sometimes referred to as rabbit calicivirus, which has been endemic in commercial and wild rabbits in Europe for many years (Capucci *et al*., 1996). Phylogenetic analyses of RHDV isolates indicate that virulent strains have emerged on more than one occasion and that the outbreaks in China were caused by a European virus following the importation of Angora rabbits from Germany (Forrester *et al*., 2006). Rabbit haemorrhagic disease virus has been used for biological control of rabbits in Australia and New Zealand.

Epidemiology

Virus is shed in all excretions and secretions. Among rabbits in close contact, transmission is mainly by the faecal-oral route. Infection may also occur by inhalation or through the conjunctiva. Mechanical transmission by a variety of insects including mosquitoes and fleas has been demonstrated. The virus survives in the environment and indirect transmission through contaminated foodstuff or fomites may occur. Spread of virus between units and between countries may result from uncontrolled movement of infected rabbits or from contact with infected rabbit meat, insects or fomites. The virus was inadvertently released from a research facility in Australia during 1995 and, subsequently, was illegally introduced into New Zealand in 1997. In Europe, outbreaks of RHD have been variable in severity and in Italy this has been attributed to the presence of non-pathogenic rabbit calicivirus infection. In certain populations of rabbits, RHDV appears to circulate without evidence of disease (Forrester *et al*., 2007). Epidemiological factors may be important in determining disease outcome, with most transmission occurring during and just after the breeding season (White *et*

al., 2004). It has been shown that infection of young rabbits may induce antibodies that are protective against subsequent RHDV challenge when they reach maturity (Ferreira *et al*., 2008).

Pathogenesis and pathology

Cells of the mononuclear phagocyte lineage are considered to be the major targets of the virus (Ramiro-Ibanez *et al*., 1999). Juvenile rabbits up to 2 months of age are resistant to the disease. The reason for this resistance is unclear but it may have a physiological basis. Severe hepatic necrosis is the most obvious lesion in affected rabbits. In addition, there may be evidence of disseminated intravascular coagulation.

Clinical signs

The incubation period is up to 3 days. The disease is characterized by high morbidity and high mortality. The course is short, with death occurring within 36 hours of the onset of clinical signs. Acutely affected animals are pyrexic and depressed and have an increased respiratory rate. A serosanguineous nasal discharge, haematuria and neurological signs including convulsions may be present. Rabbits may be found dead or die in convulsions. A few rabbits may present with mild, subacute signs during the later stages of an epizootic. Some animals may survive for a few weeks with jaundice, weight loss and lethargy.

Diagnosis

High mortality in rabbits along with characteristic gross lesions including necrotic hepatitis and congestion of spleen and lungs are suggestive of RHD. Culture of RHDV has not been successful. High concentrations of virus are present in affected livers. Confirmation is based on detection of virus by electron microscopy or of viral antigen by ELISA, immunofluorescence or haemagglutination using human erythrocytes. Reverse transcriptase PCR and real time RT-PCR have been developed for the detection of RHDV nucleic acid (Moss *et al*, 2002; Gall *et al*, 2007). Suitable serological tests for the detection of specific antibodies to the virus include haemagglutination-inhibition and ELISA.

Control

In countries where RHD is endemic, control is achieved by vaccination.

Inactivated and adjuvanted vaccines prepared from clarified liver suspensions of experimentally infected rabbits are usually administered at about 10 weeks of age. Novel vaccines are being developed, based on recombinant myxoma virus expressing RHDV capsid protein or on virus-like particles from capsid protein produced in baculovirus expression systems.

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Further reading

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Chapter 78

Astroviridae

The family *Astroviridae* (Greek *aster*, star) contains viruses with a surface structure that imparts a star-like appearance. Astroviruses, 28 to 30 nm in diameter, are non-enveloped and have icosahedral symmetry (Fig. 78.1). The genome consists of a single molecule of positive-sense, linear, single-stranded RNA. These viruses are resistant to low pH values, various detergents and heating at 60°C for 5 minutes. Replication occurs in the cytoplasm of host cells, and virions are released by cell lysis. Trypsin is required for cultivation of these viruses.

The family contains two genera: *Avastrovirus*, whose members infect avian species, and *Mamastrovirus*, whose members infect mammalian species (Fig. 78.2). Species are designated according to the host of origin while serotypes are defined by cross-neutralization tests. Two serotypes of bovine astrovirus and two serotypes of turkey astrovirus are recognized.

Clinical infections

Astroviruses, which are distributed worldwide, have been detected in the faeces of humans, cattle, pigs, sheep, dogs, cats, deer, chickens, ducks and turkeys. Transmission occurs by the faecal-oral route. Isolates from different host species are antigenically distinct and host species-specific. Infections are mild in most species. Mamastroviruses are associated with self-limiting gastroenteritis in animals and humans. Following an incubation period of up to 4 days, diarrhoea may develop. Infections with avastroviruses may be severe and frequently involve a number of organs (Koci and Schultz-Cherry, 2002). In ducklings, a severe hepatitis may develop, while kidney lesions have been found in birds infected with chicken astro-virus (avian nephritis virus); turkey astro virus 2 is associated with poult enteritis mortality syndrome (PEMS).

Key points

- Small, single-stranded RNA viruses with icosahedral symmetry
- Replicate in the cytoplasm
- Two genera, *Avastrovirus* and *Mamastrovirus*, containing viruses which generally produce mild gastroenteritis in most domestic species; in ducks serious disease may occur

Diagnosis is based on the detection of astroviruses in faeces using electron microscopy or ELISA. Detection of viral RNA using reverse transcriptase PCR (Koci *et al*., 2000; Day *et al*., 2007) and virus isolation in primary cell lines or embryonated eggs are also possible. Apart from a vaccine for duck astrovirus, vaccines for other astroviruses have not been developed as diseases caused by these viruses are usually mild. Control is based on husbandry practices appropriate for the prevention of enteritis in young animals.

Figure 78.1 Astrovirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).

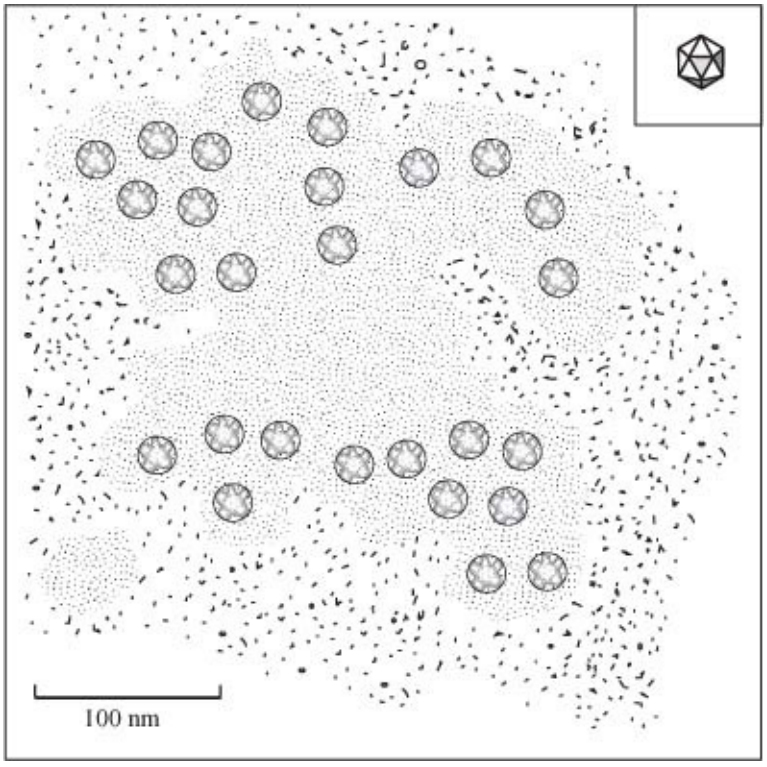
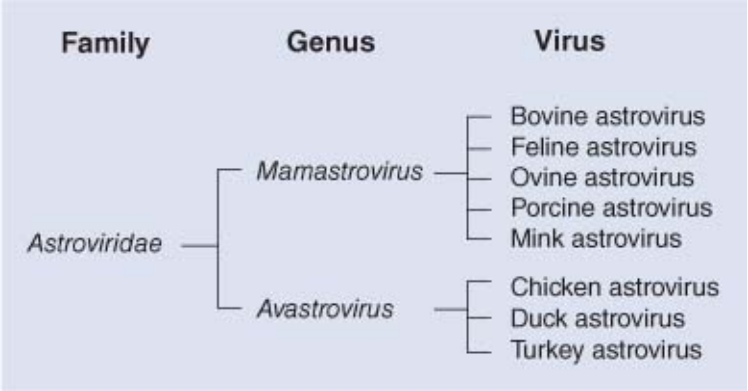


Figure 78.2 Viruses of veterinary importance in the family *Astroviridae*.



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Chapter 79

Coronaviridae

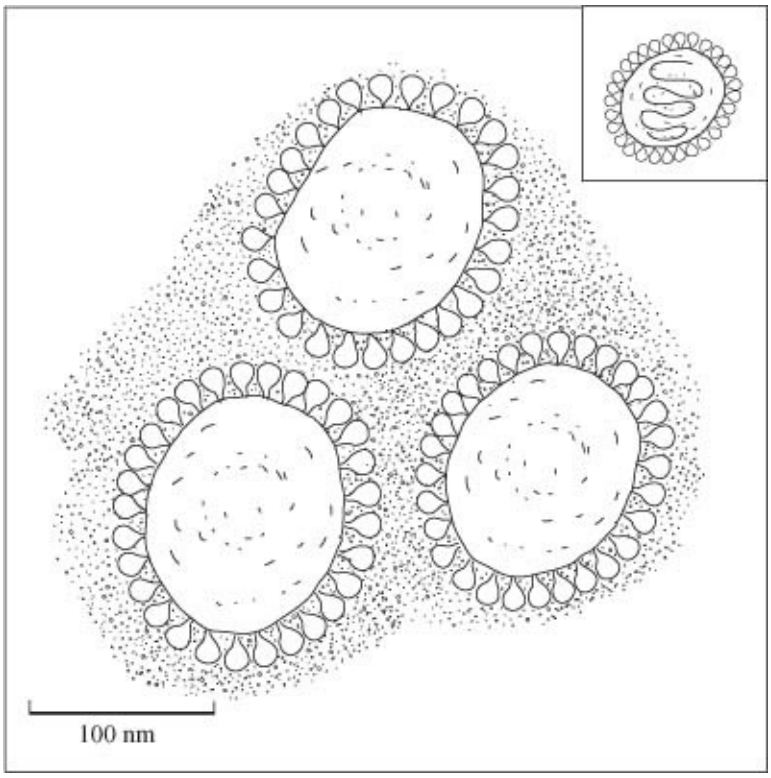
Members of the family *Coronaviridae* (Latin *corona*, crown) are large, pleomorphic, enveloped viruses. They contain a single molecule of linear, positive-sense, single-stranded RNA. Club-shaped glycoprotein peplomers projecting from the envelope impart a crown-like appearance to the virus (Fig. 79.1). Each peplomer is composed of a large viral trimeric glyco-protein (spike or S protein) which is responsible for attachment to cells and fusion between the envelope and plasma membrane. The S protein is the main antigenic component which induces the production of neutralizing antibodies during natural infection. Hypervariable domains in the S protein facilitate the production of virus escape mutants capable of evading the host immune response. Along with the family *Arteriviridae*, the family *Coronaviridae* belongs to the order *Nidovirales*. A major reorganization of the *Coronaviridae* has been accepted recently in which the family will be divided into two subfamilies, *Coronavirinae* and *Torovirinae* (Fig. 79.2). Coronaviruses, which are almost spherical with a diameter of 120 to 160 nm, have helical nucleocapsids. Toroviruses, which have a tubular nucleocapsid, may be disc-shaped, kidney-shaped or rod-shaped and are 120 to 140 nm in diameter. The *Coronavirinae* subfamily will comprise three genera, *Alphacoronavirus*, *Betacorona-virus* and *Gammacoronavirus*. Closely-related virus species are to be grouped together and renamed as follows: alphacoronavirus 1 (comprising feline coro-navirus, canine coronavirus and transmissible gastroenteritis virus); betacoronavirus 1 (comprising human enteric coronavirus, human coronavirus OC43, bovine coronavirus, porcine haemagglutinating encephalomyelitis virus, equine coronavirus and the newly-recognized canine respiratory coronavirus); avian coronavirus (comprising infectious bronchitis virus, turkey coronavirus, pheasant coronavirus, duck coronavirus, goose coronavirus and pigeon coronavirus). The subfamily *Torovirinae* will comprise two genera, *Torovirus* and the newly-created *Bafinivirus*, which contains a virus of fish.

Key points

- Enveloped, pleomorphic, single-stranded RNA viruses
- Replicate in the cytoplasm
- Labile in the environment
- Two genera:
  - *Coronavirus*, helical nucleocapsid
  - *Torovirus*, tubular nucleocapsid
- Coronaviruses:
  - Systemic disease in cats
  - Enteric and systemic disease in pigs
  - Respiratory disease in poultry
  - Enteric disease in cattle

Coronaviruses replicate in the cytoplasm of cells. Newly synthesized virions acquire their envelopes from the membranes of the endoplasmic reticulum and the Golgi complex. They are incorporated into vesicles and transported to the cell surface where the virions are released following fusion of the vesicles with the plasma membrane. Coronaviruses readily mutate leading to the emergence of genetically divergent strains. Genetic recombination can occur at high frequency between related coronaviruses and is believed to be mediated by a ‘copy choice’ mechanism.

Figure 79.1 Coronavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



With the exception of infectious bronchitis virus, coronaviruses are usually difficult to grow in cell culture. The virions are sensitive to heat, lipid solvents, formaldehyde, oxidizing agents and non-ionic detergents. The stability of coronaviruses at low pH values is variable; some are stable at values as low as pH 3.0.

Clinical infections

Coronaviruses can infect a number of mammalian and avian species and many display tropisms for respiratory and intestinal epithelium. The coronaviruses of veterinary importance and the clinical consequences of infection are indicated in Table 79.1. Infections, which are usually mild or inapparent in mature animals, may be severe in young animals. Coronaviruses are aetiologically important in humans as a cause of the common cold. A recently described member of the family that is capable of causing serious illness in humans, severe acute respiratory syndrome (SARS) virus, is believed to have been acquired from another animal species. The Chinese horseshoe bat may have been the source of infection.

Although evidence of torovirus infection has been found in pigs, sheep, goats and cats (Muir *et al.*, 1990), the clinical significance of these infections is

questionable. Two toroviruses have been implicated in enteric diseases of domestic animals (Table 79.2).

Feline infectious peritonitis

The disease feline infectious peritonitis (FIP), caused by certain strains of feline coronavirus (alphacorona-virus 1), is a worldwide and invariably fatal, sporadic, immune-mediated disease of domestic cats and other Felidae. Strains of feline coronavirus vary in patho-genicity. The term feline enteric coronavirus (FECV) has been used to describe strains that cause mild or inapparent enteritis, while the term feline infectious peritonitis virus (FIPV) was applied to those strains aetiologically implicated in FIP. It is now clear that FIPV arises as a mutant of the widely distributed FECV resulting in an alteration in tropism from exclusively enteric epithelial cells to the inclusion of the myeloid cells, monocytes and macrophages (Pedersen and Floyd, 1985 ; Poland *et al*., 1996; Rottier *et al*., 2005). Current thinking envisages a single virus termed feline coronavirus (FCoV) which encompasses strains of varying virulence that can be grouped broadly into two biotypes, enteric and FIP-associated. Genomic studies have shown a high degree of genetic related-ness between isolates of the two strains from the same location (Vennema *et al*., 1998). A mutation in the 3c gene, which encodes a small protein of unknown function, is considered to be responsible for the altered cell tropism of FIPV isolates (Pedersen, 2009). The parent FECV displays a strong tropism for mature apical epithelium of the intestine, while FIPV isolates display an enhanced ability to replicate in macrophages. Two serotypes of FCoV are described, both of which can cause FIP. Serotype 1 accounts for most field infections in Europe and North America whereas serotype 2 predominates in Japan. Serotype 2 is thought to have arisen as a result of a recombinational event between FCoV and canine coronavirus (Herrewegh *et al*., 1998).

Epidemiology

Feline infectious peritonitis occurs sporadically in catteries or multicat households. The incidence is reported to be higher in pedigree cats (Sparkes *et al*., 1992). Although cats of any age may be affected, those less than 1 year of age appear to be most susceptible. A second peak of disease in cats over 10 years of age has been noted (Barr, 1998). Infected cats shed virus in faeces and oronasal secretions. Transmission is mainly by the faecal – oral route. Litter

boxes are considered to be the main source of infection in groups of cats. Infection is acquired by young kittens from their mothers or from other adult cats (Addie and Jarrett, 1992). In infected multiple-cat households, about 40% of cats shed virus in their faeces at any given time, while about 15% of cats are persistently-infected carriers, responsible for maintaining the infection. While the majority of cats may be transiently infected with a particular strain of virus, reinfection with a different strain can occur. Shedding and transmission of the mutated, FIP-associated virus does not appear to occur under natural circumstances.

Figure 79.2 Taxonomic reorganization of the *Coronaviridae* submitted to the ICTV Executive Committee. The proposed changes include the introduction of new subfamilies and realignment of closely-related species. Viruses in red cause OIE-listed diseases.

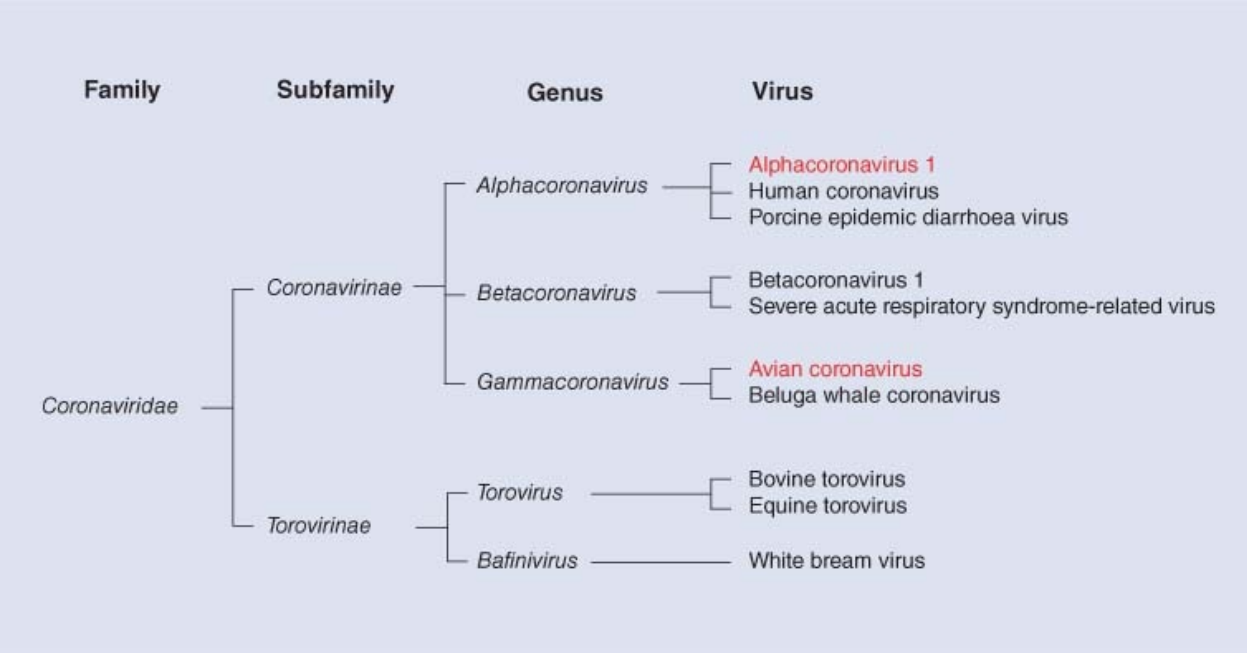


Table 79.1 Coronaviruses of veterinary significance.

Virus	Consequences of infection
Feline coronavirus (FCoV)	Replicates in enterocytes; subclinical infection common. May produce mild gastroenteritis in young kittens; also referred to as feline enteric coronavirus (FECV). Feline infectious peritonitis virus (FIPV) is considered to have evolved through mutation from strains of FCoV which initially replicated in enterocytes and subsequently in macrophages; causes sporadic fatal disease of young cats, often presenting clinically as an effusive peritonitis
Transmissible gastroenteritis virus (TGEV)	Highly contagious infection with vomiting and diarrhoea in piglets; high mortality in newborn piglets. A deletion mutant of TGEV, porcine respiratory coronavirus, induces partial immunity to TGEV
Porcine epidemic diarrhoea virus	Causes enteric infection similar to that caused by TGEV but with lower neonatal mortality
Porcine haemagglutinating	Nervous disease or vomiting and emaciation (vomiting and wasting disease) in young pigs. Infection is widespread but

encephalomyelitis virus	clinical disease is uncommon
Infectious bronchitis virus	Acute, highly contagious, respiratory infection in young birds; causes a drop in egg production in layers
Turkey coronavirus	Infectious enteritis (bluecomb disease)
Bovine coronavirus	Diarrhoea in calves; associated with winter dysentery in adult cattle
Canine coronavirus	Asymptomatic infection or diarrhoea in dogs characterized by high morbidity and low mortality
Canine respiratory coronavirus	Associated with respiratory disease in kenneled dogs

Table 79.2 Toroviruses of possible veterinary significance.

Virus	Hosts	Comments
Equine torovirus (Berne virus)	Horses	Isolated from rectal swab of a horse with diarrhoea in Berne, Switzerland. Clinical disease appears to be rare
Bovine torovirus (Breda virus)	Calves	Diarrhoea in newborn calves, particularly if deprived of colostrum

Pathogenesis

The pathogenesis of FIP is outlined in Fig. 79.3. Infection with FIPV does not always result in clinical disease; about one in nine FCoV-infected cats go on to develop FIP. Factors which may influence the development of the disease include the age, immune status and genetic characteristics of the host and the emergence of virulent virus strains (Addie *et al.*, 1995). Any stressful episode experienced during FCoV infection may predispose a cat to develop FIP. Following mutational changes in the virus, the emergence of a virulent FIPV strain results in systemic invasion with replication in macrophages. In most infected kittens, the development of effective cell-mediated immunity (CMI) restricts viral replication and ultimately eliminates infection. Some individual animals with less effective CMI may shed virus intermittently while remaining clinically normal. When CMI is severely impaired or defective, virus replication continues leading to B cell activation and the production of non-protective antibodies. The immune complexes, formed from these antibodies and FIPV, activate complement leading to immune-mediated vasculitis. The severity of this vasculitis influences the clinical presentation and the rate of progression of the disease. In addition to type III hypersensitivity, there is evidence of type IV hypersensitivity in FIPV-induced lesions (Paltrinieri *et al.*, 1998a,b). In experimentally induced FIP, an enhanced form of the disease may occur in cats that already possess antibodies to FCoV. It is thought that this antibody-dependent enhancement (ADE) is the result of opsonizing antibodies facilitating

the uptake of FCoV by macrophages (Hartmann, 2005). Failure to develop effective vaccines for FIP has been attributed to the development of antibodies which promote uptake of virus by macrophages rather than neutralizing antibodies.

Clinical signs

The incubation period ranges from weeks to months. The onset of clinical signs may be either sudden or slow and insidious. Early signs, which are generally non-specific, include anorexia, weight loss, listlessness and dehydration. Affected cats often present with icterus.

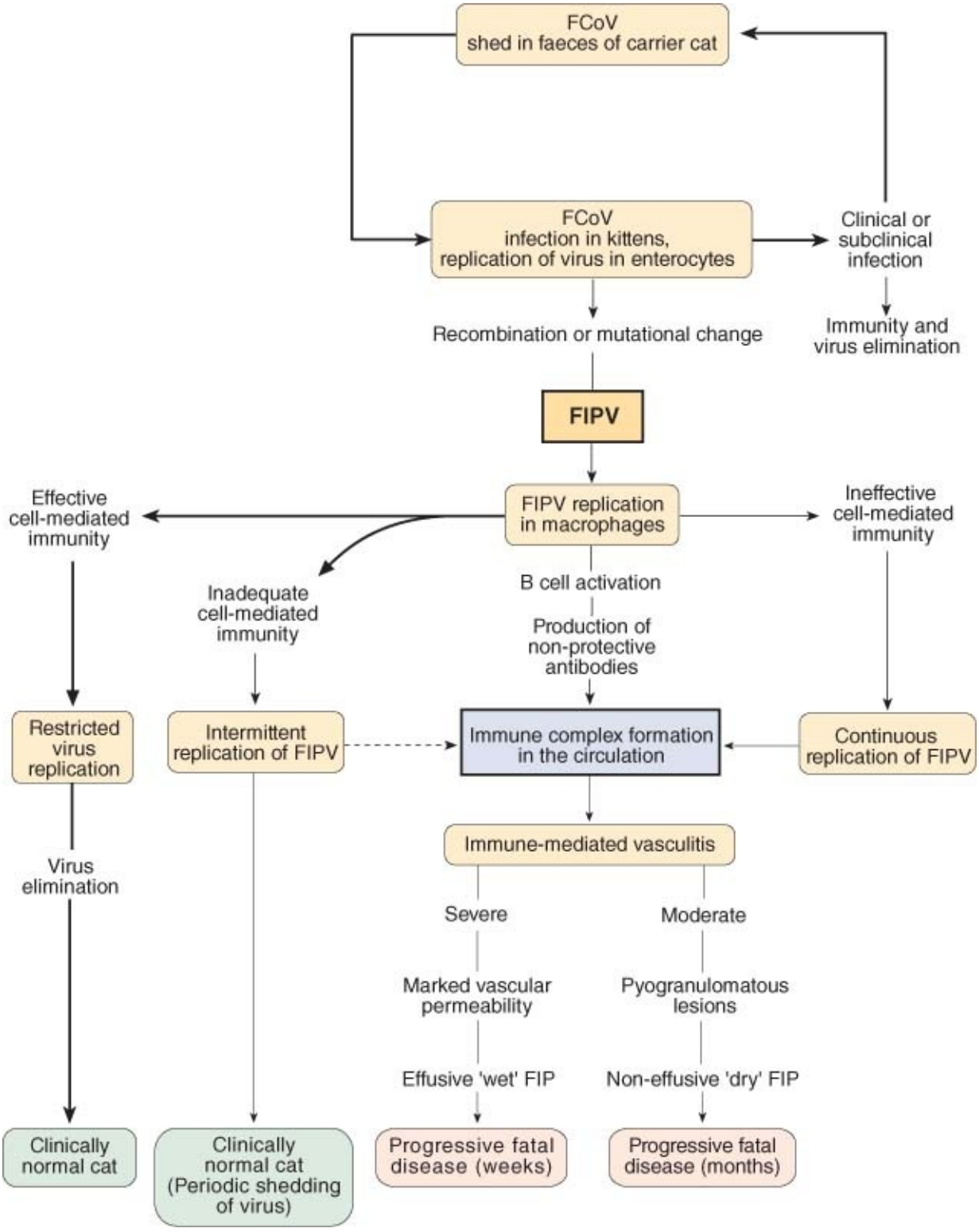
Cats with the effusive form of the disease have fibrin-rich exudates in the abdominal or thoracic cavities. If the pleural effusion is marked, dyspnoea develops. The effusive form of the disease usually leads to death within 8 weeks.

In the non-effusive form of FIP, clinical findings are less characteristic. Signs referable to lesions in organs or tissues in the abdominal cavity are present in about 50% of affected cats. Anterior uveitis, chorioretinitis and neurological signs may be evident in up to 30% of cases. The course of the disease is usually protracted with animals surviving for weeks or months. Infection with feline leukaemia virus or with feline immunodeficiency virus may increase susceptibility to FIPV and contribute to the severity of the clinical signs.

Diagnosis

- Currently, histological examination of affected tissues is the only procedure available for the definitive diagnosis of FIP.
- Immunohistochemical demonstration of FCoV antigen in tissue macrophages is confirmatory. However, invasive techniques are required to obtain the appropriate tissue samples.
- Pleural or peritoneal fluid, which may contain fibrin strands, clots on standing. It has a high protein content. A gamma globulin content exceeding 32% of total protein is suggestive of FIP (Weiss, 1991). Detection of FCoV antigen in macrophages in the body fluid correlates with FIP but in some cases macrophage numbers in effusion smears are too low for detection of the virus.
- Typical haematological changes include neutrophilia, lymphopenia and, in chronic cases, a normocytic, normochromic, non-regenerative anaemia.

Figure 79.3 Proposed relationship between infection with feline enteric coronavirus and the emergence of feline infectious peritonitis virus leading to the development of feline infectious peritonitis.
FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; FIP, feline infectious peritonitis



- A serum hyperproteinaemia is frequently present due to a hypergammaglobulinaemia. Serum liver enzymes and total bilirubin may also be raised.

- Diagnostic serological tests, including IFA and ELISA can be used to quantify antibody levels to FCoV. Considering that a large proportion of the cat population is antibody-positive and that high titres may be found in healthy cats, particularly cats from multiple-cat households, titres should be interpreted with caution. When determined by indirect immunofluorescence, antibody titres may be high in some FIP cases, while antibody titres are negligible in other cases (Sparkes *et al*., 1991).
- Reverse transcriptase PCR can be used to detect viral RNA in blood and in pleural/peritoneal effusions (Hartmann, 2005). It has also been used to detect the shedding of virus in faeces and for the identification of carriers (Addie and Jarrett, 2001 ; Addie *et al*., 2004). Unfortunately it has not been possible to design primers capable of distinguishing FIP-causing FCoV from enteric FCoV. In terms of ante-mortem testing of suspect FIP cases, the detection of messenger RNA within circulating monocytes by RT-PCR is considered the most reliable (Simons *et al*., 2005)

Treatment and control

Specific treatment for FIP is not available, and cats with clinical FIP eventually die. Supportive therapy and broad-spectrum antibiotics may be useful for treating affected cats in good physical condition (Weiss, 1994). Immunosuppressive and antiinflammatory treatments may slow the progression of the disease but do not prevent the inevitable fatal outcome.

An intranasal vaccine employing a temperature-sensitive mutant strain of serotype 2 FIPV has been developed. It is considered to be safe and does not induce ADE. Although reports of some efficacy trials have been favourable (Postorino Reeves *et al*., 1992; Hoskins *et al*., 1994; Fehr *et al*., 1997), other studies have failed to demonstrate significant protective immunity. The vaccine is licensed for cats older than 16 weeks. However, kittens in multiple-cat households where FCoV is present are often already infected and seropositive by that age.

The creation and maintenance of coronavirus-negative catteries is an effective method of control but is extremely difficult to achieve. Measures aimed at reducing the incidence of the disease include observing strict hygiene, avoiding large numbers of cats in single households, breeding from bloodlines free of FIP, rearing litters of kittens in isolation (Addie and Jarrett, 1990) and reducing stress in catteries.

Canine coronavirus infection

The ability of canine coronavirus (CCoV), now referred to as alphacoronavirus 1, to cause disease is variable and the agent can be isolated from normal dogs and those with diarrhoea (Tennant *et al*., 1993). Two genetic types have been recognized: CCoV type 1 and CCoV type 2 (Pratelli *et al*., 2003). Highly virulent variants of CCoV have occasionally been described (Decaro and Buonavoglia, 2008). The virus is related antigenically to the feline coronaviruses. A genetically and antigenically distinct virus, canine respiratory coronavirus (CRCoV), has been described and is associated with mild respiratory disease, particularly in kennelled dogs (Erles and Brownlie, 2008)

Epidemiology

Serological studies indicate that infection is common (Tennant *et al*., 1991). Infections with CCoV may spread rapidly among susceptible dogs at shows and in kennels. Seroprevalence may approach 100% in kennels and range from 6% to 75% in pet dog populations. The prevalence of CCoV in a cross-section of dogs presenting at veterinary clinics, as determined by RT-PCR, was 2.8% (Stavisky *et al*., 2010).

Infection is acquired from the faeces of infected animals. Infected dogs usually shed the virus for up to 9 days and intermittent shedding may continue for months. The virus is not particularly resistant in the environment and carrier dogs are required for its maintenance. Mucosal immunity appears to be more important than circulating antibody for protection of dogs from reinfection. In the absence of frequent re-exposure to the virus, the duration of immunity may be relatively short.

Pathogenesis

Canine coronaviruses withstand the acidic environment of the stomach and infect enterocytes in the duodenum. Infection spreads rapidly involving other parts of the small intestine. Diarrhoea may follow loss of digestive and absorptive capacity in the small intestine as a result of damage to mature enterocytes at the tips of villi. Recovery is rapid in uncomplicated cases.

Clinical signs

Although clinical disease has been recorded in dogs, foxes and coyotes, infection

with CCoV is often asymptomatic. Dogs of all ages can become infected; serious illness is most likely to occur in pups. The incubation period is up to 3 days. Clinical signs, which are variable and non-specific, include anorexia, depression, vomiting and diarrhoea. Most animals recover in 7 to 10 days. Occasionally, illness may be protracted due to secondary bacterial, parasitic or other viral infections. The mortality rate is low.

Diagnosis

- Virus may be detected in faeces by electron microscopy.
- Virus can be isolated in a number of cell lines but the procedure is slow and unreliable.
- A number of PCR assays have been described for the detection of canine coronavirus in faecal samples (Pratelli *et al*., 1999; Naylor *et al*., 2001).
- Serum neutralization or indirect immunofluores-cence tests can be used to demonstrate an increasing antibody titre.

Treatment and control

- Supportive treatment, including fluid replacement therapy and antibiotic administration, should be instituted when required.
- Although inactivated vaccines are available and can be used in pregnant bitches to boost colostral immunity, the degree of protection induced by these vaccines is uncertain. A modified live vaccine administered oronasally has been shown to be effective but only against the homologous CCoV type (Pratelli *et al*., 2004).
- Contact with infected animals and faeces should be minimized.
- Effective disinfection of premises and utensils can be achieved with 3% sodium hypochlorite or 2% formalin.

Transmissible gastroenteritis

Transmissible gastroenteritis (TGE) is a highly contagious, coronaviral disease of young pigs which occurs worldwide. There is one serotype of transmissible gastroenteritis virus (TGEV) which is closely related antigenically to feline coronavirus and canine coronavirus and all three viruses are now considered a single species and referred to as alphacoronavirus 1. A relatively non-pathogenic respiratory variant of TGEV, referred to as porcine respiratory coronavirus

(PRCV), was first recognized in 1984. This virus, a deletion mutant of TGEV, spread to pig populations in many European countries and has now been identified in the USA and in some Asian countries. Infection with PRCV is usually subclinical.

Epidemiology

Transmission of TGEV is usually by the faecal – oral route. The virus is moderately stable in the presence of proteolytic enzymes and at pH 3.0, ensuring survival in the stomach and small intestine. Viral shedding in faeces can persist for up to 2 weeks. Outbreaks of TGE tend to occur in winter. In fully susceptible herds, the virus spreads rapidly infecting animals of all ages. The disease is, however, most severe in newborn piglets. Outbreaks usually terminate in a few weeks if no new susceptible animals are introduced into the herd.

Pathogenesis

Following ingestion, the virus replicates mainly in mature enterocytes at the tips of the villi in the small intestine. Viral replication results in villous atrophy throughout the length of the small intestine. Digestion and cellular transport of nutrients and electrolytes are severely disrupted resulting in the accumulation of fluid in the intestinal lumen and diarrhoea. Young piglets are particularly susceptible to the ensuing dehydration and metabolic acidosis.

Clinical signs

The incubation period is up to 3 days. Vomiting and watery diarrhoea may be evident in affected piglets less than 7 days old. Rapid dehydration and weight loss follow. The disease is usually confined to piglets under 3 weeks of age and, in newborn piglets, mortality may approach 100%. Inappetence and transient diarrhoea may be observed in older pigs. Subclinical infections also occur. Sows quickly become immune, and maternally-derived immunity, lactogenic IgA, reduces the severity of clinical signs in piglets. Outbreaks usually last a few weeks. However, TGEV infection may become endemic in a herd if consecutive litters become infected as maternally-derived immunity wanes. Clinically, such infections are usually mild.

Diagnosis

The sudden onset and rapid spread of diarrhoea among newborn pigs along with almost 100% mortality is highly suggestive of TGE. Post-mortem examination of washed small intestine discloses paper-thin walls due to villous atrophy. The walls of the jejunum and ileum are affected whereas those of the duodenum are usually normal.

- Viral antigen can be detected in mucosal smears or cryostat sections of the small intestine by immunofluorescence. It may be necessary to euthanize some piglets in the early stages of the disease in order to obtain suitable specimens for laboratory examination. Viral antigens can be demonstrated in faeces by ELISA.
- Virus can be isolated from faeces in a swine testis cell line.
- Reverse transcriptase PCR assays for the detection of TGEV and differentiation from PRCV are available (Paton *et al*., 1997).
- Serological testing for antibodies can be carried out using virus neutralization. However, virus neutralization does not distinguish antibodies to TGEV from those induced by PRCV infection. Competitive blocking ELISAs, which are based on the use of monoclonal antibody directed against a glycoprotein epitope present in TGEV but absent from PRCV, can be used to distinguish infections caused by these two viruses. These assays are available commercially.

Treatment and control

- Specific treatment is not available but fluid replacement therapy may be beneficial. Maintaining the farrowing house at an optimal temperature may enhance survival.
- In acute outbreaks of TGE, deliberate exposure of pregnant sows to the virus may reduce neonatal mortality. After exposure, sows due to farrow should be moved to clean premises. Newborn piglets born to exposed sows will usually receive passive antibody protection through colostrum.
- Modified live and inactivated vaccines are available. Modified live vaccines are administered orally to sows 5 to 7 weeks before farrowing and a booster inoculation is administered parenterally 1 week before parturition. Vaccination reduces mortality but does not eliminate infection.
- Serious outbreaks of TGE have become rare in European pig populations endemically infected with PRCV. Porcine respiratory coronavirus is spread in pig herds by aerosols. Sows infected with PRCV usually transfer

substantial colostral protection to their litters (Wesley and Woods, 1993).

Porcine epidemic diarrhoea

This porcine disease, which is clinically similar to TGE, occurs in Europe and Asia. There is only one serotype of porcine epidemic diarrhoea virus (PEDV), a coronavirus serologically unrelated to TGEV.

Epidemiology

The virus is transmitted by the faecal – oral route. Spread of the virus to susceptible herds occurs directly through infected pigs and indirectly through contaminated fomites or vehicles. The rate of spread of infection within a farm is slower than that of TGEV.

Pathogenesis

Virus replication occurs in epithelial cells in the small intestine and colon. In the small intestine, shortening of villi occurs. The rate and severity of cell destruction is less marked than with TGEV.

Clinical signs

The incubation period is up to 4 days. The age of animals affected and the associated morbidity and mortality are variable. On some farms, animals of all ages become sick and the mortality rate in piglets under 1 week may approach 50%. Watery diarrhoea, which may be preceded by vomiting, is the main clinical presentation. Occasionally a few animals die suddenly, with back muscle necrosis evident post mortem. The virus may persist on large breeding farms by infecting consecutive litters of pigs. Most affected pigs recover after about 1 week and mortality rates are usually low.

Diagnosis

- Direct immunofluorescence, using cryostat sections of small intestine from pigs euthanized during the phase of acute diarrhoea, is sensitive and reliable, particularly in specimens from newborn piglets.
- Viral antigen may be detected by ELISA in faecal material or intestinal contents collected during the acute phase of the disease.
- A duplex RT-PCR designed to detect and to differentiate PEDV and TGEV

has been described (Kim *et al*., 2001, 2007).

- Antibodies can be detected in paired serum samples using a blocking ELISA or by indirect immunofluo-rescence on PEDV-positive cryostat sections of intestine.

Treatment and control

- Specific treatment is not available. Vaccines are not generally available but an oral attenuated vaccine has been developed in South Korea (Song *et al*., 2007).
- Appropriate hygienic measures and control of animal and human movement between farms are necessary measures for disease prevention.
- During an outbreak of the disease on a breeding farm, good hygiene slows the spread of infection. Deliberate spread of the virus to pregnant sows using infected faecal material stimulates colostral immunity and shortens the course of a disease outbreak.

Porcine haemagglutinating encephalomyelitis virus infection

This coronavirus disease of young pigs, also known as vomiting and wasting disease, is caused by porcine haemagglutinating encephalomyelitis virus of which there is only one serotype. The virus agglutinates red cells of several animal species.

Epidemiology

Infection is common and probably worldwide. The virus is shed in nasal secretions and readily transmitted by aerosols. Infection persists on breeding farms as a subclinical respiratory condition. In herds where infection is endemic, immune sows transfer protective antibodies to their offspring and piglets are protected until they have developed an age-related resistance. Pigs which become subclinically infected develop an active immunity at 8 to 16 weeks of age.

Pathogenesis

The virus replicates locally in the upper respiratory tract and tonsils before spreading via the peripheral nervous system to the medulla oblongata. It then

spreads to other parts of the central nervous system. Viral damage to the vagal sensory ganglion and to the intramural plexus of the stomach are considered to be responsible for vomiting and delayed gastric emptying, respectively.

Clinical signs

Clinical signs develop in pigs less than 3 weeks of age after an incubation period of up to 7 days. Signs range from acute encephalomyelitis characterized by incoordination, convulsions and high mortality to vomiting and wasting, where the principal signs include vomiting, dehydration and emaciation. Newborn piglets become severely dehydrated and may die. The mortality rate is often 100% in young pigs. Older pigs continue to vomit and become emaciated. Survivors may be permanently stunted.

Diagnosis

- For the isolation of virus or demonstration of viral antigen in cryostat sections by immunofluorescence, samples of brain stem must be collected within 2 days of the onset of clinical signs. Porcine thyroid cells are suitable for virus isolation.
- Lesions of non-suppurative encephalomyelitis may be evident.
- Viral RNA may be detected using reverse transcriptase and nested PCR assays (Sekiguchi *et al*., 2004).
- A significant rise in antibody titre may be demonstrable in paired serum samples by virus neutralization or haemagglutination-inhibition tests.

Treatment and control

- Specific treatment is not available.
- Due to the sporadic nature of the disease, vaccination is unwarranted.
- Appropriate measures should be taken to prevent the introduction of infection into breeding units. If infection is introduced, it is important to ensure that infection of sows results in an adequate antibody response for the protection of litters. To ensure that they are exposed to the virus in advance of pregnancy, gilts should be integrated into the herd before breeding commences.

Infectious bronchitis

Infectious bronchitis, caused by avian coronavirus (infectious bronchitis virus, IBV), is a highly contagious, economically important, worldwide disease of poultry which affects the respiratory, reproductive and renal systems. Many serotypes are recognized, often with different virulence and tissue tropisms, probably as a result of mutation or recombination.

Epidemiology

The chicken is the main host although IBV has been isolated from pigeons and pheasants. The most important route of transmission is by aerosols, and spread of infection occurs rapidly among susceptible birds. Morbidity may approach 100%. Virus, shed from the respiratory tract for a few weeks after infection, may be recovered over a period of weeks from the faeces and from eggs of infected birds. Infection may persist in the digestive tract of individual birds. Several serotypes can co-circulate in a region and, as serotypes cross-protect poorly, chickens can acquire infection on more than one occasion (Cavanagh, 2007).

Pathogenesis

The respiratory system is the primary site of virus replication. Viraemia follows within 1 to 2 days of exposure. The virus becomes widely distributed throughout the body, particularly in the oviducts, kidneys and bursa of Fabricius. The distribution and severity of lesions in these tissues are influenced by the virulence of the infecting strain and the breed of chicken (Cavanagh, 2007).

Clinical signs

The incubation period is up to 48 hours. Age, immune status and strain of virus strongly influence the nature and severity of disease observed in a flock. In general, disease is most severe in young birds, particularly when secondary infections are present. In chickens less than 3 weeks of age, there is gasping and nasal exudate present. Infection may result in stunting and some birds may die suddenly from occluded bronchi. In older birds, rales and gasping are usually observed. Mortality rates are generally low in the absence of secondary infections. The course of the disease is up to 7 days in individual birds and outbreaks last about 10 to 14 days in flocks. Layers show signs of rales followed by a marked reduction in egg production which slowly returns to normal. Poor egg quality, with soft-shelled and misshapen eggs, may continue for several weeks. A relatively new strain, termed QX, was first described in China in 1996

and is associated with cystic oviducts. Affected birds stand with their heads held up due to an enlarged abdomen, the result of large fluid-filled cysts in the oviducts. Infection with nephrotropic strains of IBV is associated with interstitial nephritis and mild respiratory signs with moderate to high levels of mortality.

Diagnosis

- Virus isolation is often feasible in the acute stage of the disease. Although specimens from the respiratory tract are preferred for virus isolation, samples from kidney, oviduct and faeces can also be used. Material is usually inoculated into the allantoic sac of 9 to 10-day-old embryonated eggs. A number of passages may be required to produce the characteristic stunting and curling of the embryo. Tracheal explants from day-old specific-pathogen-free chicks may be used for antigenic typing of isolates by virus neutralization.
- The detection and typing of isolates can be accomplished using real-time RT - PCR (Callison *et al*., 2005).
- Serological tests, including virus neutralization, agar gel immunodiffusion, haemagglutination inhibition and ELISA, can be used to demonstrate a rise in antibody titre between acute and convalescent serum samples.

Treatment and control

- Specific treatment is not available. The administration of antibiotics may reduce mortality due to secondary bacterial infections.
- Both live and killed vaccines with adjuvant are available. Protection tends to be short-lived and serotype-specific. Consequently, vaccination on a number of occasions and with more than one sero-type is usual. Live vaccines are usually administered in the drinking water or by aerosol to chicks up to 14 days of age and again at about 4 weeks of age. A high-passage vaccinal virus, which is less virulent, is used for primary immunization. A more virulent strain of virus is used for booster vaccination. Following primary immunization with live vaccines, killed vaccines inoculated shortly before the birds start laying eggs are useful in layer and breeder flocks to prevent losses in egg production and to ensure a high level of yolk-sac-derived immunity in chicks.

Bovine coronavirus infection

Bovine coronavirus (BCV) is one of the causes of calf diarrhoea and is also associated with winter dysentery in adult housed cattle. There is evidence of its involvement in the bovine respiratory disease complex (Kapil and Goyal, 1995). The virus, which exists as a single serotype, haemagglutinates red cells of mice, rats and hamsters.

Epidemiology and pathogenesis

Virus is mainly transmitted by the faecal – oral route. However, coronaviruses have also been recovered from the respiratory tract of calves (McNulty *et al*., 1984) and infected calves often harbour BCV in both the enteric and respiratory tracts (Thomas *et al*., 2006). Infection is usually endemic on farms, maintained by clinically-affected calves and persistently-infected, but clinically normal, calves and cows. The virus replicates and destroys mature enterocytes in the small intestine and colon, resulting in a malabsorptive diarrhoea. The severity of disease is influenced by the age of the animal at the time of infection and type of management. Risk factors, which include changes in diet, cold temperatures, close confinement and the presence of other microorganisms such as *Campylobacter jejuni*, appear to be particularly important in the development and pathogenesis of winter dysentery.

Clinical signs

In calves, the incubation period is up to 2 days and clinical signs are usually observed between 3 and 30 days of age. There is profuse diarrhoea which may result in dehydration, acidosis and death. With appropriate treatment, the diarrhoea usually ceases in a few days. Respiratory tract infections are generally mild but may predispose to more severe secondary infections.

In adult animals the incubation period of winter dysentery is 3 to 7 days. There is a sudden onset of diarrhoea accompanied by a dramatic drop in milk yield. The faeces of some animals may contain blood or blood clots. A nasolacrimal discharge and cough may accompany the diarrhoea. Herd outbreaks can last for 2 weeks.

Diagnosis

- Faeces or intestinal contents for laboratory examination should be collected early in the course of the disease.
- Typical coronavirus particles can be demonstrated in faecal samples by

direct electron microscopy (EM). Immune EM is preferable as it is more sensitive and specific. Alternative diagnostic methods of detection include ELISA and reverse passive haemagglutination.

- Immunofluorescence can be used to detect viral antigen in cryostat sections of distal small intestine or colon.
- RT-PCR, nested PCR and real-time PCR assays are available for detection of bovine coronavirus RNA in clinical samples (Cho *et al*., 2001; Decaro *et al*., 2008).
- Isolation of virus in tissue culture is difficult.
- Antibodies to BCV are widespread in cattle, and serological assays, including virus neutralization, ELISA and haemagglutination inhibition, are of limited value for diagnosis.

Treatment and control

Treatment is supportive but non-specific. Control of the disease in calves is based on vaccination and good management practices including thorough cleaning and disinfection, good ventilation to reduce airborne transmission and ensuring an adequate intake of colostrum. To avoid transmission to susceptible calves, animals of different age groups should not be mixed. Both live and inactivated vaccines have been developed and can be used orally in calves to stimulate active immunity. The presence of maternal antibodies to BCV in colostrum-fed calves may interfere with the efficacy of oral vaccination. Alternatively, vaccines may be administered parenterally in cows to increase the level of antibody in colostrum and milk. A vaccine is not available at present for the prevention of winter dysentery in adult animals.

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Chapter 80

Arteriviridae

Arteriviruses, formerly classified as members of the family *Togaviridae*, have been assigned to the family *Arteriviridae*. Their genome organization and mode of replication are similar to those of members of the *Coronaviridae* and these two families are part of the order *Nidovirales*. In the family *Arteriviridae* there is a single genus, *Arterivirus*. The name of the family and genus derives from the disease, equine arteritis, which is caused by the type species. Arteriviruses are spherical, 40 to 60 nm in diameter and possess a lipid- containing envelope carrying small projections (Fig. 80.1). There are two major and four minor envelope proteins, four of which are glycoproteins. The icosahedral nucleocapsid contains a molecule of linear, positive-sense, single-stranded RNA. Replication takes place in the cytoplasm of infected cells and is characterized by the formation of double-membrane vesicles that probably carry the replication complex. Preformed nucleocapsids bud into the lumen of smooth intracellular membranes of the endoplasmic reticulum and/or the Golgi complex. The enveloped virions accumulate in intracellular vesicles, which are transported to the cell membrane for release by exocytosis. Arteriviruses are relatively labile, being sensitive to heat, low pH, lipid solvents, detergent treatment, UV irradiation and many disinfectants.

Clinical infections

Members of the genus are host-specific and antigenically unrelated. Infections have been described in horses, pigs, mice and monkeys. The primary target cells are macrophages. Infection is spread horizontally by aerosol, by biting or by venereal transmission. Infections are frequently persistent.

Key points

- Medium-sized, enveloped, single-stranded RNA viruses
- Icosahedral symmetry

- Replicate in cytoplasm of macrophages and endothelial cells
- Cause equine viral arteritis and porcine reproductive and respiratory syndrome

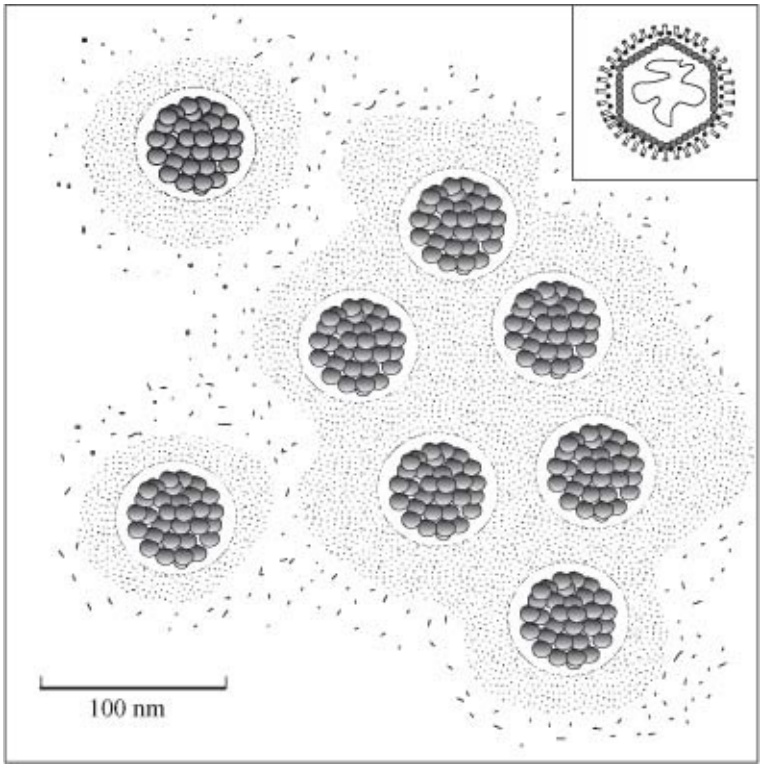
Equine viral arteritis

Although infection with equine arteritis virus (EAV) occurs worldwide, outbreaks of clinical disease are comparatively rare. Upper respiratory tract infection, ventral oedema and abortion are prominent clinical features. Biological and genomic differences have been demonstrated in isolates of EAV but antigenic variation is limited. Only one serotype has been recognized.

Epidemiology

Horses, donkeys and mules are susceptible to infection. The percentage of seropositive animals is higher in Standardbreds than in Thoroughbreds. Whether this reflects differences in susceptibility or in degree of exposure as a result of management practices is unclear. Although infection is prevalent in some horse populations, outbreaks of disease are sporadic. The frequency of confirmed outbreaks of equine viral arteritis has increased in recent years. Factors which may have contributed to this increase include increased international movement of horses, more extensive use of artificial insemination and greater awareness of the disease.

Figure 80.1 Arterivirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



During the acute phase of infection, virus is spread primarily by aerosols from the respiratory tract. Virus is also shed in faeces, urine and vaginal secretions. Close contact facilitates spread of infection. Virus is usually eliminated from mares and geldings within 1 to 2 months but may persist in about 35% of infected stallions. Carrier stallions are asymptomatic and shed virus continuously in semen. More than 80% of mares covered by carrier stallions may become infected. Persistent infection, which does not impair the fertility of stallions, appears to be testosterone- dependent (McCollum *et al*., 1994). Mares infected venereally may spread virus horizontally via the respiratory route to in-contact susceptible animals. Mares do not become persistently infected. Abortion or infection of the foal may result when pregnant mares are infected.

Pathogenesis and pathology

Following aerosol transmission, replication occurs in pulmonary macrophages. There is subsequent spread to the bronchial lymph nodes and viraemia. Pathological changes, arising from infection of endothelial cells and widespread necrotic arteritis, include oedema, congestion and haemorrhage in many tissues. Aborted foetuses, which often exhibit autolysis, rarely display characteristic lesions.

Clinical signs

The incubation period ranges from 3 to 14 days. Many infections are subclinical. The disease tends to be more severe in very young and aged animals and in those subjected to stress. Affected animals present with fever, anorexia, depression, conjunctivitis, rhinitis and stiff gait. Oedema, which is usually prominent, may involve the eyelids, ventral abdomen and limbs, particularly the hind limbs. Urticarial-type lesions commonly affect the skin of the neck. Acute, often fatal, respiratory disease has been recorded in foals (Del Piero *et al*., 1997). Affected horses almost invariably make a full recovery and are usually immune for several years. Clinical signs in donkeys are similar to those in horses but generally milder.

Diagnosis

Because equine viral arteritis resembles a number of other equine diseases in its clinical presentation, definitive diagnosis requires laboratory confirmation. Internationally accepted testing procedures have been published (Timoney, 1996; OIE, 2008).

- Virus isolation is carried out in appropriate cell lines such as rabbit or equine kidney cells. Samples suitable for isolation procedures include nasopharyngeal and conjunctival swabs, placental tissue and foetal tissue and fluids.
- Viral RNA can be detected in semen and other specimens using the reverse transcriptase polymerase chain reaction. A variety of protocols have been published (OIE, 2008).
- Acute and convalescent blood samples can be submitted for serology. The virus neutralization test, considered to be sensitive and highly specific, is the most widely used test and is usually carried out in microtitre plates with the addition of complement to enhance sensitivity.
- Carrier stallions can be identified by serological testing. If stallions are seropositive, virus isolation from semen should be attempted. The sperm-rich fraction of semen is suitable for virus isolation or RT-PCR. Alternatively, carrier animals can be mated to seronegative mares which are monitored for seroconversion.

Treatment

Supportive therapy is indicated in severe cases. The carrier state in stallions cannot be eliminated by chemotherapy.

Control

Persistently infected stallions should be identified and either castrated or euthanized. In order to reduce the risk of colt foals becoming carriers, vaccination at 6 to 12 months of age is recommended (Timoney and McCollum, 1996). Two types of vaccine are available commercially:

- A modified live tissue-culture-adapted vaccine induces good protection against clinical disease but not against infection. Use of this vaccine is contrain- dicated in pregnant mares and in foals under 6 weeks of age. Vaccinated stallions do not appear to develop the carrier state with vaccinal virus.
- An inactivated whole-virus vaccine with adjuvant is reported to be safe for pregnant mares but requires booster injections at 6-month intervals (Fukunaga, 1994).

Replicon particles, derived from a vaccine strain of Venezuelan equine encephalitis (VEE) virus expressing the two major envelope proteins, have been shown to be capable of inducing protective, neutralizing antibodies (Balasuriya *et al*., 2002).

Porcine reproductive and respiratory syndrome

This economically important condition is characterized by reproductive failure in sows and pneumonia in young pigs. The syndrome was first described in the USA in 1987. Despite attempts at controlling spread, the disease is now endemic in many countries. The aetiological agent, originally called Lelystad virus, was first isolated in the Netherlands (Wensvoort *et al*., 1991). It was characterized as an arterivirus and renamed porcine reproductive and respiratory syndrome virus (PRRSV). Significant antigenic and genomic differences between European and American isolates of the virus are evident, referred to as type I and type II subspecies, respectively. However, type I isolates have now been found in America, while type II isolates have been introduced into Europe through the use of modified live vaccine made from a North American isolate.

Epidemiology

Natural infection occurs in pigs and wild boars. Virus, which is shed in saliva, urine, semen and faeces, is highly infectious. Nose-to-nose contact is considered to be the most likely route of transmission. Airborne transmission between farms was important during the early acute outbreaks of the disease when large quantities of virus were excreted. Now it appears to be important only when pig population densities are high and when weather conditions are suitable. Virus survival, which is prolonged in winter months when low temperature and high humidity prevail, facilitates transmission. Infection is generally introduced on to farms by infected pigs or by infected semen. On endemically-infected farms, virus is transmitted either continuously or in waves. Maintenance of infection on farms is multifactorial (Albina, 1997). Maternally- derived immunity is of such short duration that piglets become susceptible to infection at 4 to 10 weeks of age. Susceptible replacement pigs maintain infection in endemic herds. Infection may spread in a slow and unpredictable manner with the result that some animals in infected herds remain susceptible. Immunocompetent pigs, which display a progressive decline in antibody levels over a period of several months, may become susceptible to reinfection. In experimentally infected pigs, infection persisted for up to 157 days (Wills *et al*., 1997).

Pathogenesis and pathogenicity

Infection occurs most frequently by the respiratory route. The virus has an affinity for pulmonary alveolar macrophages and the lungs are probably the target organs (van Reeth, 1997). Early antibody responses are not effective in clearing virus infection. Antibody- dependent enhancement of infection of pulmonary alveolar macrophages has been described in the disease. After transportation to regional lymph nodes, virus spreads to tissue macrophages throughout the body. Transplacental infection of foetuses occurs. For reasons that are unclear, reproductive failure is more difficult to induce experimentally in early gestation than in late gestation (Kranker *et al*., 1998). Foetal and placental abnormalities are not consistently present and the mechanism of foetal death and reproductive failure is uncertain. Although the virus does not appear to have a systemic immunosuppressive effect, it predisposes to infection with other microorganisms such as *Streptococcus suis*, porcine respiratory corona- virus and *Haemophilus parasuis* (Albina *et al*., 1998).

Clinical signs

Introduction of PRRSV to a breeding herd is usually followed by reproductive failure which may take the form of abortions, early farrowing, increased numbers of stillborn and mummified foetuses, weak neonatal pigs and delayed return to service in affected sows. A ‘rolling inappetence’, progressively affecting animals in an infected herd, has been described. In some cases, cyanosis of the ears and vulva along with erythematous plaques on the skin (‘blue-eared disease’) have been described. Respiratory distress and increased preweaning mortality are important features of the disease in neonatal pigs. Subclinical infection is common. Factors which may exacerbate clinical disease include concentrated numbers of pigs, the virulence of the strain of PRRSV and the presence of slatted floors. Although sporadic respiratory and reproductive problems are the main clinical manifestations in most affected herds, in a few endemically- infected herds, chronic disease problems predominate (Zimmermann *et al*., 1997). However, more severe outbreaks with deaths occurring in both young and adult pigs, and mortality rates averaging 20%, have been described in China (Tian *et al*., 2007).

Diagnosis

- Laboratory confirmation is usually required because the clinical presentation is variable, particularly in endemically-infected herds.
- Serology is the most widely used diagnostic method. Several serological tests are available including ELISA and an immunoperoxidase monolayer assay. However, these tests do not distinguish carrier from vaccinated animals. Commercial assays are available (Okinga *et al*., 2009).
- The presence of PRRSV may be demonstrated by virus isolation, direct FA staining, *in situ* hybridization or reverse transcriptase polymerase chain reaction (Kleiboeker *et al*., 2005). Virus isolation is difficult, requiring the use of porcine alveolar macrophages. Suitable samples for submission include serum, foetal fluids, spleen, tonsils, lymph nodes and lung tissue. A multiplex PCR assay has been designed to differentiate North American and European PRRSV isolates (Gilbert *et al*., 1997).

Treatment

Specific treatment is not available. Supportive therapy and antibiotic administration to suppress secondary infections may be beneficial.

Control

Vaccination and effective hygiene and health management are important for controlling infection.

- A commercial modified live vaccine is available for use in pigs 3 to 18 weeks of age and is suitable for use in non-pregnant sows before breeding. It is not suitable for use in boars and pregnant sows or in herds free from PRRSV infection. Spread of vaccinal virus to non-vaccinated animals, in some instances resulting in clinical disease, has been reported. An inactivated vaccine with adjuvant is also available (Plana-Duran *et al*., 1997). Vaccination provides reasonable protection from the clinical effects of infection. Vaccines may contain type I or type II viruses. It is considered that vaccines containing virus closely related to the circulating field virus provide the greatest benefit (Scortti *et al*., 2007 ; Okuda *et al*., 2008).
- Stabilization of the sow herd is required to avoid subpopulations of non-immune sows and to break the cycle of reinfection. Effective isolation and acclimatization procedures should be introduced before replacement sows are added to the herd. Other control measures, relating to the weaning and rearing of piglets on infected premises and to strategies for the elimination of infection from herds, have been proposed (Dee and Joo, 1997) Dee and Molitor, 1998).

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Chapter 81

Flaviviridae

The family name of the *Flaviviridae* (Latin *flavus*, yellow) is derived from yellow fever, a disease of humans caused by a flavivirus with jaundice as a major clinical feature. Members of the family are 40 to 60 nm in diameter with icosahedral capsids and tightly adherent envelopes which contain either two or three virus-encoded proteins depending on the genus (Fig. 81.1). The genome is composed of positive-sense, single-stranded RNA. Replication of virus occurs in the cytoplasm with assembly and envelopment taking place at intracellular membranes, probably endoplasmic reticulum. Viral particles are transported in cytoplasmic vesicles through the secretory pathway before release by exocytosis. The labile mature virions are sensitive to heat, detergents and organic solvents.

The family comprises three genera, namely *Flavivirus*, *Pestivirus* and *Hepacivirus* (Fig. 81.2). Two genera, *Flavivirus* and *Pestivirus*, contain viruses of veterinary importance. The genus *Flavivirus* contains more than 50 members assigned to several serologically-defined groups. Most members of the genus are arboviruses, which require either mosquitoes or ticks as vectors. Viruses in the genus agglutinate goose red cells. The genus *Pestivirus* contains four viruses of veterinary importance, namely bovine viral diarrhoea virus 1 and 2, border disease virus, and classical swine fever virus. Pestiviruses possess four structural proteins: a capsid protein and three envelope glycoproteins designated E^{rns} (soluble ribonuclease), E1 and E2. The immunodominant major glycoprotein, E2 (gp55) induces neutralizing antibodies.

Key points

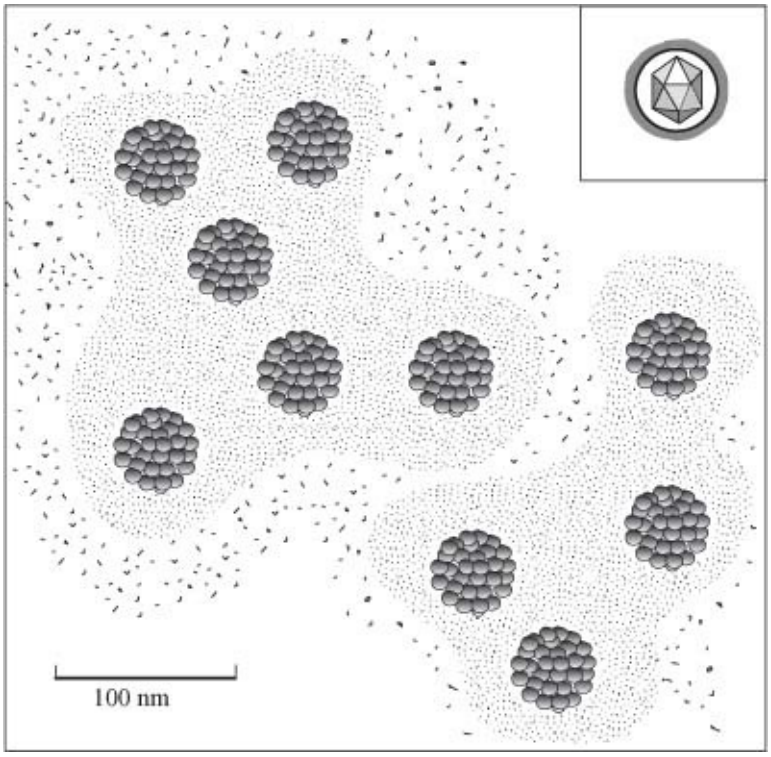
- Positive-sense, single-stranded RNA
- Replicate in the cytoplasm
- Two genera of veterinary importance, *Flavivirus* and *Pestivirus*
- Most viruses in the genus *Flavivirus* are transmitted by arthropods and cause encephalitis
- Pestiviruses, which are transmitted directly or indirectly, cause bovine viral diarrhoea, border disease and classical swine fever

Clinical infections

In the genera *Flavivirus* and *Pestivirus*, there are several viruses of particular veterinary importance (Table 81.1). Four members of the genus *Flavivirus*, louping ill virus, Japanese encephalitis virus, Wesselsbron virus and Israel turkey meningoencephalitis virus, cause disease in domestic animals. In addition, infection with West Nile virus, an important human pathogen, causes fatal disease in horses and, since 1999, has become a disease of major importance following its dramatic spread across North America. Other members of the genus which are important human pathogens include yellow fever virus, dengue virus, Japanese encephalitis virus, tick-borne encephalitis virus and St. Louis encephalitis virus. The sole member of the *Hepacivirus* genus, hepatitis C virus, is an important cause of hepatitis in humans.

The four recognized members of the *Pestivirus* genus which infect domestic species are closely related antigenically. Bovine viral diarrhoea virus can infect both cattle and sheep, as well as other ruminants and pigs. On the basis of sequence differences in the gene encoding the envelope glycoprotein E2, six distinct genotypes have been defined within the genus (van Rijn *et al*., 1997): classical swine fever virus, border disease virus, classical bovine viral diarrhoea virus (isolates predominantly from cattle), atypical bovine viral diarrhoea virus (isolates from cattle, sheep and pigs), deer pestivirus, and giraffe pestivirus. A novel pestivirus genotype has been identified recently in pronghorn antelope (Vilcek *et al*., 2005), while phylogenetic studies have suggested that there may be nine species of pestiviruses (Liu *et al*., 2009). Pestivirus infections may be inapparent, acute or persistent and are economically important worldwide.

Figure 81.1 Flavivirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Louping ill

The name ‘louping ill’ derives from the Scottish vernacular for ‘leaping’ or ‘bounding’, an allusion to the abnormal gait of some affected animals. Louping ill is a viral disease primarily of sheep. Although the virus is pathogenic for humans, infection is rare. The disease, which is largely confined to Britain and Ireland, has also been described in Norway, Spain, Bulgaria and Turkey. Isolates from Spain and Turkey are distinct from each other and also from isolates in Britain, Ireland and Norway (Marin *et al*., 1995). Louping ill virus belongs to a group of serologically-related viruses, the mammalian tick-borne encephalitis group or complex. Members of this group are distributed in northern temperate latitudes and are mainly human pathogens.

Figure 81.2 Viruses in the family *Flaviviridae*, with emphasis on those of veterinary importance. Viruses in red cause OIE-listed diseases.

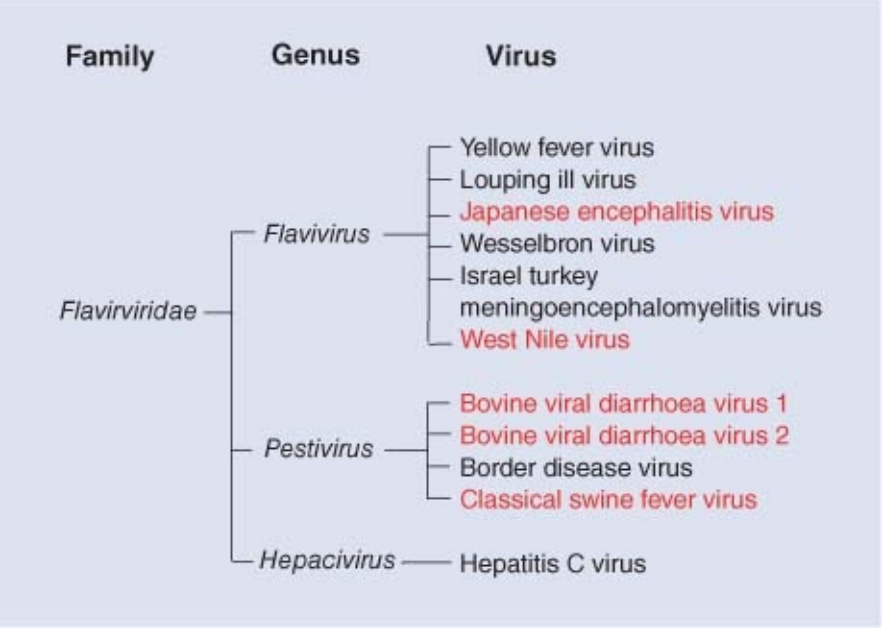


Table 81.1 Viruses of veterinary importance in the genera *Flavivirus* and *Pestivirus*.

Genus	Virus	Hosts	Comments
Flavivirus	Louping ill virus	Sheep, cattle, horses, red grouse and humans	Present in defined regions of Europe. Transmitted by the tick *Ixodes ricinus* and produces encephalitis in sheep and other species
	Japanese encephalitis virus	Waterfowl, pigs, horses and humans	Widely distributed in Asia. Transmitted by mosquitoes. Waterfowl are reservoir hosts. Infection in pigs results in abortion and neonatal mortality
	Wesselsbron virus	Sheep	Occurs in parts of sub-Saharan Africa. Transmitted by mosquitoes. Produces generalized infection, hepatitis and abortion
	Israel turkey meningo-encephalomyelitis virus	Turkeys	Reported in Israel and South Africa. Transmitted by mosquitoes. Progressive paresis and paralysis
	West Nile virus	Birds, humans, horses	Birds are the natural hosts. Transmitted by mosquitoes. Serious nervous disease reported sporadically in humans and horses. Leading cause of human arboviral encephalitis in the United States
Pestivirus	Bovine viral diarrhoea virus types 1 and 2	Cattle (sheep, pigs)	Occurs worldwide. Causes inapparent infection, bovine viral diarrhoea and mucosal disease. Congenital infection may result in abortion, congenital defects and persistent infection due to immunotolerance
	Border disease virus	Sheep	Occurs worldwide. Infection of pregnant ewes may result in abortion and congenital abnormalities
	Classical swine fever (hog cholera) virus	Pigs	Highly contagious, economically important disease with high mortality. Generalized infection with nervous signs and abortion; congenital tremors in piglets

Epidemiology

Louping ill virus is transmitted by the tick *Ixodes ricinus* and the seasonal incidence and regional distribution of the disease reflect periods of tick activity in suitable habitats such as upland grazing. Two main periods of tick activity occur, the first in spring and a second in late summer or early autumn. The host range of *I. ricinus* is wide, and infection with louping ill virus can occur in many vertebrate species including sheep, cattle, horses, deer, red grouse and humans. Red grouse are particularly susceptible to infection, with mortality reaching 80% in experimental infections. In areas where louping ill is endemic, infection can result in a dramatic reduction in the population of red grouse. They are not, therefore, considered to be important maintenance hosts. Louping ill virus is maintained in endemic areas through a sheep–tick cycle. It has been suggested that the mountain hare may be an important reservoir host (Laurenson *et al*., 2003). Trans-stadial but not transovarial transmission of the virus occurs in the tick. Rarely, contaminated instruments may be responsible for virus transmission. On farms where infection is endemic, losses occur mainly in sheep under 2 years of age. Most sheep acquire life-long immunity. Young lambs are protected by colostral antibody.

Pathogenesis

Viral replication occurs initially in lymph nodes draining sites of inoculation. Viraemia follows with dissemination to other lymphatic organs and to the brain and spinal cord. The speed and onset of the immune response are important in preventing spread of virus and limiting the degree of damage in the central nervous system. Immunosuppression caused by infection with *Anaplasma phagocytophilum*, the agent of tickborne fever, is considered to be responsible for increased mortality in sheep with louping ill.

Clinical signs

Following infection, sheep develop a febrile response, which may go unnoticed. The temperature then returns to normal and, in a proportion of animals, rises again as neurological signs develop. Signs of clinical disease are highly variable ranging from ataxia to the rapid onset of coma and death. Signs include hyperexcitability, fine muscular tremor, incoordination and exaggerated limb movements. Most affected animals develop convulsions prior to coma and death. Some sheep recover but exhibit mild, residual neurological signs. In cattle, the course of louping ill is more protracted. Affected animals may become

recumbent but usually remain bright and many eventually recover. In humans, influenza-like clinical signs are followed, in most cases, by mild neurological disturbance.

Diagnosis

- A history of neurological signs or unexplained deaths in sheep in endemic areas during periods of tick activity may indicate louping ill. Laboratory confirmation is usually required.
- A non-suppurative encephalomyelitis is usually detectable histologically. Lesions are most pronounced in the brain stem and spinal cord. A specific diagnosis may be possible using an immunoperoxidase technique to detect viral antigen.
- Specimens of brain, collected aseptically into 50% glycerol saline, may be inoculated into tissue culture or intracerebrally into baby mice in order to isolate the virus.
- Detection of louping ill virus using an RT-PCR protocol has been described (Gaunt *et al*., 1997; Marriott *et al*., 2006).
- Antibody to the virus can be detected using complement fixation and gel diffusion tests. The virus agglutinates goose red cells. Detection of IgM antibodies is indicative of acute infection.

Treatment

Specific treatment is not available. Careful nursing and sedation may aid recovery.

Control

- Inactivated vaccines are protective. In the past, formalized vaccines produced from infected sheep brains were responsible, in some instances, for causing scrapie in vaccinated animals. Tissue- culture-derived virus is now used for inactivated vaccines.
- Animals to be retained for breeding are vaccinated at 6 to 12 months of age. Colostrum from vaccinated ewes usually protects lambs during the first year of life. A booster injection may be advisable for ewes in their second pregnancy to enhance colostral antibody levels.
- Land improvement may help to reduce tick populations. Dipping of sheep

may also reduce risk of infection.

Japanese encephalitis

This disease, which mainly affects humans, has a wide geographical distribution in Asia. Infection can occur in a number of animal species including horses and pigs. The virus is transmitted by mosquitoes (*Culex* species) and is maintained by a mosquito–aquatic bird (egrets, herons) cycle. The disease is of reduced importance in horses because of their declining numbers in endemic areas and the use of effective vaccines. The pig is an important amplifying host because of its close association with human populations in parts of Asia. Boars can transmit the virus in semen. Infection can cause reproductive failure in sows. Litters from infected sows may include mummified and stillborn foetuses, weak piglets with neurological signs and clinically-normal piglets. Confirmation is based on virus isolation, detection of viral RNA by RT-PCR or the demonstration of specific antibody. Both inactivated and live attenuated vaccines have been used to control the disease.

West Nile virus

West Nile virus (WNV) is a member of the Japanese encephalitis virus serogroup. There are five distinct genetic lineages: lineage 1 is the most widespread (1a Europe, Africa, Americas and Asia; 1b Kunjin virus, Australia); lineage 2 has occurred in Africa and has also been linked to avian mortality in central Europe; lineages 3 and 4 have been found in Russia; lineage 5 (formerly 1c) has been identified in India. The viruses responsible for recent epidemics generally belong to lineage 1. In 1999 WNV was introduced to the Western hemisphere from the Old World and spread rapidly. An increased number of cases with severe and fatal neurological disease in humans, horses and birds have been associated with this spread. The virus is transmitted in enzootic cycles involving *Culex* species of mosquitoes and birds. Secondary transmission cycles occur, typically in late summer, involving *Aedes* species (‘bridging’ vectors) of mosquitoes, which preferentially feed on mammals, and result in incidental infections in humans and domestic animals. Bird migrations may carry the virus into new geographical areas. Infections in birds are frequently asymptomatic but clinical signs and high mortality can occur in crows, ravens, jays and geese. Only a small percentage of infected horses develop neurological disease. Clinical signs in horses include anorexia, depression, ataxia, circling, head

pressing and convulsions. Paralysis, coma and death may follow in approximately 30% of cases.

Diagnosis and control

Cases of neurological disease in birds, particularly *Corvidae* species, during the warm months of the year are suggestive of WNV infection. Sick birds are often viraemic and virus may be present in blood, saliva and droppings. Diagnostic samples should be handled at containment level three. Virus can be recovered from a wide range of tissues in avian species but brain and spinal cord are the preferred specimens from horses. Virus isolation can be carried out in cell culture. Alternatively, infection can be confirmed by detection of viral antigen by immunoassay and immunohisto- chemistry or by detection of viral RNA by RT-PCR (Johnson *et al*., 2001; Tewari *et al*., 2004). The primary method of diagnosis of WNV infection is by virus demonstration using PCR. Suitable serological tests include ELISA and plaque reduction neutralization test (PRNT). Antibody cross-reactions with related flaviviruses can occur; the PRNT is the most specific assay. Control is based on vaccination and a range of vaccines are commercially available for horses including inactivated, canarypox-vectored vaccine, DNA vaccine and a chimeric vaccine based on a yellow fever virus vector.

Wesselsbron disease

This disease, caused by a flavivirus, has a wide host range including domestic species, wild mammals and humans. However, clinical disease is usually encountered in sheep; infections in other species tend to be mild or subclinical. Infection in humans may result in febrile influenza-like symptoms. The virus is transmitted by mosquitoes. Infection is widespread in sub- Saharan Africa. The disease in sheep is similar to Rift Valley fever but is clinically less severe. It is characterized by abortion, neonatal mortality and congenital abnormalities such as hydranencephaly and arthrogryposis. The disease is most severe in newborn lambs, which present with fever, depression, general weakness and polypnoea. Confirmation is based on virus isolation, intracerebral inoculation of baby mice and the demonstration of specific antibody. An attenuated vaccine, which provides life-long immunity, is available. Pregnant animals should not be vaccinated because of the risk of abortion.

Bovine viral diarrhoea and mucosal disease

Infection with bovine viral diarrhoea virus (BVDV), also known as bovine virus diarrhoea virus, is common in cattle populations throughout the world. The virus can cause both acute disease, bovine viral diarrhoea (BVD), and a protracted form of illness, mucosal disease, arising from persistent infection. Using cell culture, cytopathic and non-cytopathic biotypes are recognized. The biotype most often isolated from cattle populations is non-cytopathic. Cytopathic isolates can arise from non-cytopathic BVDV as a result of recombination events including incorporation of host RNA and duplication of viral RNA sequences in the *NS2-3* gene resulting in cleavage of NS2-3 and increased production of NS3 (Meyers *et al*., 1996). Two genotypes, now considered separate species, BVDV 1 (classical BVDV isolates) and BVDV 2 (atypical BVDV isolates), are recognized on the basis of differences in the 5′ untranslated region of the viral genome. Both genotypes contain cytopathic and non-cytopathic isolates and produce similar clinical syndromes in cattle. However, only type 2 isolates have been associated with thrombocytopenia and a haemorrhagic syndrome, first described in North America (Rebuhn *et al*., 1989). Based on phylogenetic analysis, 13 subgenotypes of BVDV 1 and two subgenotypes of BVDV 2 have been described.

Epidemiology

When cattle are infected initially with BVDV, they shed virus for a short period and may transmit virus to other animals. Persistently-infected (PI) animals, which shed virus in secretions and excretions, are particularly important sources of infection. Persistent infection develops when infection of the foetus with a non-cytopathic strain occurs before day 120 of gestation. About 1% of animals in an infected population are persistently infected and viraemic. Although persistently-Infected cows may breed successfully, they can transmit virus transplacentally to calves during successive gestations. This form of disease transmission is relatively common. The presence of cattle with persistent infection in a herd results in constant exposure of the other cattle to virus, producing a high level of herd immunity. In such herds, more than 80% of animals are serologically positive.

Virus is excreted in the semen of both persistently and transiently-infected bulls. Infection may be transmitted through natural service or by artificial insemination. Embryo transfer from animals with persistent or transient infection

can lead to infection in recipient cows. If pregnant animals are inoculated with live vaccines, their calves may develop persistent infection. Due to the instability of the virus, indirect transmission rarely occurs through farm workers, equipment and biting flies. Although cattle are the primary hosts, the virus can infect most even-toed ungulates. Interspecies spread of bovine and ovine pestiviruses has been demonstrated under natural conditions but its epidemiological significance is uncertain.

Pathogenesis

The virus is usually acquired by the oronasal route and initial replication occurs in the oronasal mucosa. In the subsequent viraemia, virus spreads throughout the body either free in the plasma or in association with leukocytes. Both B and T lymphocyte numbers decrease. As the virus has an immunosuppressive effect, infection may predispose calves to respiratory and enteric disease. The outcome of transplacental spread depends on the age of the foetus at the time of infection. During the first 30 days of gestation, infection may result in embryonic death with return of the dam to oestrus. The effects of foetal infection between 30 and 150 days of gestation include abortion, mummification and congenital abnormalities of the CNS, often cerebellar hypoplasia. Foetuses, which become infected after day 120 of gestation, can mount an active immune response and are usually normal at birth. If virus invades the foetus before the development of immune competence, immunotolerance to the agent develops with persistent infection for the lifetime of the animal. The virus involved in this persistent infection is non-cytopathic. Later, usually between 6 months and 2 years of age, a cytopathic biotype emerges as a consequence of mutation of the non-cytopathic virus or of recombination with nucleic acid of the host cell or other non-cytopathic biotypes. The presence of cytopathic virus, which is antigenically homologous to the resident non-cytopathic biotype and is not cleared by the immune system, leads to the development of widespread lesions and mucosal disease. In some cases the source of the cytopathic virus may be external, as occurs in vaccine-associated outbreaks. If the superinfecting virus is antigenically heterologous, it will be cleared by the immune system but may give rise to a new cytopathic mutant following recombination with the resident non-cytopathic virus.

Cytopathic isolates differ from their non-cytopathic counterparts by the continual production of an 80-kDa non-structural protein (NS3), the result of cleavage of the NS2-3 gene product. The role of NS3 in the pathogenesis of

mucosal disease is unclear, but cellular destruction by cytopathic BVDV appears to be as a result of apoptosis. The NS3 protein is also detectable in leukocytes and several tissues in cattle persistently infected with non-cytopathic virus (Kameyama *et al*., 2008) and may account for signs of reduced immuno-ompetence, poor development and respiratory signs observed in some persistently-infected animals. Cytopathic isolates have a particular tropism for gut-associated lymphoid tissues.

Clinical signs

Most BVDV infections are subclinical. Outbreaks of BVD are usually associated with high morbidity and low mortality. When present, clinical signs include inappetence, depression, fever and diarrhoea. Significant mortality has been described in some outbreaks of BVD (David *et al*., l 994). Peracute BVD is characterized by high fever, severe diarrhoea and dehydration. Ulceration of the oral mucous membrane and the epithelia of the interdigital cleft and coronary band may also be present. In some cases, thrombocytopenia results in bloody diarrhoea, epistaxis and petechiae in the mouth, conjunctiva and sclera.

Although a significant proportion of persistently- infected animals are clinically normal, some are born undersized and exhibit retarded growth rate and poor viability. Increased susceptibility to enteritis and pneumonia has been reported. Mucosal disease is usually sporadic in occurrence. The condition affects persistently-infected animals, usually between 6 months and 2 years of age. Clinical signs include depression, fever, profuse watery diarrhoea, nasal discharge, salivation and lameness. Ulcerative lesions are present in the mouth and interdigital clefts. Case fatality rate is 100%; death usually occurs within weeks of the onset of clinical signs. A few animals may survive for several months before dying from severe debilitation.

Diagnosis

A tentative diagnosis may be possible on the basis of clinical signs and pathological findings. Laboratory confirmation requires demonstration of antibody, viral antigen or viral RNA. Seroconversion and the presence of viraemic animals are necessary for confirmation of established infection in a herd.

- Specimens suitable for laboratory examinations include serum, spleen, tissue from the pinna (‘ear notch’ test), lymph node and lesions from the

gastrointestinal tract.

- Virus can be isolated in cell cultures. Sequential samples taken 3 weeks apart should be used to confirm persistent infection. Before foetal calf serum is used in cell culture medium, it should be screened for the presence of virus or antibody.
- Viral antigen can be detected by ELISA or an immunoperoxidase technique. Immunohistochemical staining of skin sections ('ear notch' test) has been shown to correlate well with results from blood- based tests (Njaa *et al*., 2000).
- Dot blot, *in situ* hybridization and PCR techniques (Letellier and Kerkhofs, 2003) for the detection of viral RNA have been described. A multiplex PCR for the detection and differentiation of BVDV 1 and BVDV 2 has been described (Gilbert *et al*., 1999). Detection of viral nucleic acid in pooled samples such as bulk milk (Drew *et al*., 1999) and serum (Weinstock *et al*., 2001) is possible using RT- PCR.
- Virus neutralization and ELISA are the most commonly used methods for the detection of antibodies to BVDV. Demonstration of a four-fold increase in antibody titre of paired serum samples is necessary to confirm recent infection. Most commercial ELISAs utilize the NS3 protein, alone or in combination with other BVDV protein, as antigen because it is highly conserved among the pestiviruses. It has been shown that NS3-specific antibody levels in serum and milk are low or undetectable following vaccination with an inactivated BVDV vaccine and that it is possible to monitor infection in vaccinated herds provided a suitable vaccine and NS3 antibody test combination is applied (Makoschey *et al*., 2007; Kuijk *et al*., 2008).

Treatment and control

Supportive therapy may be of benefit in outbreaks of bovine viral diarrhoea. Most losses arising from BVDV infections in herds result from the effects of prenatal infections and mucosal disease. Control strategies are directed at preventing infections which can lead to the birth of persistently-infected animals.

- Killed, attenuated live and temperature-sensitive mutant virus vaccines have been developed. Live vaccines induce both cellular and humoral immunity but may cause foetal infections and immuno- suppression. In addition, they may precipitate mucosal disease in some persistently-infected

animals. Killed vaccines primarily elicit a humoral immune response and may be used in pregnant animals but require regular boosters to maintain protection. The extent to which killed vaccines protect the foetus is unclear. Vaccines produced from a single strain or genotype of virus may not be fully protective due to antigenic variation, a feature of BVDV isolates. Vaccines have largely been evaluated for their ability to prevent acute disease, and manufacturers generally do not claim that vaccination prevents foetal infection (van Campen and Woodard, 1997). Exposure of replacement stock to a persistently-infected animal before breeding commences may help to maintain herd immunity but is less reliable than vaccination.

- The elimination of BVDV from a herd requires the identification and removal of persistently-Infected animals. The dam, sire and progeny of persistently- infected animals should be tested since the virus can be passed from parent to offspring.
- Based on successful control and eradication programmes carried out in northern Europe, a two- step vaccination in conjunction with the test and removal of persistently-Infected animals in areas with high cattle densities and high seroprevalence has been recommended (Moennig *et al*., 2005). This involves inoculation with an inactivated vaccine followed by administration of a modified live vaccine 4 weeks later.
- Herd immunity wanes following the removal of persistently-infected animals. Therefore, all newly acquired cattle should be tested before being introduced to the herd.
- Systematic testing of bulk milk or pooled blood samples for antibodies from herds plays an important role in identifying herds with persistently-infected animals and is important in national eradication programmes.

Border disease

This congenital disorder of lambs, also known as hairy shaker disease, occurs worldwide. Border disease, which was first reported from the Welsh/English border, is caused by infection of the foetus with a non- cytopathic pestivirus. Border disease virus (BDV) is closely related to bovine viral diarrhoea virus and it has been suggested that they may be a single species. Pestivirus isolates from sheep can infect other domestic ruminants and pigs. Moreover, pestivirus isolates from a number of domestic species, particularly cattle, can infect pregnant sheep

causing border disease in their offspring.

Epidemiology

Persistently-infected animals shed virus continuously in excretions and secretions. These animals tend to have a low survival rate under field conditions, although some may survive for several years without developing clinical signs. Persistently-infected ewes may give birth to persistently- infected lambs. Acute infections in susceptible sheep are transient and result in immunity to challenge with homologous strains of BDV. Infected rams shed virus in semen and may infect susceptible ewes. In addition to sheep-to-sheep contact, transmission can occur through contaminated needles during flock vaccination. Other ruminant species shedding pestivirus are potential sources of infection for sheep.

Pathogenesis

Virus is probably acquired by the oronasal route. In susceptible pregnant ewes, infection results in placentitis and invasion of the foetus. The immune response of the ewe does not protect the developing foetus. The age of the foetus at the time of infection ultimately determines the outcome. The foetus develops immune competence between 60 and 85 days of gestation. Foetal death may follow infection prior to the development of immune competence, the outcome being resorption, abortion or mummification. Foetuses which survive become immunotolerant and remain persistently infected. These animals may be clinically normal at birth or may display tremors and hairy birthcoat, consequences of viral interference with organogenesis. Congenital defects in affected lambs include skeletal growth retardation, hypomyelinogenesis and enlarged primary hair follicles with reduced numbers of secondary follicles. Infection after day 85 of gestation induces an immune response with elimination of the virus and the birth of a healthy lamb. Foetal infection during mid-gestation, when the immune system is developing, may result in lesions in the central nervous system including cerebral cavitation and cerebellar dysplasia. Immune-mediated reactions have been suggested as the possible explanation for these severe lesions. Some persistently-infected sheep may develop a condition similar to mucosal disease of cattle. Cytopathic isolates of BDV have been recovered from the intestines of such affected animals.

Clinical signs

In flocks infected with BDV, there may be an increase in the number of abortions and weak neonatal lambs. Characteristic signs of infection in newborn lambs include altered body conformation, changes in fleece quality and tremors. Hairs projecting above the wool, particularly along the neck and back, impart a halo effect that is most noticeable in fine-coated breeds. Affected lambs are often small and their survival rate is poor. The survival rate is influenced not only by the severity of the neurological dysfunction but also by the standard of animal care. In well-nursed lambs, the neurological signs gradually abate and such animals may eventually become clinically normal.

Diagnosis

- The characteristic clinical signs are diagnostic.
- Dysmyelination may be demonstrable histologically in the central nervous system. Immunohistochemical staining can be used to demonstrate virus in brain tissue.
- Virus isolation is possible in susceptible bovine or ovine cell lines. Immunohistochemical staining is used to demonstrate the presence of non-cytopathic virus.
- Samples suitable for virus isolation include whole blood and tissues from affected lambs. Pre-colostral blood from lambs is preferable because antibody acquired from colostrum may interfere with virus isolation.
- Viral antigen can be detected by immunofluorescent staining of frozen sections or by immunoperoxidase staining of fixed sections. An ELISA can be used to detect viral antigen in the blood of persistently-infected lambs.
- Reverse transcriptase polymerase chain reaction can be used to detect viral RNA and to genotype the virus (Vilcek and Paton, 2000; Willoughby *et al*., 2006).
- Serological testing, employing methods such as virus neutralization and ELISA, can be used to determine the extent of infection in a flock.

Control

Control should be based on identification and removal of persistently-infected animals and precautions to avoid introduction of infected animals into a flock. Where such a policy is not feasible, breeding stock should be deliberately mixed with persistently-infected animals at least 2 months before mating. A commercial inactivated, adjuvanted vaccine is available, containing BDV and

BVDV-1 (Nettleton *et al*., 1998).

Classical swine fever (hog cholera)

This highly contagious, potentially fatal disease of pigs, although still present in many countries, has been eradicated from North America, Australia and most European countries. It is classed as a listed (formerly List A) disease by the Office International des Epizooties. In recent years, sporadic outbreaks have occurred in the United Kingdom, Italy, Belgium, The Netherlands and Germany. It is still enzootic in wild boar in some European countries. Isolates of classical swine fever virus (CSFV), the causal agent of the disease, can be placed in three major groups on the basis of nucleotide sequence data (Lowings *et al*., 1996; Moennig *et al*., 2003). Recent European isolates, placed in Group 2, are distinct from those that caused swine fever outbreaks during the 1940s and 1950s (Group 1) and those circulating in Asia (Group 3). Isolates, although conforming to a single major antigenic type and being mostly non-cytopathic, differ substantially in virulence.

Epidemiology

Pigs, both domestic and feral, are the natural hosts of CSFV and direct contact between infected and susceptible animals is the main means of transmission. In endemic areas, the disease is spread principally by movement of infected pigs. Shedding of virus may begin before clinical signs become evident. Virulent virus is shed in all excretions and secretions until the time of death at about 20 days post infection. Virus strains of moderate virulence may result in chronic infection with continuous or intermittent shedding by infected pigs. In addition, congenital infections with strains of low virulence may result in the birth of persistently-infected piglets. Infected animals occur in wild boar populations in Europe, which act as reservoirs of infection leading to spread by direct contact and indirectly by infected meat. Spread between premises can occur indirectly, particularly in regions with a high density of pig farms. Transmission can occur mechanically via personnel, vehicles and biting arthropods. The virus, which is relatively fragile and does not persist in the environment, is not spread over long distances by air movement. Despite its lability, CSFV can survive for long periods in protein-rich biological materials such as meat or body fluids, particularly if chilled or frozen. Although legislation prohibiting the feeding of uncooked swill is in place in most European countries, recent outbreaks of

classical swine fever can still be traced to waste food fed to pigs.

Pathogenesis and pathogenicity

Pigs are usually infected by the oronasal route. The tonsil is the primary site of viral multiplication. Virus spreads to regional lymph nodes and viraemia develops after further viral multiplication. Virus, which has an affinity for vascular endothelium and reticuloendothelial cells, can be isolated from all major organs and tissues. In acute swine fever, vascular damage in conjunction with severe thrombocytopenia results in widespread petechial haemorrhages. A non- suppurative encephalitis with prominent perivascular cuffing is present in most CSFV-infected pigs. Virus strains of reduced virulence can cause a mild form of the disease. In pregnant sows, virus may be transmitted to foetuses. The outcome of transplacental infection is determined by the age of the foetus and the virulence of the invading strain of virus. Infection early in gestation results in foetal death with resorption or abortion. *In utero* infection may also result in stillbirths, weak newborn piglets with congenital tremors and, occasionally, clinically normal piglets. Piglets with immune tolerance to the virus remain persistently infected and excrete virus continuously. Persistently-infected animals, which are clinically normal when born, may subsequently develop late-onset disease following several weeks or months of virtual absence of clinical signs. The factors that precipitate late-onset disease are unclear.

Clinical signs

Following an incubation period of up to 10 days, affected animals develop high fever and become inappetent and depressed. Sick pigs are inclined to huddle together. Vomiting and constipation are followed by diarrhoea. Some animals may die soon after developing convulsions. A swaying gait usually precedes posterior paresis. Most cases of acute classical swine fever succumb within 20 days after infection.

Signs of disease are milder in infections caused by strains of low virulence. Partial recovery from an initial phase of acute illness may be followed by relapse and death. Some pigs may survive for several months but exhibit marked growth retardation. Abortion, mummification, malformations and stillbirths may be encountered in breeding herds. Liveborn infected piglets, often exhibiting tremors, may die soon after birth. Some affected piglets may present with haemorrhages in the skin. Congenital malformations include deformities of the

head and limbs and cerebellar hypoplasia.

Diagnosis

Although clinical signs and history may provide evidence for a tentative diagnosis, laboratory confirmation is essential, particularly with infections caused by strains of reduced virulence.

- In acute disease, haemorrhages are present in many internal organs and on serosal surfaces. Petechiae are often present on kidney surfaces and in lymph nodes. Other gross pathological features of diagnostic significance are splenic infarction and ‘button’ ulcers in the mucosa of the terminal ileum near the ileocaecal valve.
- Rapid confirmation is possible using direct immunofluorescence on frozen sections of tonsillar tissue, kidney, spleen, distal ileum and lymph nodes. As pigs can be infected with BVDV, monoclonal antibodies specific for CSFV may be required to reach a definitive diagnosis. Antigen-capture ELISAs are available commercially and are suitable for detection of viral antigen in blood or organ suspensions. These assays are less sensitive than virus isolation and are best used on a herd basis.
- Virus isolation can be carried out in porcine cell lines using homogenates of spleen and tonsil. As most isolates are non-cytopathic, immunostaining is necessary for the demonstration of viral antigen.
- The reverse transcriptase polymerase chain reaction assays for detection of CSFV RNA are sensitive and rapid (Dewulf *et al*., 2004 ; Hoffmann *et al*., 2005). They are now replacing most other methods used for virus detection.
- Serological testing is useful on farms infected with strains of low virulence or for serological surveys. Virus neutralization and ELISA are the tests most widely used. A blocking ELISA has been developed for distinguishing CSFV from BVDV (Wensvoort *et al*., 1988) but the definitive method for differentiation is the comparative neutralization test, which compares the level of antibodies to the different *Pestivirus* species.

Control

- The disease is notifiable in many countries which have adopted slaughter policies and banned vaccination. Pigs and pig products should not be imported from countries where infection with CSFV is present. Swill must be boiled before being fed to pigs. Contact between domesticated pigs and

wild pigs/boars should be avoided.

- In countries where the disease is endemic or during the early stages of an eradication programme, vaccination may be used. Live vaccines attenuated either by serial passage in rabbits (Chinese strain) or in tissue culture (Japanese guinea-pig strain or French Thiverval strain) are currently used. These vaccines are safe and effective. Vaccinated animals cannot be distinguished serologically from naturally-infected animals. The use of recombinant E2 glycoprotein marker vaccine in conjunction with a specific ELISA capable of detecting antibodies to the envelope glycoprotein, E^{rns}, offers a means of distinguishing vaccinated from naturally-infected pigs (Baars *et al*., 1998; Langedijk *et al*., 2001).

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Chapter 82

Togaviridae

Viruses in the family *Togaviridae* (Latin *toga*, cloak) are enveloped RNA viruses, approximately 70 nm in diameter, with icosahedral symmetry. The envelope, which contains glycoprotein spikes, is closely bound to an icosahedral capsid (Fig. 82.1). Togaviruses agglutinate goose and chick erythrocytes. There are two genera, *Alphavirus* and *Rubivirus*, in the family. The sole member of the genus *Rubivirus* is rubella virus, which does not require an arthropod vector and causes German measles in children and young adults.

The genus *Alphavirus* includes more than 25 species, a number of which are important animal pathogens. Alphaviruses are divided, on the basis of genomic composition, into a number of antigenically-related groups including Venezuelan equine encephalitis virus (VEEV) complex, eastern equine encephalitis virus (EEEV) complex, Semliki forest virus complex and western equine encephalitis virus (WEEV) complex. Western equine encephalitis virus has been shown to have arisen by recombination between EEEV and Sindbis-like viruses, probably between 1,300 and 1,900 years ago (Weaver *et al*., 1997). Recombination is infrequent among alphaviruses as these viruses undergo genetic change primarily by accumulation of point mutations.

Replication of alphaviruses, which contain positivesense single-stranded RNA, occurs in the cytoplasm, and nucleocapsids are assembled in the cytosol. In vertebrates, alphavirus infection results in cytolysis. The viral envelope, which contains virus-derived glycoprotein spikes, is acquired as the nucleocapsid buds into the plasma membrane. Viral infection of invertebrate cells is usually non-cytolytic and is persistent. In this instance, virus assembly occurs in association with intracellular membranes rather than through the plasma membrane.

Key points

- Enveloped, RNA viruses with icosahedral symmetry
- Replicate in cell cytoplasm
- Labile in the environment

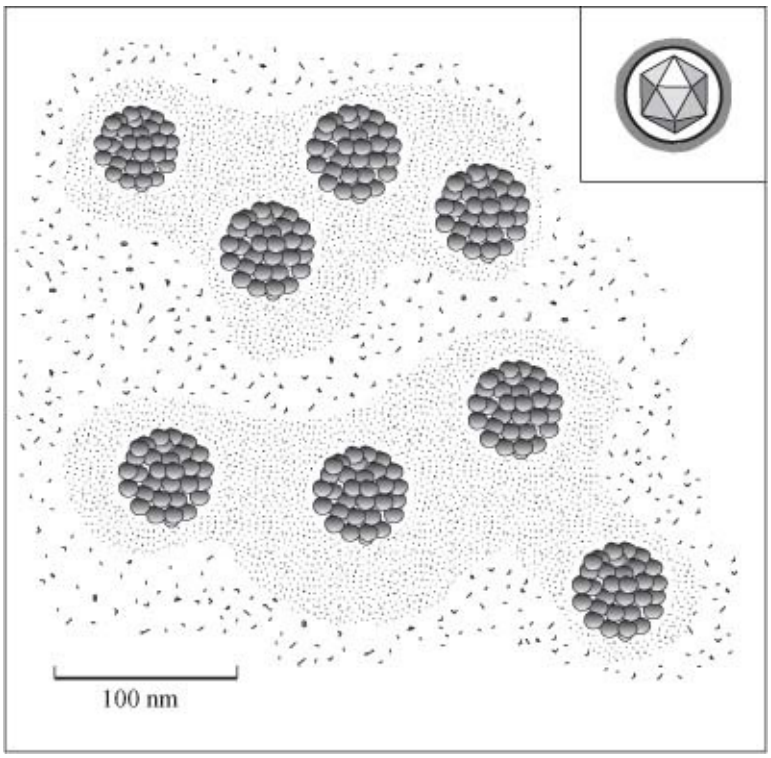
- *Genus Alphavirus*
  – arthropod-borne
  – cause eastern equine encephalitis, western equine encephalitis and Venezuelan equine encephalitis
  – major cause of zoonotic infections

Mature virions of alphaviruses are sensitive to pH changes, heat, organic solvents, detergents and disinfectants and are not stable in the environment. Alphaviruses, in common with certain members of the *Flaviviridae, Reoviridae, Rhabdoviridae* and *Bunyaviridae*, are termed arboviruses indicating that they are arthropod-borne. This term, however, does not have taxonomic significance. Alphaviruses primarily infect and are transmitted by mosquito species. Although they are capable of infecting a variety of vertebrate hosts, they usually have a principal invertebrate vector and an amplifying or reservoir vertebrate host. It is this enzootic cycle that tends to determine the geographical distribution of alphaviruses.

Clinical infections

Domestic animals and humans are usually considered to be ‘dead-end’ hosts of alphaviruses because they do not develop a sufficiently high titre of circulating virus to act as reservoir hosts. A number of important equine diseases are caused by infection with members of the genus *Alphavirus* (Table 82.1). The three equine encephalitis viruses (Venezuelan, eastern and western), which are confined to the western hemisphere, are transmitted by mosquitoes. Getah virus occurs mainly in south-east Asia and in Australia. A number of outbreaks of disease caused by this virus have been recorded in Japan. The recent addition of alphaviruses that infect fish, salmon pancreas disease virus (SPDV), and elephant seals, southern elephant seal virus, has added to the complexity of the organization of the genus. It is considered unlikely that SPDV is transmitted by an arthropod vector.

Figure 82.1 Togavirus particles as they appear in an electron micrograph and a diagrammatic representation (inset).



Equine encephalitides

The viruses that cause Venezuelan, eastern and western encephalitis are important in the Americas. These three viruses produce similar clinical signs although infections caused by the virus of western equine encephalitis tend to be mild. Venezuelan equine encephalitis virus (VEEV) is considered the most important of the three viruses with major disease outbreaks occurring at approximately 10 to 20-year intervals in South America.

Epidemiology

The equine encephalitides share some common epidemiological features. The peak periods of these diseases coincide with times of maximum vector numbers, usually in late summer following heavy rainfall. The regional distribution of the viruses is related to that of mosquito vectors. Numbers of clinical cases drop dramatically when vector numbers decrease due to cold weather or drought.

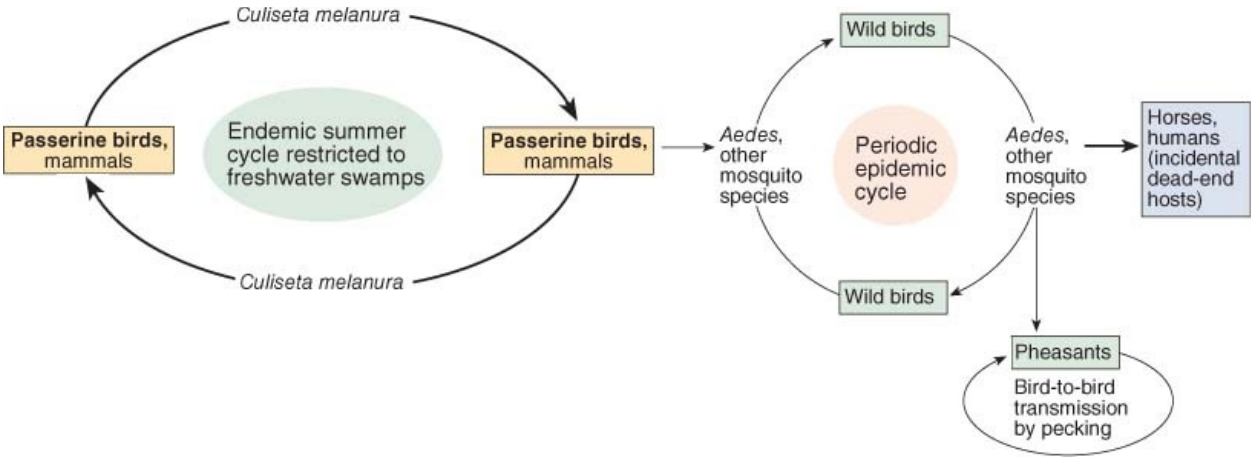
Eastern equine encephalitis virus (EEEV) occurs principally in Atlantic coastal areas of North America. However, EEEV has also been isolated in Michigan, the Caribbean islands and South America. Antigenic and genetic differences exist between isolates from North and South America indicating two distinct lineages. North American isolates are highly conserved (Group I). In contrast, South

American isolates comprise several genotypes, and at least three different subgroups (Groups IIa, IIB, III) can be delineated. South American EEEV infections are associated with equine disease but rarely human disease. Accordingly, these viruses are considered to be less virulent than North American isolates. The virus is maintained in cycles of infection involving passerine birds and the irrigation ditch mosquito (*Culiseta melanura*), which inhabits freshwater swamps and is ornithophilic (Fig. 82.2). Following infection, a high titre of virus develops in many wild birds without evidence of disease. High mortality rates have, however, been recorded in pheasants, emus and whooping cranes. Virus can be transmitted between pheasants by pecking and cannibalism. Periodic epidemic outbreaks of infection in wild birds, which may lead to infection of humans and horses, involve additional mosquito species such as *Aedes sollicitans* and *Coquillettidia perturbans* which feed both on birds and on mammals. Infection usually results in sporadic disease in humans, horses and pheasants. Epizootics, which tend to occur in the autumn, disappear with the arrival of the first frosts. Overwintering mechanisms for virus maintenance are unclear, although wild birds are considered to be possible reservoirs. Transovarial transmission in mosquitoes has not been demonstrated.

Table 82.1 Alphaviruses of veterinary significance.

Virus	Vector	Comments
Eastern equine encephalitis virus	Mosquito (*Culiseta melanura*, *Aedes* species)	Infection endemic in passerine birds which frequent freshwater swamps of eastern North America, Caribbean islands and parts of South America. Causes disease in horses, humans and pheasants
Venezuelan equine encephalitis virus	Mosquito (*Culex* species)	Infection endemic in small mammals in Central and South America. Causes outbreaks of disease in horses, donkeys and humans in endemic regions, occasionally spreading to southern USA
Western equine encephalitis virus	Mosquito (*Culex tarsalis* and other *Culex* species, *Aedes* species)	Infection of passerine birds widespread in the Americas. Causes mild disease in horses and humans
Getah virus	Mosquito	Causes sporadic disease in horses in south-east Asia and Australia characterized by fever, urticaria and oedema of the limbs.
		Subclinical infection occurs in pigs

Figure 82.2 Endemic and epidemic transmission cycles of eastern equine encephalitis virus in North America. The endemic cycle, which occurs in summer, is restricted to swampy regions. The epidemic cycle, which occurs periodically, involves mosquito species and wild birds not usually associated with swamps. Infection in horses and humans, which are ‘dead-end’ hosts, is a ‘spill over’ from the epidemic cycle. In farmed pheasants, bird-to-bird transmission may occur through pecking.



Isolates of VEEV comprise a complex of six subtypes (I to VI). Within subtype I, there are five antigenic variants or serotypes (AB–F). Originally, subtypes I-A and I-B were considered to be distinct, but they are now believed to be identical (I-AB). Epizootic forms of Venezuelan equine encephalitis are caused by two highly virulent subtype I serotypes of the virus (I-AB and I-C). The serotypes I-D, I-E and I-F, as well as the other five subtypes (II to VI) of VEEV, comprise enzootic viruses and are considered to be non-pathogenic for *Equidae*. However, enzootic I-E viruses have been shown to be responsible for a small number of limited outbreaks of neurological disease in horses in Mexico. It is noteworthy that the level of viraemia produced by these enzootic strains in horses is generally low. The viruses are maintained in sylvatic cycles involving rodents and mosquitoes (*Culex* species) in swampy habitats. Phylogenetic studies suggest that the viruses implicated in epizootics are derived from mutation of viruses involved in enzootic cycles (Weaver *et al*., 1992), giving rise to increased infectivity for other mosquito vector species and/or an increased level of viraemia in horses. *Equidae* serve as amplifying hosts for these epizootic strains. Genetic studies indicate a close relationship between I-AB, I-C and I-D viruses. Epizootics of VEE occurred regularly between 1962 and 1972 in the northern part of South America and in Central America, extending at one stage as far as Texas. Some of these outbreaks resulted from residual live virus contained in formalinized vaccine preparations. Following a quiescent period of 20 years, a limited outbreak of disease occurred in Venezuela in 1992 followed by an extensive epizootic in Venezuela and Columbia in 1995 (Weaver *et al*., 1996). Horses inoculated with a virulent subtype of VEEV are important amplifiers of virus activity, developing a viraemia of sufficiently high titre to allow transmission by feeding mosquitoes.

Although western equine encephalitis (WEE) has traditionally occurred in the

USA west of the Mississippi, it is also present in many other parts of the North American continent. Infections tend to recur in certain areas. The cycle of infection involves mosquitoes, usually *Culex tarsalis*, and indigenous wild birds in which infection is inapparent. Horses are infected incidentally and are 'dead-end' hosts because levels of virus in the blood remain low. Epizootics are rare and epizootic strains probably arise from nonpathogenic enzootic strains. Overwintering mechanisms of the virus are unclear but may involve birds, reptiles or mosquitoes. Highlands J virus is a closely related virus that belongs to the WEEV complex and is found in the eastern United States. It is a rare cause of encephalitis in horses but an important pathogen of a number of bird species such as emus and turkeys.

Pathogenesis

Following inoculation by a feeding mosquito, viral replication occurs near the site of entry and in the regional lymph nodes. Viraemia, ranging from barely detectable to high levels, is accompanied by fever. When disease is severe, the virus invades the central nervous system resulting in neuronal necrosis and perivascular lymphoid cuffing.

Clinical signs

Diseases caused by the three equine encephalitis viruses are clinically similar. The incubation period may be up to 9 days. Clinical signs, which usually last from 4 to 9 days, range from mild fever and depression to fatal febrile encephalomyelitis. Neurological signs include photophobia, blindness, head pressing, circling, ataxia and inability to swallow. Affected horses exhibit severe depression with low carriage of the head and a wide-based stance. Terminally, animals become recumbent and semicomatose with convulsions prior to death. The case fatality rate is 90% for EEE, 50 to 80% for VEE and 20 to 40% for WEE.

Diagnosis

Clinical signs along with a history of previous cases of equine encephalitis in the same region may be suggestive of the disease. However, laboratory confirmation is usually required. Due to the possibility of human infection, care must be taken during specimen collection.

- Virus isolation provides a definitive diagnosis. Isolation is carried out in

cell culture or in suckling mice. Whole blood or serum, collected during the pyrexic phase of the disease, is suitable for virus isolation. Brain or cerebrospinal fluid can be collected post mortem. When VEE is suspected, isolates should be typed using monoclonal antibodies or nucleic acid sequencing in order to distinguish virulent from non-virulent subtypes.

- An immunohistochemical staining technique for the detection of EEEV antigen in fixed brain sections has been described (Patterson *et al*., 1996).
- Detection of viral RNA in tissues of affected animals and in mosquitoes can be achieved using RT-PCR (Pfeffer *et al*., 1997; Linssen *et al*., 2000).
- Diagnosis of WEE or EEE is usually based on serology. Paired serum samples should be collected to demonstrate a rising titre. Suitable testing methods include ELISA, plaque reduction neutralization assay, haemagglutination-inhibition and complement fixation. An IgM capture ELISA has been used to provide evidence of infection in single serum samples. The vaccination status of an animal must be considered in interpreting the results of serological tests. The interpretation of serological results for VEEV is complicated by the presence of antibodies produced in response to inapparent infections with non-virulent subtypes.

Treatment and control

Although supportive palliative treatments may be beneficial, the prognosis is generally poor. Control is based on vaccination of horses and implementation of measures aimed at reducing mosquito populations.

- Monovalent, bivalent and trivalent vaccines are available. Vaccines for EEE and WEE are inactivated. A live attenuated TC-83 VEEV vaccine provides effective protection and has been used successfully to prevent epizootics of VEE.
- Vector control measures include spraying of vector habitats, destruction of mosquito breeding areas, use of insect repellants and stabling of horses at night in insect-proof buildings.

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Chapter 83

Prions: unconventional infectious agents

The microbial pathogens responsible for the majority of infectious diseases in mammals conform to longestablished criteria used to describe infectious agents. These criteria include their size, morphology, biochemical characteristics, mode of replication and their susceptibility to physical and chemical methods of inactivation. The realization that some infectious agents did not conform to this system of evaluation was initially treated with scepticism by many scientists. Gradually it became evident that a particular group of infectious agents, termed unconventional infectious agents, had attributes which set them apart from typical microbial pathogens such as viruses, bacteria and fungi. These unconventional infectious agents appear to be devoid of nucleic acid, can infect animals and humans without inducing an immune response and exhibit resistance to physical and chemical methods of inactivation far beyond the most resistant forms of conventional infectious agents. The diseases in which unconventional infectious agents are implicated are termed transmissible spongiform encephalopathies (TSEs), a unique group of neurodegenerative diseases characterized by neuronal degeneration. A proposal that the agents responsible for TSEs be grouped together as distinctly different infectious agents gradually gained acceptance and the term prions was introduced into the scientific literature (Prusiner, 1982). Two types of prions are described, one associated with mammals, the other with fungi (Wickner, 1994). The ‘prion theory’ proposes that this unique group of infectious agents are derived from native glycoproteins which have become altered by a number of different biological mechanisms (Prusiner *et al*., 1999). As a consequence of these alterations, it is suggested that prions can induce changes in the animal’s body which lead to the development of TSEs. The native glycoprotein, PrP^{C} (host cellular prion protein), which is highly conserved across most species, is composed of 252 to 264 amino acids and is proteinase K-sensitive and detergent-soluble (Wopfner *et al*., 1999). The C-terminal half of the molecule comprises two short β-sheets and three longer α-helices. The first 23 amino acids of the N

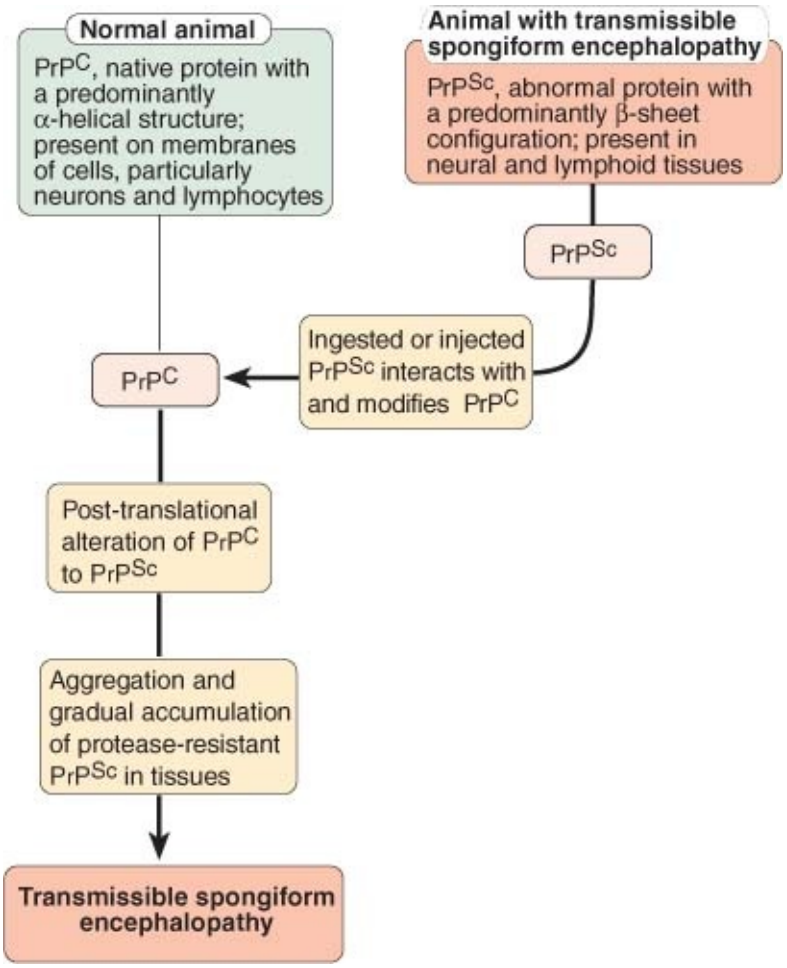
terminus of PrP^{C} act as a signal peptide that targets the protein to the cell membrane where the C terminus becomes attached to a glycosyl phosphatidylinositol (GPI) anchor. PrP^{C} is associated with the plasma membrane of many cell types, particularly neurons and lymphocytes. A number of different functions have been ascribed to PrP^{C} including regulation of immune responses, signal transduction, copper binding, synaptic transmission and apoptotic functions. Following exposure to abnormal prion protein (PrP^{Sc}, scrapie prion protein), PrP^{C} is altered post-translationally to a structure similar to that of the PrP^{Sc} in which the protein is folded mainly into β-sheets, is partially resistant to proteinase K and has a high propensity to aggregate (Fig. 83.1). Both PrP^{C} and PrP^{Sc} occur in three main states of glycosylation: unglycosylated, monoglycosylated and diglycosylated. The mechanism by which PrP^{Sc} induces the structural alteration in native PrP^{C} has not yet been defined. However, the newly formed PrP^{Sc} closely resembles the 'infecting' PrP^{Sc} in its three-dimensional structure, implying that the latter has a central role in the initiation of the chain reaction which results in the intracellular accumulation of large amounts of PrP^{Sc}. The nucleation-dependent polymerization model envisages PrP^{Sc} acting as a seed which binds to PrP^{C} and catalyses its conversion into a misfolded, aggregation-prone form (Jarrett and Lansbury, 1993). As more PrP^{C} is converted to PrP^{Sc}, this molecule which is partially resistant to proteinase K gradually accumulates, especially in the long))ived cells of the CNS. At some point, fission occurs and the long PrP^{Sc} polymers break into smaller pieces. This fragmentation results in an increase in the number of effective nuclei which promote further conversion of PrP^{C} to PrP^{Sc}. Recent studies suggest that PrP^{Sc} is formed from PrP^{C} on cell membranes in caveolae-like structures before these fuse with endosomes. During normal metabolic turnover in cells, most membrane glycoproteins are transported in endosomes to lysosomes for degradation. However, because it is partially proteaseresistant, PrP^{Sc} accumulates in cytoplasmic vesicles, particularly lysosomes (Prusiner *et al*., 1999). It is thought that vacuolation is brought about by destruction of the neuronal cytoskeleton as a result of the liberation of hydrolytic enzymes from lysosomes overloaded with PrP^{Sc} (Laszlo *et al*., 1992). Accumulation of PrP^{Sc} leads to neuronal apoptosis, synaptic dysfunction and neurodegeneration. It is considered that prions themselves are not directly neurotoxic but that both soluble misfolded intermediates and amyloidlike fibril deposits are toxic, most

likely by different mechanisms (Soto and Estrada, 2008).

Key points

- Prions are proteinaceous particles apparently devoid of nucleic acid
- Aetiologically implicated in the transmissible spongiform encephalopathies, fatal neurodegenerative diseases with long incubation periods
- Neuropathological changes, which include vacuolation of both neurons and neuropil without evidence of an inflammatory response, are associated with the accumulation of abnormally-folded host-derived prion protein
- Transmissible spongiform encephalopathies include:
  - Scrapie in sheep
  - Bovine spongiform encephalopathy
  - Feline spongiform encephalopathy
  - Transmissible mink encephalopathy
  - Kuru and Creutzfeldt–Jakob disease in humans

Figure 83.1 Outline of the proposed mechanisms involved in the pathogenesis of transmissible spongiform encephalopathies. PrP^{C}, Native cellular prion protein. PrP^{Sc}, Abnormal prion protein present in scrapie and other transmissible spongiform encephalopathies.

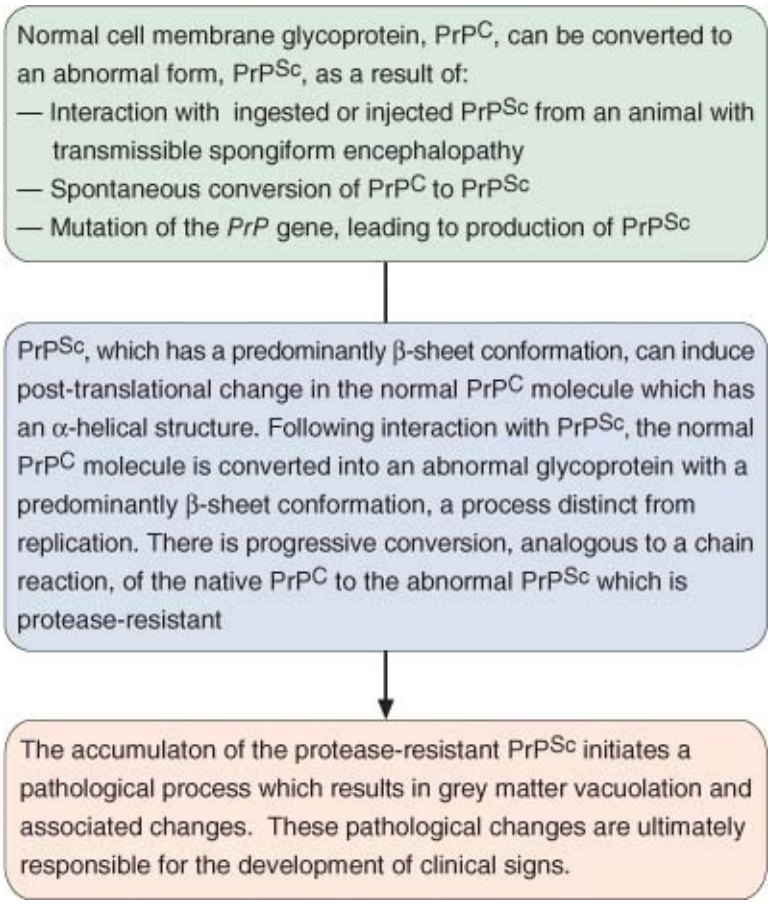


Experimentally, mild acidification and reduction procedures induce structural rearrangement in PrP^C producing a highly soluble monomeric form of PrP (β-PrP) which is rich in β-sheets (Jackson *et al.*, 1999; Zou and Cashman, 2002). This β-PrP can revert to an α-configuration or, alternatively, it can act as a stable 'seed' inducing polymerization with the *in vitro* formation of insoluble fibrillar structures similar to the prion fibrils which can be recovered from detergenttreated extracts of the brains of animals with TSEs. These experiments demonstrate that binding of several β-PrP molecules together can lead to the formation of an irreversible β-sheet configuration, the probable basis of PrP^{Sc} accumulation in TSEs.

The formation of PrP^{Sc} from PrP^C in TSEs may be initiated following exposure to an external source of PrP^{Sc}, usually by ingestion such as occurred in cases of kuru following ritualistic cannibalism in the Fore peoples of Papua New Guinea (Fig. 83.2). Random spontaneous conversion of native PrP^C to PrP^{Sc}, typified by cases of sporadic Creutzfeldt–Jakob disease (CJD), may initiate the

process in an individual. This is the most common form of human prion disease accounting for approximately 70 to 80% of all cases with an incidence of one per million of the population. A third mechanism relates to mutation in the *PrP* gene which predisposes to configurational change in PrP^{C} as occurs in the Gerstmann–Sträussler–Scheinker syndrome in humans.

Figure 83.2 An outline of the probable mechanisms which lead to the accumulation of PrP^{Sc} in neurons.



The *PrP* gene of an infected animal determines the primary amino acid sequence of the prion protein in that animal. The resistance of some species to infection by prions derived from another species is termed the ‘species barrier’. This barrier is attributed to differences between the amino acid sequences of the prion proteins in the two species. According to the conformational selection model, the host PrP^{C} primary structure influences its degree of compatibility with the PrP^{Sc} type and the ease of transmission between species is determined by the degree of overlap between the PrP^{Sc} types permitted by the PrP^{C} present in the host and donor species (Collinge and Clarke, 2007). On initial transfer of

PrP^{Sc} between species, the incubation period tends to be relatively long. Subsequent transfer between members of the recipient species leads to shorter incubation periods. The presence of a 'species barrier' may explain the resistance of humans to infection with PrP^{Sc} derived from sheep with scrapie. The prion strain responsible for bovine spongiform encephalopathy (BSE) is considered to be highly 'promiscuous' and may represent a thermodynamically highly favoured PrP^{Sc} conformation that is readily imprinted on PrP^{C} from a range of different species.

Infectious TSE isolates which, following inoculation of syngeneic hosts, cause disease with distinct characteristics and exhibit distinct prion-disease phenotypes, are defined as prion strains (Aguzzi *et al*., 2007). Prion strains from infected sheep have been described on the basis of bioassays in mice. Strain differentiation is based on incubation periods, mortality patterns, PrP^{Sc} deposition patterns, lesion profiles, proteinase K sensitivity and titratable infectivity in the brains of mice of known genotypes. Prion diversity is thought to be determined by the conformation and glycosylation patterns of PrP^{Sc} and may account for observed differences in prion strains. The 'protein only' theory of prion composition is contested (Chesebro, 1998) and the existence of strains has been cited as evidence which supports a requirement for the presence of nucleic acid in prions.

Prions are stable at a wide pH range and are remarkably resistant to most methods of biological inactivation. Early evidence of the resistance of these agents to chemical inactivation was provided by retrospective studies on 18,000 sheep, which had been inadvertently exposed to infection when inoculated with a formalized louping ill vaccine. The vaccine at that time was prepared from brains, spinal cords and spleens of sheep. It also contained the scrapie agent which was not inactivated by treatment with formaldehyde (Greig, 1950). Approximately 10% of the vaccinated animals developed scrapie. Treatment of prions with alcohols and aldehydes that fix proteins may help to stabilize rather than inactivate these agents. The inclusion of a formic acid step in formaldehyde fixation of brain tissue is used to reduce the infectivity of the agents of scrapie, BSE and Creutzfeldt–Jacob disease in histological sections. Physical methods for inactivating the agents of BSE and scrapie have been intensively investigated because safe disposal of infected carcasses is mandatory. Although autoclaving at 132°C has been recommended, it does not ensure prion inactivation. High concentrations of sodium hypochlorite or hot solutions of sodium hydroxide

have been shown to inactivate a thermostable strain of scrapie agent (Taylor, 2000).

Clinical infections

Diseases attributed to prions occur sporadically and are significantly influenced by the genome of the affected animal. They are viewed as part of a diverse group of neurodegenerative diseases including Alzheimer's disease and Parkinson's disease in humans, referred to as 'protein-folding diseases'. These diseases share a number of features in common including appearance late in life, neuronal loss and the accumulation of cerebral deposits of misfolded protein aggregates (Soto and Estrada, 2008). Transmissible spongiform encephalopathies are slowly progressive neurodegenerative diseases, which are characterized by long incubation periods and spongiform changes in the brain. They have been described in many animal species, including ruminants and certain carnivores (Table 83.1). Similarities in the neuropathological features of scrapie in sheep and kuru in humans suggested that the two diseases had a similar aetiology (Hadlow, 1959). Subsequently, it was established that kuru was also caused by an unconventional transmissible agent. A number of other similar neurodegenerative diseases of humans, some of which are genetically determined, are classified as TSEs. The TSEs that have been described in humans are presented in Table 83.2. In humans, these TSEs may occur as infectious, genetic or sporadic diseases. In scrapie, there is convincing evidence for the importance of the genetic constitution of certain breeds of sheep in determining susceptibility to the disease.

Table 83.1 Transmissible spongiform encephalopathies of animals.

Disease	Comments
Scrapie	Recognized in sheep in parts of Europe for 300 years; apart from Australia and New Zealand, now occurs worldwide. Occurs also in goats
Bovine spongiform encephalopathy	First reported in England in 1986; developed into a major epidemic over a 10-year period. Prevalence declined with the implementation of effective control measures. Occurs at lower frequency in many other European countries. Cases also described in USA, Japan and Canada
Feline spongiform encephalopathy	First recorded during the bovine spongiform encephalopathy epidemic in the early 1990s. Most cases reported in the UK
Transmissible mink encephalopathy	First recognized in caged mink in Wisconsin in 1947; attributed to the feeding of scrapie-infected sheep meat
Spongiform encephalopathy in captive ruminants	First recorded during the bovine spongiform encephalopathy epidemic in 1986. Reported in greater kudu, nyala, oryx and some other captive ruminants in zoological collections
Chronic wasting disease	First recognized in captive mule deer in Colorado in 1980. Occurs in deer and elk populations in the wild in North America. Horizontal transmission occurs; shedding of prions in saliva. Clinical signs include severe generalized wasting, trembling and ataxia

Table 83.2 Transmissible spongiform encephalopathies of humans.

Disease	Comments
Kuru	Described in members of the Fore population in Papua New Guinea. Acquired through ritualistic cannibalism; brain tissue was the primary source of infection
Creutzfeldt–Jakob disease (CJD)	
Sporadic CJD	Aetiology unclear but may be due to somatic mutation in *PrP* gene or to random conversion of PrP^{C} into PrP^{Sc}
Iatrogenic CJD	Transmitted during procedures in which contaminated instruments or infected human tissues are used
Variant CJD	Considered to be a consequence of exposure to PrP^{Sc} from cattle with bovine spongiform encephalopathy
Familial CJD	Germline mutations in *PrP* gene
Gerstmann-Sträussler-Scheinker syndrome	Germline mutations in *PrP* gene
Fatal familial insomnia	Germline mutation in *PrP* gene

Scrapie

This insidious, fatal, neurological disease of adult sheep, goats and moufflon occurs worldwide except in Australia and New Zealand.

Epidemiology

The mode of transmission of scrapie is not clearly understood but epidemics are self-sustaining due to horizontal transmission and environmental persistence. Potential modes for natural infection include ingestion, entry through superficial abrasions and transmission from ewe to lamb. There is evidence that transmission tends to occur during early life and that exposure to placental material and milk from affected ewes may be important (Konold *et al*., 2008). Pastures grazed by affected animals appear to remain contaminated for years.

Particular polymorphisms of the *PrP* gene are associated with an increased incidence of scrapie in certain breeds of sheep. Some research workers have inferred from breeding experiments that scrapie is an exclusively genetic disease. However, Australia and New Zealand are free of scrapie despite the presence of animals with scrapie-associated PrP alleles (Hunter *et al*., 1997). The *PrP* gene-coding sequence in sheep is highly polymorphic. Polymorphisms at codons 136, 154 and 171 of the *PrP* gene, producing amino acid substitutions in PrP protein, are important in influencing susceptibility to scrapie. In many breeds, the valine 136 glutamine 171 arginine 154 (denoted VRQ) allele is strongly associated with susceptibility to scrapie (Laplanche *et al*., 1999). An atypical form of scrapie, Nor98, which has a different tissue distribution and can occur in animals genetically resistant to classical scrapie, has been described in

Europe and the United States (Benestad *et al*., 2003).

Pathogenesis

Following natural infection, PrP^{Sc} is usually first detected in tissues of the lymphoreticular system including the spleen, the palatine tonsil and the retropharyngeal and mesenteric lymph nodes. In lymph nodes, replication apparently occurs in follicular dendritic cells. After oral exposure it is thought that the portal of entry to neural tissues is in the duodenum and ileum. The agent then spreads through fibres of the autonomic nervous system to the spinal cord and the medulla oblongata (van Keulen *et al*., 2008). Neuronal and neuropil vacuolation and astrogliosis are characteristic features of this disease.

Clinical signs

The disease has a long incubation period. Neurological signs develop predominantly in sheep of breeding age with a peak incidence between 3 and 4 years of age. Initially, affected animals may present with restlessness or nervousness, particularly after sudden noise or movement. Fine tremors of the head and neck and incoordination with a tendency to exhibit jerky movements are characteristic signs. Pruritus may result in loss of wool. In some affected sheep, a nibbling reflex can be elicited by scratching the back. Progression of the disease leads to emaciation. Death usually occurs within 6 months from the onset of clinical signs. The annual mortality rate due to scrapie in an affected flock is typically 3 to 5% although the rate can reach 20% in severely affected flocks.

Diagnosis

Clinical signs and histopathological examination of the CNS form the basis for diagnosis. Characteristic microscopic changes include neuronal vacuolation and degeneration, vacuolar change in the neuropil, and astrocytosis, particularly in the medulla. However, additional samples of all major brain regions may need to be processed given that strain-specific targeting of other parts of the brain has been described (Benestad *et al*., 2003). No obvious inflammatory response is evident. Confirmatory methods include immunohistochemical staining for PrP^{Sc}, immunoblotting to detect proteinase-K-resistant PrP^{Sc} and electron microscopy to detect scrapie-associated fibrils in detergent-treated extracts of brain. Ante-

mortem detection methods, based on demonstration of PrP^{Sc} in lymphoid tissues of the palatine tonsil, nictitating membrane and rectal biopsies by histochemical methods, have being reported but do not detect prions in all infected sheep. A number of rapid immunodiagnostic tests based on western blot methods or ELISA methodology, originally developed for BSE, are now available for the diagnosis of scrapie (Novakofski *et al*., 2005). Positive or inconclusive results require confirmation by immunohistochemistry or western blot methods. Serology is not useful diagnostically as antibodies specific for the scrapie agent that do not cross-react with PrP^{C} have not been demonstrated.

Control

In the European Union, scrapie has been designated a notifiable disease. Countries free of the disease impose strict quarantine procedures. Slaughter policies have been enforced with different degrees of success in several countries. In Australia and New Zealand, an eradication policy, implemented soon after the introduction of the disease, was successful. Eradication was abandoned in the United States because of the cost and difficulties in implementation. A control policy involving flock certification and movement restrictions is now in place. Breeding scrapie-resistant sheep may be a realistic method for reducing the frequency of the disease (Parry, 1983).

Bovine spongiform encephalopathy

This condition is a progressive, neurodegenerative disease of adult cattle, first recognized in England in 1986 (Wells *et al*., 1987). More than 180,000 cases of the disease were subsequently confirmed and an estimated one million animals were infected. This common source epidemic peaked in 1992 when more than 36,000 cases were identified. Since then, there has been a steady decline in numbers of confirmed cases. The disease has been reported in several countries in animals imported from Great Britain. In addition, indigenous cattle in many European countries, including Switzerland, Ireland, France and Portugal, have developed the disease. Cases of BSE in indigenous cattle have also been reported in Canada, USA, Israel and Japan.

The prion strain causing bovine spongiform encephalopathy (BSE) is not considered to be species-specific; infection has been reported in exotic ungulates in zoological collections following ingestion of feed derived from contaminated bovine tissues. In addition, feline spongiform encephalopathy was first recorded

in the early 1990s in association with the BSE epidemic. In 1996, a novel form of human prion disease termed variant Creutzfeldt–Jakob disease (vCJD) was recognized in Great Britain. Molecular strain-typing studies and experimental transmission in transgenic and conventional mice indicated that vCJD and BSE are caused by indistinguishable prion strains. The extent of exposure of the human population to the agent cannot be accurately estimated because of uncertainties about risk factors and about the length of the incubation period in vCJD (Collinge, 1999). Susceptibility to vCJD is strongly associated with a methionine homozygous genotype at codon 129 of the human prion protein (*PRNP*) gene. The number of deaths due to vCJD peaked in the United Kingdom in 2000 at 28 cases, while the total number of deaths caused by vCJD reached 170 in 2010.

The BSE epidemic in Great Britain is considered to have been caused by a single major strain of BSE. However, following large-scale active surveillance studies in several countries, atypical forms of BSE have been identified based on variant features of pathology and/or molecular characteristics. One type (‘H-type’) is characterized by higher molecular mass fragments than classical BSE, while the other has a lower molecular mass and is referred to as ‘L-type’ or bovine amyloidotic spongiform encephalopathy (BASE). The discovery of atypical forms of BSE suggests that this neurodegenerative disease may sometimes arise spontaneously and, accordingly, sporadic cases will continue to occur.

Epidemiology

The BSE epidemic, which started simultaneously at several geographical locations in Great Britain, was attributed to contaminated meat-and-bone meal (MBM) prepared from slaughterhouse offal and fed as a protein dietary supplement to cattle. It is postulated that the scrapie agent crossed the species barrier into cattle in the early 1980s following changes in the rendering process which allowed survival of increased amounts of scrapie PrP (PrP^{Sc}) in the MBM. Recycling of infected tissues from animals with BSE, prior to the recognition of the disease and the imposition of control measures, resulted in extension of the epidemic. Because of the high ratio of sheep to cattle, the frequency of endemic scrapie and the heavy reliance on MBM as a supplement for dairy cattle in Great Britain, the epidemic has been largely confined to that country (Nathanson *et al*., 1999). However, MBM was exported from the UK during the early part of the

epidemic and the subsequent introduction of rapid testing across Europe has revealed small numbers of cases in many countries. As a result of the banning of ruminantderived MBM in 1988, there was a marked decline in the prevalence of BSE in Great Britain after 1993. Despite this decline, animals born after imposition of the ban have developed BSE. This was ascribed to the continued use of ruminant-derived MBM and also to cross-contamination in feed mills by rations specified for pigs and poultry. Additional stringent regulations, introduced in 1996, banned the inclusion of mammalian-derived MBM from farm animal feed.

Horizontal transmission of BSE does not appear to occur and, although maternal transmission may occur at a low rate, it is considered to be of minimal importance in the spread of disease. Susceptibility of cattle to BSE appears to be independent of sex, breed and genotype.

Pathogenesis

The pathogenesis of BSE is poorly defined. The agent of BSE has been found in the distal ileum following experimental oral exposure. In naturally occurring cases of the disease, the agent has been demonstrated mainly in the CNS, retina and distal ileum. Characteristic changes in the CNS include vacuolation and glial proliferation.

Clinical signs

The mean incubation period is about 5 years. Neurological signs, which are highly variable, include changes in behaviour and deficits in posture and movement. Loss of weight and decreased milk production also occur. Other clinical signs include tremors, hyperaesthesia, apprehension, bruxism, exaggerated menace reflex and head shyness. Ataxia, hypermetria and a tendency to fall become increasingly evident in the later stages of the disease. The clinical course may extend over many days or months.

Diagnosis

Bovine spongiform encephalopathy can be confirmed by histopathological examination of brain tissue and appropriate additional methods. Examination of brain tissue may be confined to a coronal section of the medulla at the obex. Characteristic neuropathological changes including neuropil vacuolation and astrocytosis are consistently present at this site. Additional confirmatory

methods include immunohistochemical staining for PrP^{Sc}, immunoblotting to demonstrate proteinase K resistant PrP^{Sc}, and electron microscopy to detect prion fibrils in detergent-treated extracts of brain. Commercial, automated immunoblotting and ELISA techniques suitable for large-scale screening of animals are now available (Novakofski *et al*., 2005). The application of protein misfolding cyclic amplification (PMCA) technology to increase the amount of PrP^{Sc} in a sample (Saborio *et al*., 2001; Soto *et al*., 2005) may enhance detection and lead to the development of more sensitive assays for the testing of presymptomatic animals and humans.

Control

Bovine spongiform encephalopathy is a notifiable disease in countries of the European Union with compulsory testing of fallen animals and cattle over 48 months of age. In some countries, the entire herd in which an affected animal is detected is slaughtered. In other countries, clinically-affected animals only, or affected and cohort animals, are slaughtered. Ruminant-derived protein should be excluded from ruminant rations. Carcasses of infected animals should be incinerated at high temperatures to ensure destruction of the thermostable agent. Buildings and equipment can be decontaminated by the application of high concentrations of sodium hypochlorite or of heated strong solutions of sodium hydroxide (Taylor, 2000). Tissues considered to have a high risk of transmitting BSE such as skull, spinal column, tonsils, intestines and mesentery are excluded from the human food chain in many countries.

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Section VII
Microbial Agents and Disease Production

Chapter 84

Tissue and system preferences of bacterial, fungal and viral pathogens and the nature of the diseases caused by these infectious agents

Pathogenic microorganisms are frequently associated with diseases in particular organs or in particular host systems. Many bacterial pathogens have well recognized predilection sites in tissues or organs where, presumably, the microenvironment, availability of nutrients, an inadequate protective immune response or other factors allow colonization by these pathogens. Susceptibility of the host is influenced by the species, breed, age, sex, genotype and immune status of the animal and also by characteristics of the infectious agent and by management and environmental factors. Irrespective of the host's age or immunological competence, some virulent bacteria and viruses can invade and produce disease. In contrast, opportunistic pathogens can produce disease in susceptible animals lacking immunological experience with the aetiological agent, or when rendered susceptible to infection due to immunological incompetence or immunosuppression. These opportunistic infectious agents sometimes produce disease when they gain entry into tissues following traumatic injury. Many opportunistic bacteria colonizing mucous membranes avail of the changes in the host's immune response, which derive from stress-induced management conditions such as overcrowding, inadequate ventilation or transportation, to invade tissues and produce disease in such immunosuppressed animals. Circumstances in which a primary viral infection may lead to a secondary bacterial infection are illustrated by the secondary invasion of damaged canine intestinal tissue by Gram-negative bacteria following canine parvovirus infection of the intestinal mucosa and mesenteric lymph nodes. Sudden dietary changes that alter the microflora of young animals, especially ruminants and pigs, predispose to enteric colonization by Gram- negative bacteria, such as *E. coli*, which are capable of producing local tissue damage and systemic disease. Gram-positive bacteria, such as enteropathogenic clostridia,

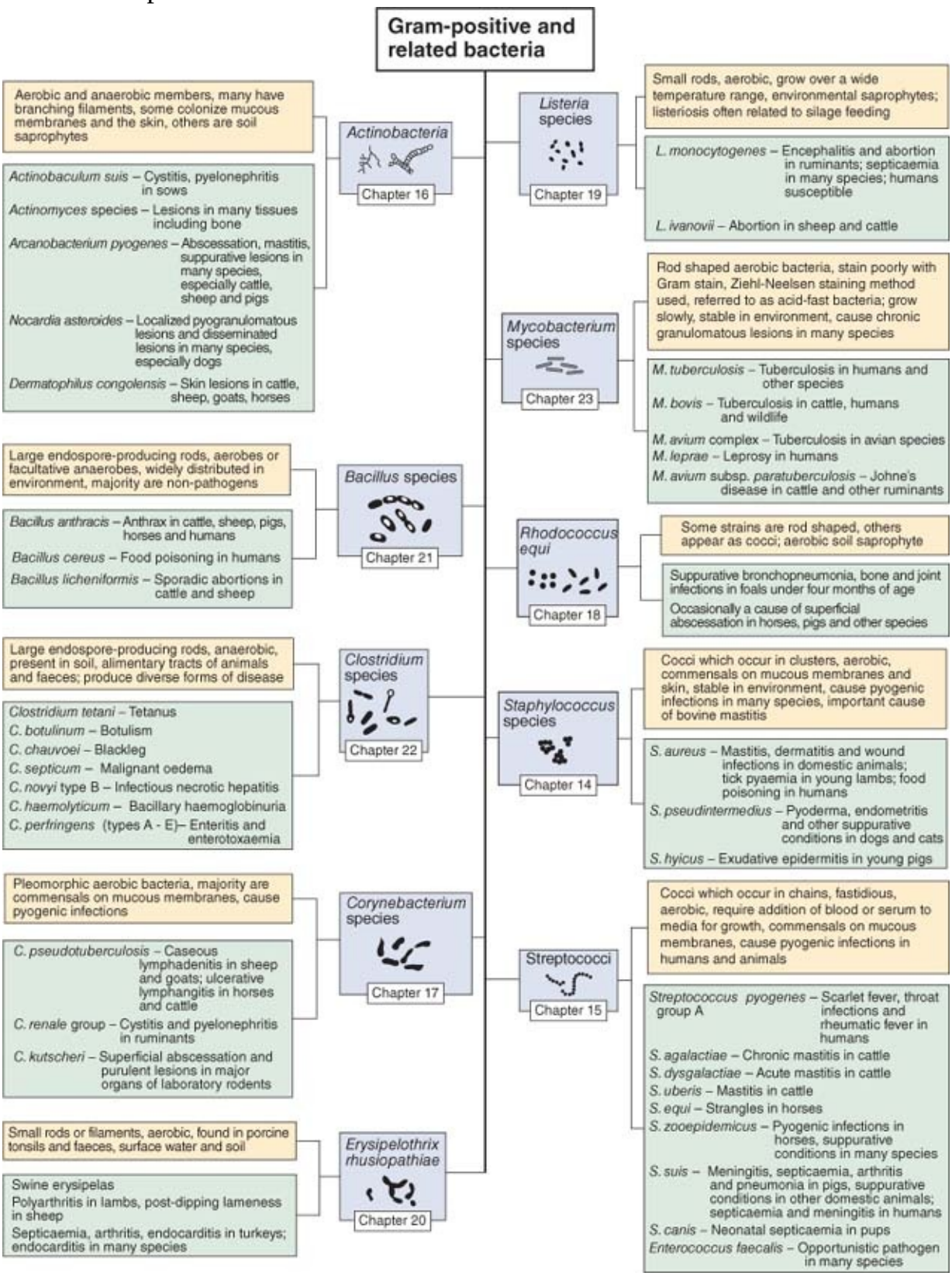
can also produce serious enteric disease when circumstances such as a high intake of grain or unrestricted access to rich pasture promote their rapid proliferation with subsequent toxin production.

Diseases caused by Gram-positive bacteria include fatal septicaemia in ruminants and other domestic animals caused by *Bacillus anthracis*, potentially fatal intoxication caused by neurotoxic clostridia, suppurative conditions associated with staphylococcal infections, and both localized and disseminated lesions caused by bacteria belonging to the *Actinobacteria* (Fig. 84.1). Pathogenic mycobacteria, which produce chronic progressive diseases in humans and animals, although cytochemically Gram-positive, stain poorly using the Gram stain. The Ziehl-Neelsen staining method is employed for demonstrating these acid-fast bacteria in smears.

Gram-positive bacteria exhibit wide diversity in their cultural characteristics, their host preferences, their ability to produce disease and their responses to chemotherapeutic agents. *Staphylococcus* species are of particular importance because of the range of virulence factors that they express and their ability to develop resistance to antibacterial drugs. Resistance of staphylococci to antibiotics is a major obstacle to the successful treatment of tissue infections in many species. Because pathogenic clostridia produce potent exotoxins which are responsible for tissue damage, neurological disturbance and enterotoxaemia, toxoids are highly effective vaccines for preventing clostridial diseases. Vaccination, however, is not an effective option at present for inducing protective immunity against infections caused by staphylococci, streptococci, listeria, corynebacteria or actinomycetes. The diverse natures of the diseases that these Gram- positive bacteria produce in animals, due to the virulence factors that they elaborate and the absence of a sustained protective immune response following infection, are among the reasons why the development of effective safe vaccines for these important pathogens has not yet been successful. Although there has been some progress in the development of vaccines for pathogenic mycobacteria, the failure to clearly identify toxic factors produced by these intracellular bacteria and the chronic nature of the diseases they produce account for some of the difficulties in explaining the pathogenesis of these acid-fast bacteria and the limitations of vaccination for the prevention of mycobacterial diseases.

Figure 84.1 Gram-positive and related bacteria arranged alphabetically, with a brief summary of their important features, the diseases which they cause and the

relevant chapter numbers.



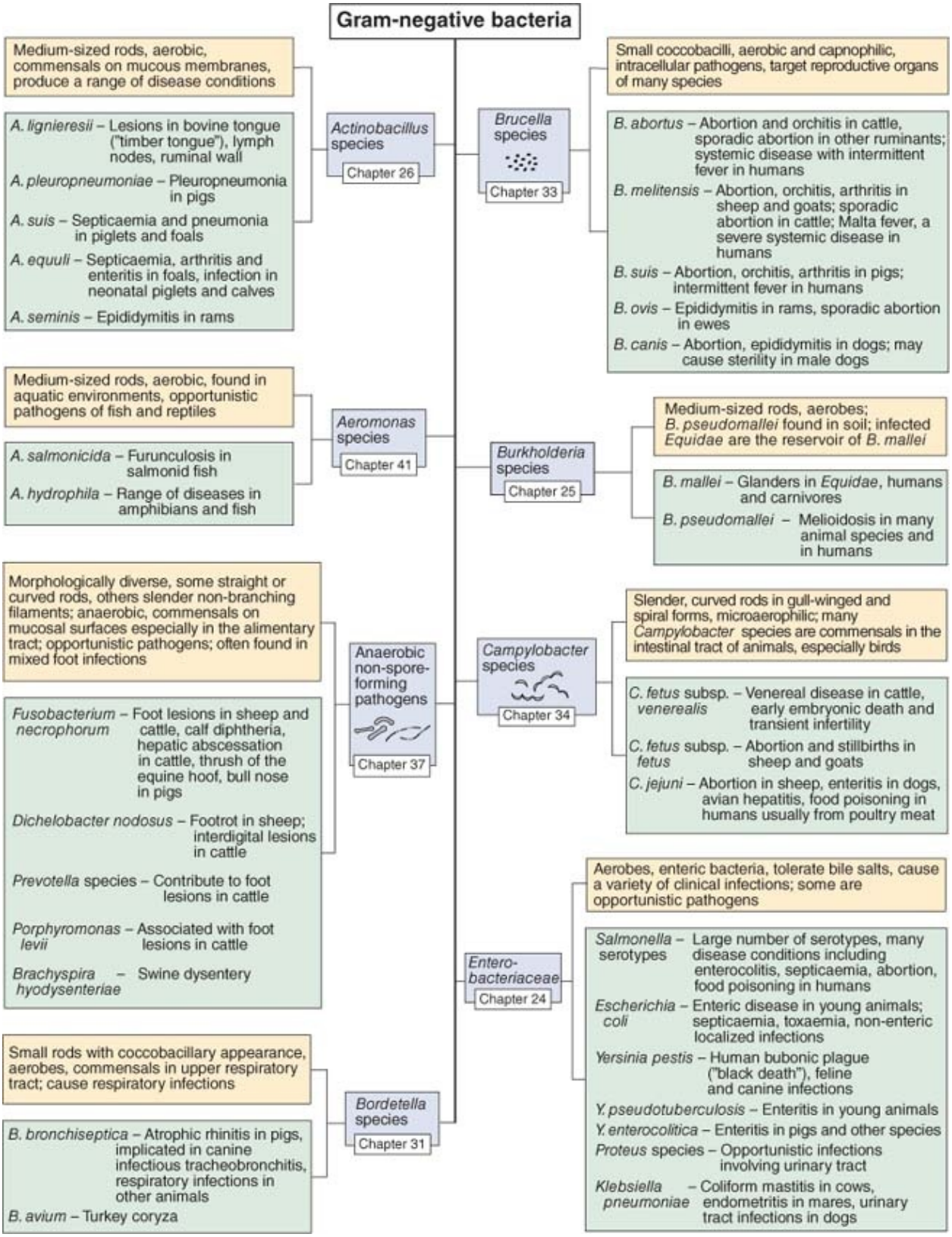
An important group of Gram-negative bacteria that inhabit the intestinal tracts of animals and humans, the *Enterobacteriaceae*, cause diseases which range from opportunistic infections to enterocolitis, septicaemia and abortion. *Klebsiella pneumoniae* and *Enterobacter aerogenes* are two opportunistic bacterial species often encountered in coliform mastitis in dairy cattle. *Proteus* species and *Klebsiella* species cause urinary tract infections in dogs, cats and other animals. *Salmonella* serotypes cause enteric disease in most species of animals, septicaemia in calves, foals and piglets and food- poisoning in humans. Serotypes vary in virulence and a number are adapted to particular hosts.

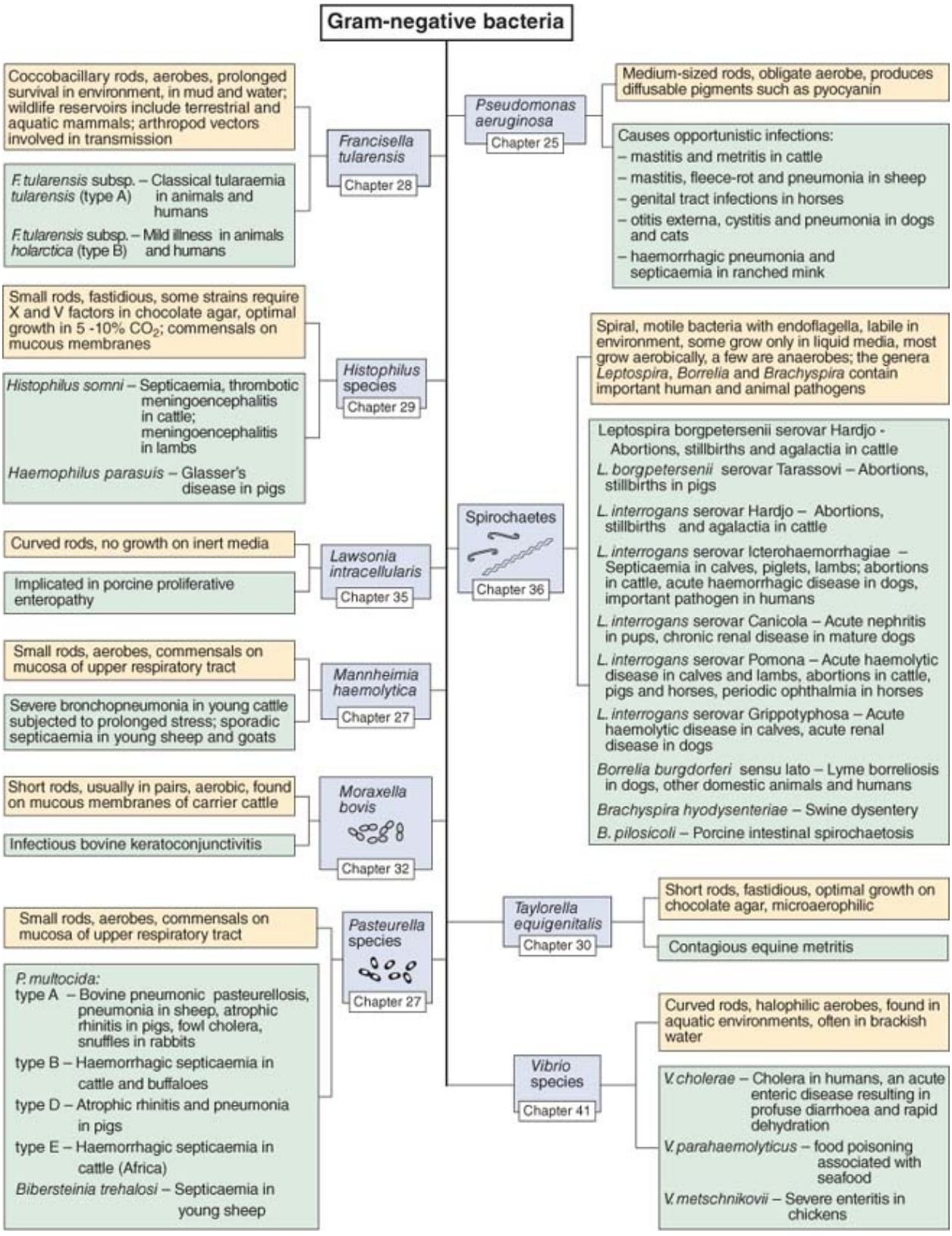
Enteric disease, septicaemia and toxaemia are conditions associated with *E. coli* infections in young animals. *Brucella* species, major intracellular pathogens, target the reproductive tract of sexually mature female and male animals. Abortion in late gestation is a feature of brucellosis in ruminants and pigs. Apart from the diseases produced in animals, many *Brucella* species cause severe systemic disease in humans characterized by intermittent fever of variable duration, profuse sweating and weight loss. Among the Gram- negative bacteria, spirochaetes are important human and animal pathogens. *Leptospira interrogans* serovar Icterohaemorrhagiae is an example of a leptospire which produces septicaemia in young animals, abortions in cattle and serious human infection. Another spirochaete, *Borrelia burgdorferi* sensu lato, causes Lyme borreliosis in dogs, other domestic animals and humans. *Brachyspira hyodysenteriae*, an anaerobic spirochaete, causes swine dysentery, a disease of weaned pigs which results in poor food conversion and economic loss. A brief summary of the characteristics of pathogenic Gram-negative bacteria, including the diseases that they cause, is presented in Fig. 84.2.

Atypical bacteria constitute a distinct grouping of microorganisms that do not conform to the convenient differentiation of bacteria into Gram-positive and Gram-negative categories based on their cell wall staining patterns. Even among these atypical microorganisms there is wide divergence in their structure, staining patterns, growth requirements and pathogenicity. The mycoplasmas do not have cell walls, are highly pleomorphic and do not stain by the Gram method. In addition, a number of mycoplasmal organisms, formerly described as rickettsiae and now referred to as haemotropic mycoplasmas, do not grow *in vitro*; many of these unusual mycoplasmas are probably arthropod-borne and they cause infectious anaemia in many mammalian species. Mycoplasmal pathogens cause respiratory diseases in ruminants, pigs and poultry; these pathogens also cause mastitis and arthritis in ruminants in many countries.

Contagious bovine pleuropneumonia is endemic in parts of Africa, the Middle East and Asia. Contagious caprine pleuropneumonia is present in northern and eastern Africa and in Turkey, while enzootic pneumonia of pigs occurs worldwide. Mycoplasmal diseases are of major economic importance not only in developing countries but also in regions of the world where ruminants, pigs and poultry are reared intensively. *Coxiella burnetii*, which occurs in two antigenic phases, is often transmitted by aerosols. This organism, which is unusually stable in the environment and resistant to disinfectants, produces an influenza-like occupational disease in farmers and abattoir workers but most infections in animals are subclinical. The obligate intracellular bacteria, chlamydiae, have a unique developmental cycle and produce respiratory, enteric and reproductive tract diseases in humans and animals. The *Anaplasma* species do not grow on inert media, are tick-borne and produce diseases in ruminants, horses, humans and dogs. *Ehrlichia* species infect ruminants, dogs and humans in many countries. These tick-borne pathogens replicate in granulocytes, monocytes and macrophages; endothelial cells are the targets of *E. ruminantium* and *E. ondiri*. *Rickettsia rickettsii* causes Rocky Mountain spotted fever, a tickborne infection. Humans, dogs and rodents are susceptible to this rickettsial disease. Vascular endothelial damage is a prominent feature of the disease in humans and in animals. A brief summary of important features of atypical bacteria and the diseases which they cause in humans and animals is presented in Fig. 84.3.

Figure 84.2 Gram-negative bacteria arranged alphabetically, with a brief summary of their important features, the diseases which they cause and the relevant chapter numbers.


Gram-negative bacteria
Medium-sized rods, aerobic, commensals on mucous membranes, produce a range of disease conditions
A. lignieresii – Lesions in bovine tongue ("timber tongue"), lymph nodes, ruminal wall
A. pleuropneumoniae – Pleuropneumonia in pigs
A. suis – Septicaemia and pneumonia in piglets and foals
A. equuli – Septicaemia, arthritis and enteritis in foals, infection in neonatal piglets and calves
A. seminis – Epididymitis in rams
Actinobacillus species
Chapter 26
Small coccobacilli, aerobic and capnophilic, intracellular pathogens, target reproductive organs of many species
B. abortus – Abortion and orchitis in cattle, sporadic abortion in other ruminants; systemic disease with intermittent fever in humans
B. melitensis – Abortion, orchitis, arthritis in sheep and goats; sporadic abortion in cattle; Malta fever, a severe systemic disease in humans
B. suis – Abortion, orchitis, arthritis in pigs; intermittent fever in humans
B. ovis – Epididymitis in rams, sporadic abortion in ewes
B. canis – Abortion, epididymitis in dogs; may cause sterility in male dogs
Brucella species
Chapter 33
Medium-sized rods, aerobic, found in aquatic environments, opportunistic pathogens of fish and reptiles
A. salmonicida – Furunculosis in salmonid fish
A. hydrophila – Range of diseases in amphibians and fish
Aeromonas species
Chapter 41
Medium-sized rods, aerobes; B. pseudomallei found in soil; infected Equidae are the reservoir of B. mallei
B. mallei – Glanders in Equidae, humans and carnivores
B. pseudomallei – Melioidosis in many animal species and in humans
Burkholderia species
Chapter 25
Morphologically diverse, some straight or curved rods, others slender non-branching filaments; anaerobic, commensals on mucosal surfaces especially in the alimentary tract; opportunistic pathogens; often found in mixed foot infections
Fusobacterium necrophorum – Foot lesions in sheep and cattle, calf diphtheria, hepatic abscessation in cattle, thrush of the equine hoof, bull nose in pigs
Dichelobacter nodosus – Footrot in sheep; interdigital lesions in cattle
Prevotella species – Contribute to foot lesions in cattle
Porphyromonas levii – Associated with foot lesions in cattle
Brachyspira hyodysenteriae – Swine dysentery
Anaerobic non-spore-forming pathogens
Chapter 37
Slender, curved rods in gull-winged and spiral forms, microaerophilic; many Campylobacter species are commensals in the intestinal tract of animals, especially birds
C. fetus subsp. venerealis – Venereal disease in cattle, early embryonic death and transient infertility
C. fetus subsp. fetus – Abortion and stillbirths in sheep and goats
C. jejuni – Abortion in sheep, enteritis in dogs, avian hepatitis, food poisoning in humans usually from poultry meat
Campylobacter species
Chapter 34
Aerobes, enteric bacteria, tolerate bile salts, cause a variety of clinical infections; some are opportunistic pathogens
Salmonella serotypes – Large number of serotypes, many disease conditions including enterocolitis, septicaemia, abortion, food poisoning in humans
Escherichia coli – Enteric disease in young animals; septicaemia, toxaemia, non-enteric localized infections
Yersinia pestis – Human bubonic plague ("black death"), feline and canine infections
Y. pseudotuberculosis – Enteritis in young animals
Y. enterocolitica – Enteritis in pigs and other species
Proteus species – Opportunistic infections involving urinary tract
Klebsiella pneumoniae – Coliform mastitis in cows, endometritis in mares, urinary tract infections in dogs
Entero-bacteriaceae
Chapter 24
Small rods with coccobacillary appearance, aerobes, commensals in upper respiratory tract; cause respiratory infections
B. bronchiseptica – Atrophic rhinitis in pigs, implicated in canine infectious tracheobronchitis, respiratory infections in other animals
B. avium – Turkey coryza
Bordetella species
Chapter 31



Pathogenic fungi can produce disease by tissue invasion, by toxin production or by induction of hypersensitivity in the host. Fungal diseases that result from

tissue invasion can be categorized according to the sites of lesions as superficial, subcutaneous or systemic mycoses. Superficial mycoses are classified as dermatomycoses or dermatophytoses. The former result from overgrowth of fungi such as *Candida* species or *Malassezia pachydermatis* on the skin or at mucocutaneous junctions. Dermatophytoses, which are clinically more important than dermatomycoses because of their communicability to other animals and also because of their zoonotic potential, are associated with the invasion and destruction of keratinized structures by dermatophytes such as *Microsporum* and *Trichophyton* species. Subcutaneous mycoses usually result from localized fungal invasion of the dermis and subcutis, often a consequence of penetration by a foreign body. Systemic mycoses, which often originate in the respiratory or digestive tracts, usually follow opportunistic infection by saprophytic fungi. Factors that predispose to fungal invasion of tissues include alteration in the normal microbial flora as a result of prolonged antimicrobial therapy, immunosuppression following corticosteroid therapy or viral infections and exposure to heavy challenge of fungal spores. In addition to invasion of tissues, pathogenic fungi can elaborate toxins, referred to as mycotoxins, which have a number of toxic effects including immuno- suppression, mutagenesis, teratogenesis and carcinogenesis. Mycotoxicoses follow the ingestion of contaminated plant material, cereals or nuts; feeds with a high moisture content stored under conditions that favour fungal growth may be a source of fungal toxins. Diseases caused by mycotoxins tend to be seasonal as climatic factors often determine the extent of fungal growth on plant material and on certain types of pasture or on stored feed harvested during a wet season. A point of distinction between mycotoxicoses and infectious diseases caused by bacteria or viruses is the absence of lateral spread to in-contact animals. A brief summary of important features of pathogenic fungi, related pathogens, mycotoxins and mycotoxicoses is presented in Fig. 84.4.

Viral pathogens, either alone or in association with bacterial agents, can initiate major outbreaks of disease involving the respiratory tract, the gastrointestinal tract or the reproductive tract. Many viruses have an affinity for particular cell types, and the stability of some viruses in the environment facilitates transmission to susceptible animals through environmental contamination. Parvoviruses, which replicate only in rapidly dividing cells, exhibit exceptional resistance to heat, pH changes and disinfectants. Canine parvovirus infection results in extensive contamination of kennels, equipment, footwear and clothing of workers. The stability of the virus and the low dose

required to establish infection in susceptible dogs are important factors in the spread of disease. Porcine parvovirus infection is an important cause of reproductive failure in pigs. Transplacental infection in pregnant sows occurs within 2 weeks of exposure, and infection of embryos in the first weeks of life leads to death and resorption. Infection later in gestation, before day 70, results in foetal death and mummification. Infection after 70 days, at a time when foetuses are immunologically competent, usually results in the birth of seropositive healthy piglets.

There is wide variation in the types of diseases that DNA virus families cause and in the range of species of animals affected (Fig. 84.5). Herpesviruses cause diseases in the respiratory, reproductive and nervous systems. As latency is a common outcome of infection with herpesviruses, these viruses establish life-long infections with reactivation and shedding of virus which may be periodic or continuous. Reactivation of infection is usually associated with various stress factors including transportation, adverse weather conditions, overcrowding and intercurrent infections. Natural infections with particular herpesviruses are usually restricted to defined host species.

Some members of the RNA virus families such as the *Arteriviridae* and *Bornaviridae* cause disease in a limited number of animal species. In contrast, viruses belonging to the *Picornaviridae* and the *Retroviridae* affect a wide range of animal species (Fig. 84.6). Retroviral infections of animals cause slowly progressing life-long infections. A feature of viruses in many genera in the *Retroviridae* is their ability to induce neoplastic changes in specific cell types. Viruses in this family include the avian leukosis virus, feline leukaemia virus, feline immunodeficiency virus, equine infectious anaemia virus and the virus that causes maedi/visna in sheep. Acquired immunodeficiency syndrome in humans is caused by human immunodeficiency virus, a lentivirus.

The ability of some retroviruses to target helper T cells results in progressive immunodeficiency and renders animals susceptible to opportunistic bacterial and fungal agents which ultimately become responsible for terminal illness in infected animals. The slow progression of lentivirus infection is in sharp contrast to the rapid progression of the aphthoviruses which cause foot-and-mouth disease. Transmission of foot- and-mouth disease virus (FMDV) can occur by direct contact, by aerosols, by mechanical means, on fomites and through animal products. Infected groups of animals, particularly pigs, shed large quantities of virus in aerosols which, under favourable conditions for virus survival, can spread over long distances. In addition to domestic ruminants and pigs, many

wildlife species are susceptible to FMDV. The short incubation period, the shedding of virus during the incubation period, the relative resistance of the virus to environmental factors and the presence of wildlife reservoirs in some countries militate against the implementation of effective control measures in many regions of the world.

Figure 84.3 Atypial bacteria arranged alphabetically, with a brief summary of their important features, the diseases which they cause and the relevant chapter numbers.

Atypical bacteria

Anaplasma species

Chapter 40

Minute, obligate intracellular pleomorphic bacteria; outer membranes structurally similar to Gram-negative bacteria; do not grow on inert media; demonstrable in blood smears by Romanowsky stains; cause systemic diseases, mainly arthropod-borne

- *A. phagocytophilum* – Infects myeloid cells mainly neutrophils, causes tick-borne fever in domestic and wild ruminants, equine granulocytic ehrlichiosis, human granulocytotropic anaplasmosis
- *A. marginale* – Bovine anaplasmosis, tick-borne disease of cattle; calves more resistant than mature animals; characterized by progressive anaemia due to destruction of erythrocytes
- *A. bovis* – Tick-borne disease referred to as bovine ehrlichiosis, pathogen infects monocytes and macrophages; occurs in Africa, Middle East and parts of Europe
- *A. platys* – Canine cyclic thrombocytopenia; tick transmission suspected; characterized by recurring thrombocytopenia and mild anaemia; usually asymptomatic

Aegyptianella pullorum – Affects poultry and wild birds, tick vector involved; affected birds have ruffled feathers,diarrhoea and anaemia

Coxiella burnetii

Chapter 40

Small pleomorphic rods with Gram-negative features and two antigenic phases; suitable staining procedures include modified Ziehl-Neelsen and immunofluorescence; replicates only in living cells, stable in environment; often transmitted by aerosols; raw milk may be responsible for some human infections

C. burnetii – Causes Q fever, an occupational disease of farmers, abattoir workers, veterinarians; replicates in the placentae of sheep, cattle and goats; although most infections in animals are subclinical, occasional abortions occur in sheep and goats; human infections usually acquired by aerosols from infected animals at parturition

Ehrlichia species

Chapter 40

Minute, obligate intracellular pleomorphic bacteria with outer membranes similar to Gram-negative bacteria; replicate only in living cells; demonstrable in blood smears by Romanowsky stains

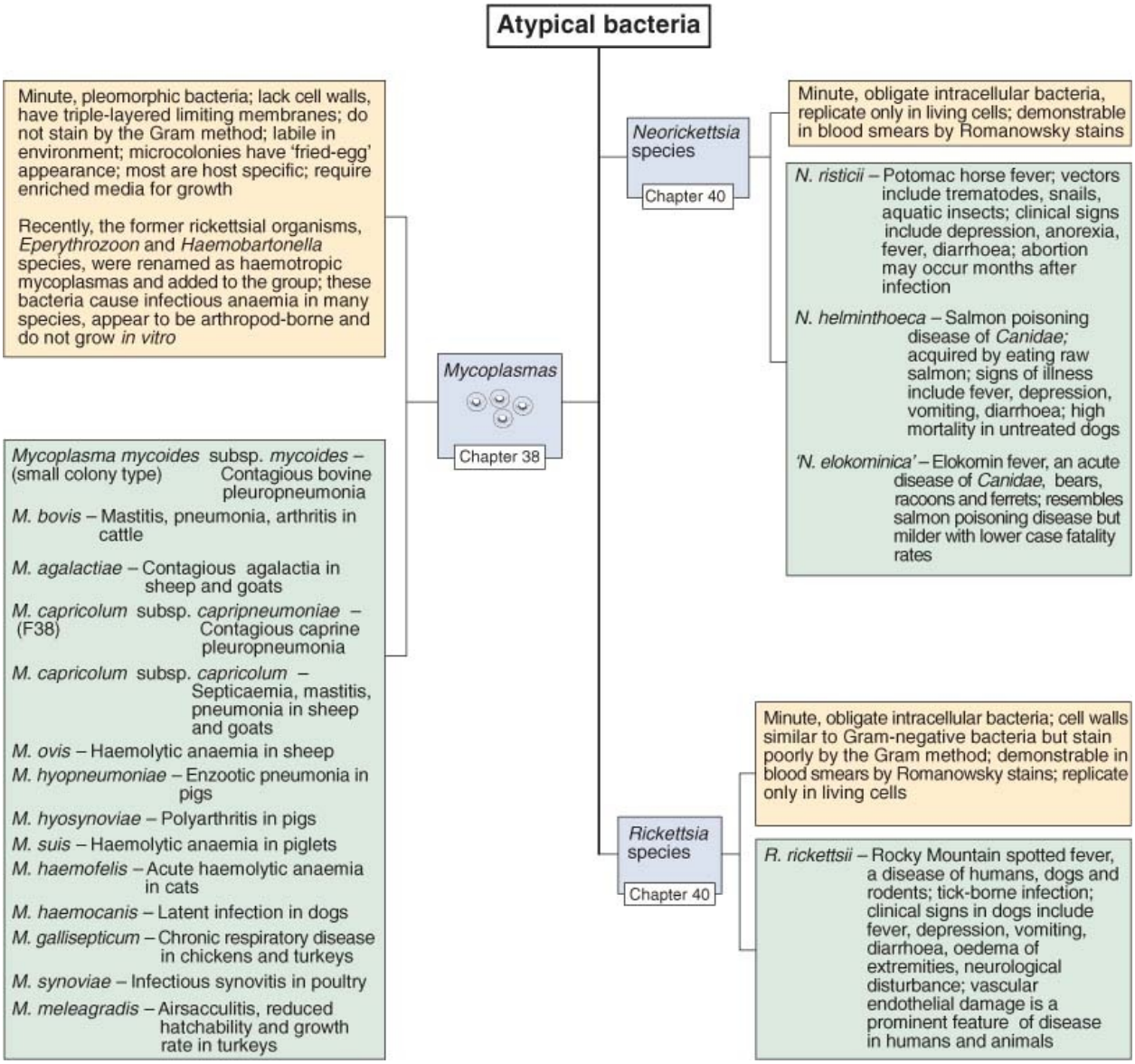
- *E. canis* – Canine monocytic ehrlichiosis, also causes human infection; tick-borne disease; fever and thrombocytopenia occur in the acute phase of disease, chronic disease occurs in some dogs
- *E. chaffeensis* – Infections in deer, dogs and humans; tick-borne infection, the pathogen causes human monocytic ehrlichiosis, a moderate to severe disease with fever and myalgia
- *E. ewingii* – Infections occur in dogs, deer and humans; tick-borne pathogen of canine and human neutrophils; usually causes mild disease in dogs; human infection can result in mild to moderate disease
- *'E. ondiri'* – Bovine petechial fever in Kenyan highlands at altitudes above 1,500m; arthropod vector suspected; may be endemic in wild ruminants; pathogen present initially in granulocytes and monocytes, later in endothelial cells; clinical signs include high fever and petechial haemorrhages on mucous membranes; pregnant animals may abort
- *E. ruminantium* – Heartwater, tick-borne disease; affects ruminants in sub-Saharan Africa and some Caribbean islands; pathogen replicates initially in macrophages and later in vascular endothelium, causing severe disease

Chlamydia and *Chlamydophila* species

Chapter 39

Spherical intracellular bacteria with unique developmental cycle; replicate only in living cells; their cell walls resemble Gram-negative bacteria; suitable staining procedures include modified Ziehl-Neelsen and Giemsa methods; species vary in virulence for particular hosts

- *Chlamydia trachomatis* – Trachoma, disease limited to humans; a chronic granulomatous process involving the eye surface which can lead to loss of vision
- *Chlamydophila psittaci* – Avian chlamydiosis, disease of domestic and wild birds; clinical signs include airsacculitis and intestinal infection; transmissible to humans
- *C. abortus* – Enzootic abortion of ewes; sporadic abortion in cattle, goats and pigs
- *C. pecorum* – Intestinal infection and conjunctivitis in sheep
- *C. felis* – Conjunctivitis in cats
- *C. pneumoniae* – Respiratory infection in humans



Viruses in the *Rhabdoviridae* and *Togaviridae* families have an affinity for cells of the central nervous system (CNS). The viruses that cause the equine encephalitides invade the CNS and induce neuronal necrosis and perivascular lymphoid cuffing. Following introduction into the tissues by the bite of a rabid animal, rabies virus enters peripheral nerve endings, is transported to the CNS by retrograde axoplasmic flow and becomes widely disseminated in nervous tissue by intra-axonal spread. Clinical signs develop following neuronal damage caused by viral replication. Viruses in the genus *Flavivirus*, which are transmitted by arthropods, cause encephalitis in many species of animals and also in humans. Following virus replication in the lymph node at the site of inoculation, viraemia follows with dissemination to lymphoid organs and

sometimes to the brain and spinal cord.

Figure 84.4 Pathogenic fungi and related pathogens arranged alphabetically, with a brief summary of their important features, the diseases which they cause and the relevant chapter numbers. A brief summary of mycotoxins and mycotoxicoses is also included.

Pathogenic fungi, related pathogens, mycotoxins and mycotoxicoses

Aspergillus species

Chapter 44

Ubiquitous, saprophytic moulds; septate,hyaline hyphae, grow rapidly, colonies pigmented; respiratory pathogens, acquired by inhalation of spores

A. fumigatus – Brooder pneumonia in newly-hatched chickens in incubators; guttural pouch mycosis in horses; mycotic abortion in cattle; nasal aspergillosis in dogs

A. flavus – Toxigenic strains produce aflatoxins; toxic effects in susceptible species include immunosuppression, neoplasia, teratogenesis

Dermatophytes

Chapter 43

Septate fungi, produce arthrospores which are the infectious form; affinity for keratinized structures; colonize and invade skin, hair, nails; grow slowly on specially formulated media; strict aerobes, majority grow at 25°C; colonies often pigmented; dermatophytosis (ringworm) affects many animal species and is zoonotic

Microsporum canis – Cats, dogs
M. equinum – Horses
M. gallinae – Chickens, turkeys
M. gypseum – Horses, dogs, rodents
M. nanum – Pigs
Trichophyton equinum – Horses
T. mentagrophytes – Rodents, dogs, horses, other animal species
T. verrucosum – Cattle

Dimorphic fungi

Chapter 46

Occur in two distinct forms, a mould form and a yeast form; they exist as moulds in the environment or when cultured at 25°C to 30°C; in animal tissues and when cultured at 37°C, most grow as yeasts; referred to as thermally dimorphic; saprophytes in soil and decaying vegetation; produce opportunistic infections in animals and humans

Blastomyces dermatitidis – Blastomycosis in dogs and humans; infection occurs by inhalation; pulmonary lesions; metastases to skin and other tissues may occur

Histoplasma capsulatum – Histoplasmosis in dogs, cats and humans; infection occurs by inhalation, granulomatous pulmonary lesions, disseminated disease may occur

H. farciminosum – Epizootic lymphangitis in horses and other *Equidae*; infection acquired from environment through skin abrasions on limbs; lymphocutaneous lesions consist of ulcerated discharging nodules

Coccidioides immitis – Coccidioidomycosis in dogs, horses and humans; respiratory infection may follow inhalation of arthroconidia

Sporothrix schenckii – Sporothrichosis in horses, dogs, cats and humans; fungal spores usually enter through skin abrasions on lower limbs, producing a chronic cutaneous or lymphocutaneous disease

Fungus-like organisms

Chapter 48

Cause sporadic infections in animals and humans; found mainly in bodies of stagnant water; *Pythium insidiosum* grows on a variety of media; *Rhinosporidium seeberi* and *Lacazia loboi* have not been cultured *in vitro*

Pythium insidiosum – Causes pythiosis, rare, sporadic condition mainly in tropical and subtropical regions; in horses, cutaneous lesions on limbs which often ulcerate; canine infections are usually associated with the gastrointestinal tract

Rhinosporidium seeberi – Rhinosporidiosis, nasal polyps, which may project from the nares; cutaneous lesions may be single or multiple; lesions are highly vascular

Lacazia loboi – Nodular or verrucose dermal lesions in humans in tropical regions and in dolphins around the coast of South America, Florida and the Gulf of Mexico

Pathogenic algae and cyanobacteria

Chapter 52

Algae, saprophytic organisms; widely distributed in the environment especially in water; some species implicated in diseases of animals; cyanobacteria form "algal" blooms on fresh water surfaces

Prototheca species widely distributed in sewage and organic matter; *P. wickerhamii*, cutaneous infections in dogs and cats; *P. zopfii*, disseminated disease in dogs, mastitis in dairy cattle

Chlorella species, green algae, occasionally associated with lymphandenitis in ruminants

Cyanobacteria, form "algal" blooms on fresh water surfaces, poisoning occurs in animals drinking contaminated water; hepatotoxins and neurotoxins produced affect fish, birds and mammals

Pneumocystis carinii

Chapter 49

Member of the kingdom Fungi; *in vitro* propagation has proved difficult; organism has been found in a wide range of domestic animals; human infection associated with severe immunosuppression

Strains of *P. carinii* from different animal species have distinct molecular and antigenic profiles

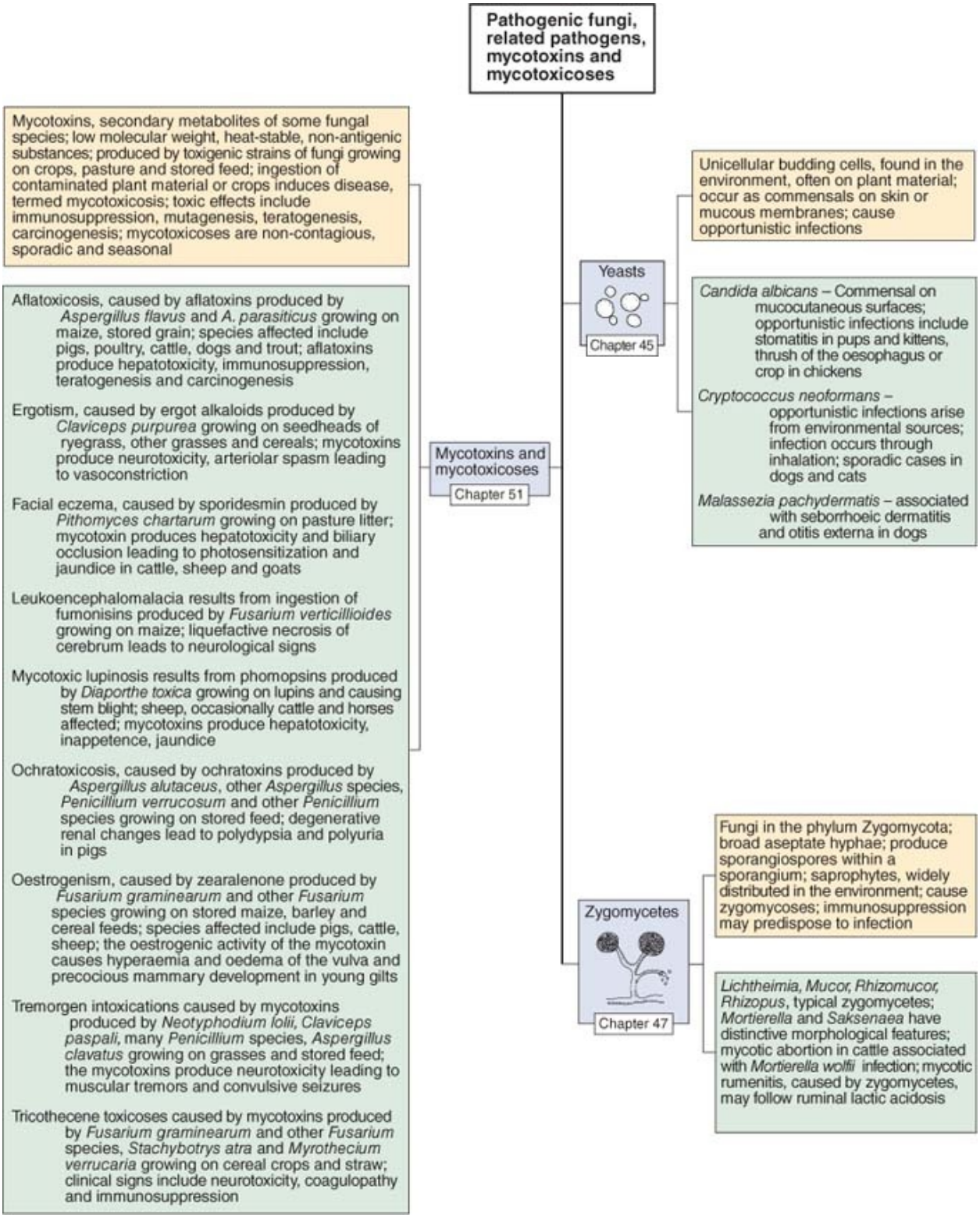
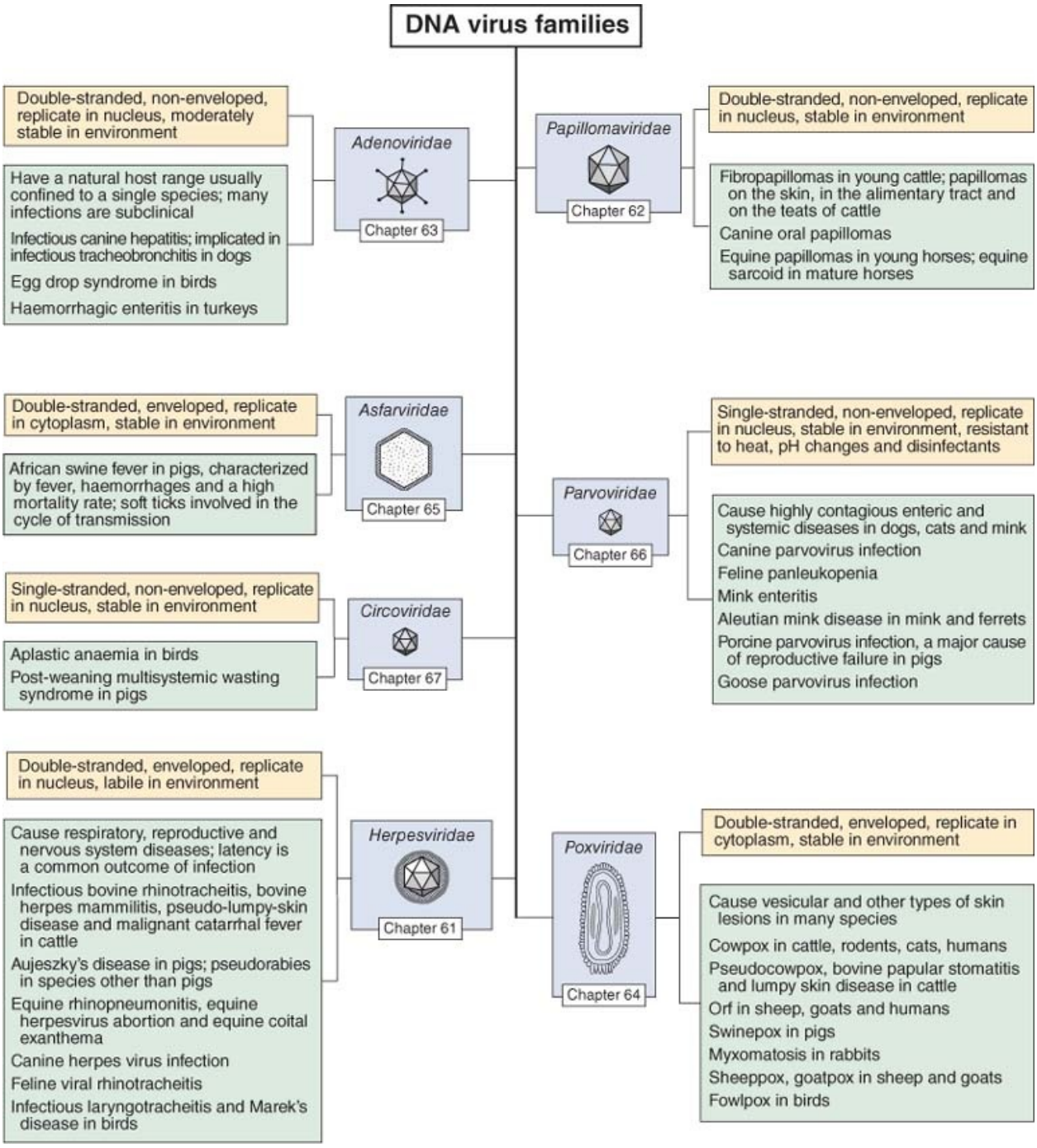


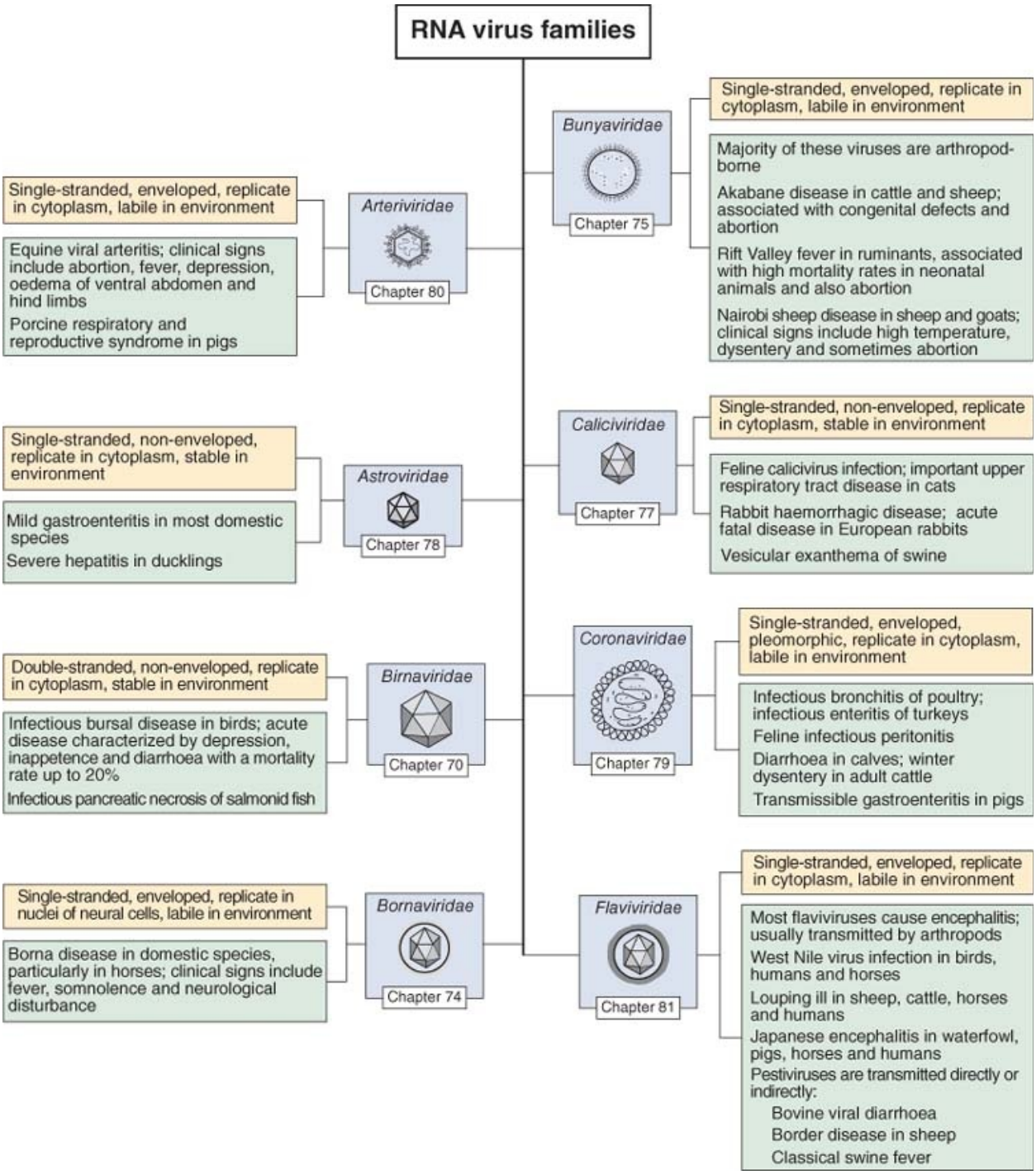
Figure 84.5 Families of DNA viruses arranged alphabetically, with a brief summary of their important features, the diseases which they cause and the relevant chapter numbers.

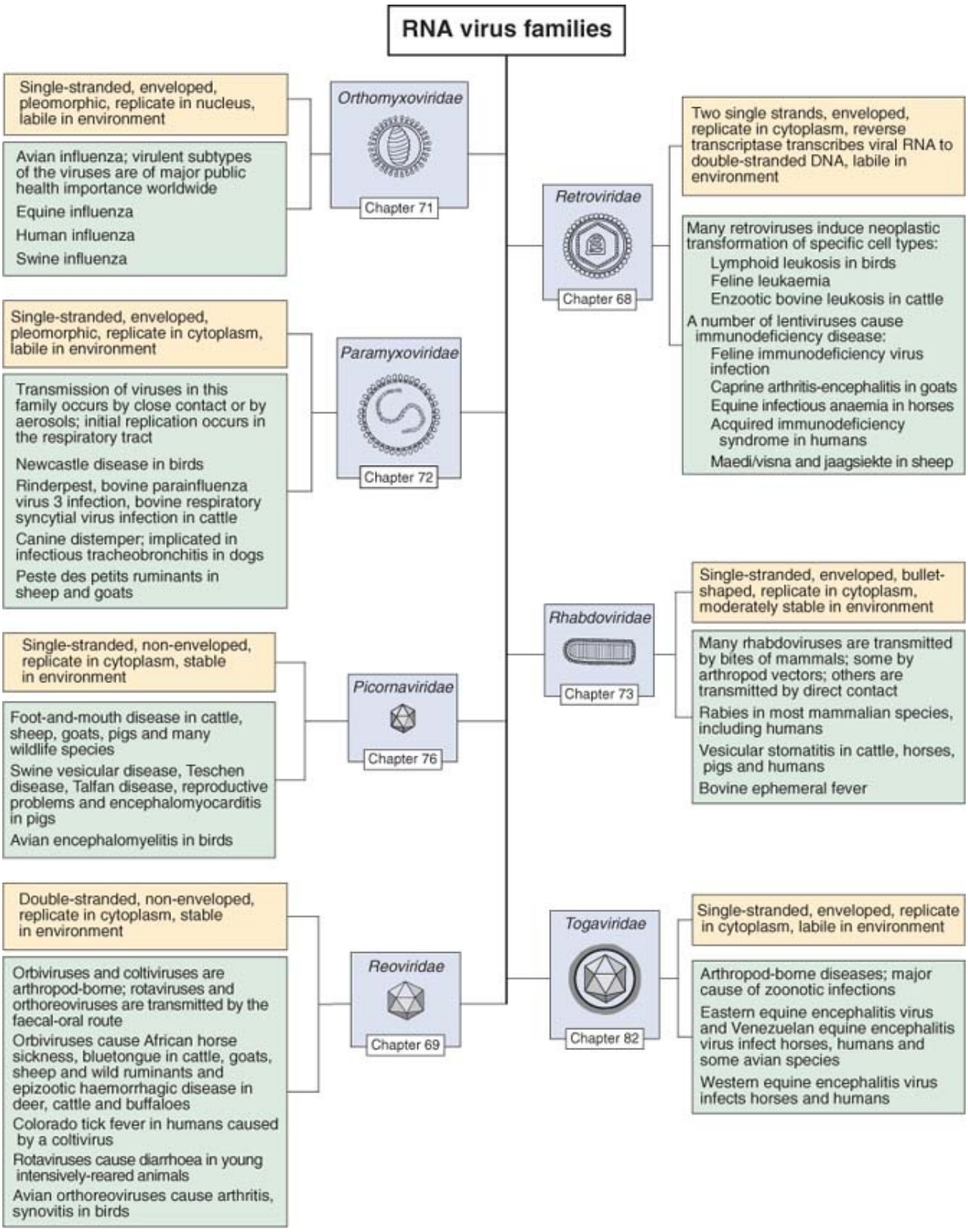


Many infectious agents of animals are of major public health importance. Among the *Enterobacteriaceae*, *Salmonella* serotypes, many of animal origin, are a constant source of human infection, often acquired by consumption of contaminated food items including ice cream, peanut butter, cereals and a range of processed foods (Maki, 2009). A number of viruses belonging to the *Orthomyxoviridae* infect birds, horses, humans and pigs. Some virulent subtypes of influenza A virus, which are well established pathogens in animal

populations, have also been implicated in human infections. A subtype of influenza A virus which caused avian influenza in the late 1990s also infected humans causing a limited number of deaths. More recently, a subtype of influenza A with genetic similarities to swine influenza isolates circulating in the pig population spread to the human population in Mexico and resulted in human-to-human transmission of the virus in a number of countries. West Nile virus, a member of the *Flaviviridae*, was first identified in Africa. It has spread throughout the world and is now the leading cause of human arboviral encephalitis in the United States (Trevejo and Eidson, 2008).

Figure 84.6 Families of RNA viruses arranged alphabetically, with a brief summary of their important features, the diseases which they cause and the relevant chapter numbers.


RNA virus families
Bunyaviridae
Chapter 75
Single-stranded, enveloped, replicate in cytoplasm, labile in environment
Majority of these viruses are arthropod-borne
Akabane disease in cattle and sheep; associated with congenital defects and abortion
Rift Valley fever in ruminants, associated with high mortality rates in neonatal animals and also abortion
Nairobi sheep disease in sheep and goats; clinical signs include high temperature, dysentery and sometimes abortion
Arteriviridae
Chapter 80
Single-stranded, enveloped, replicate in cytoplasm, labile in environment
Equine viral arteritis; clinical signs include abortion, fever, depression, oedema of ventral abdomen and hind limbs
Porcine respiratory and reproductive syndrome in pigs
Caliciviridae
Chapter 77
Single-stranded, non-enveloped, replicate in cytoplasm, stable in environment
Feline calicivirus infection; important upper respiratory tract disease in cats
Rabbit haemorrhagic disease; acute fatal disease in European rabbits
Vesicular exanthema of swine
Astroviridae
Chapter 78
Single-stranded, non-enveloped, replicate in cytoplasm, stable in environment
Mild gastroenteritis in most domestic species
Severe hepatitis in ducklings
Coronaviridae
Chapter 79
Single-stranded, enveloped, pleomorphic, replicate in cytoplasm, labile in environment
Infectious bronchitis of poultry; infectious enteritis of turkeys
Feline infectious peritonitis
Diarrhoea in calves; winter dysentery in adult cattle
Transmissible gastroenteritis in pigs
Birnaviridae
Chapter 70
Double-stranded, non-enveloped, replicate in cytoplasm, stable in environment
Infectious bursal disease in birds; acute disease characterized by depression, inappetence and diarrhoea with a mortality rate up to 20%
Infectious pancreatic necrosis of salmonid fish
Flaviviridae
Chapter 81
Single-stranded, enveloped, replicate in cytoplasm, labile in environment
Most flaviviruses cause encephalitis; usually transmitted by arthropods
West Nile virus infection in birds, humans and horses
Louping ill in sheep, cattle, horses and humans
Japanese encephalitis in waterfowl, pigs, horses and humans
Pestiviruses are transmitted directly or indirectly:
Bovine viral diarrhoea
Border disease in sheep
Classical swine fever
Bornaviridae
Chapter 74
Single-stranded, enveloped, replicate in nuclei of neural cells, labile in environment
Borna disease in domestic species, particularly in horses; clinical signs include fever, somnolence and neurological disturbance



Succeeding chapters in this section deal with the role of microbial pathogens in diseases which affect particular tissue structures or body systems. This

approach provides a clinically relevant perspective on the association between infectious agents and the diseases that they produce with emphasis on the pathogenesis of the diseases they cause and the nature and distribution of lesions in the tissues or systems affected.

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Chapter 85

Interactions of microbial pathogens with the nervous system

Microbial pathogens can damage the nervous system by invasion and replication in the tissues, by provoking immune-mediated reactions and by elaborating toxins. The species, age and immune status of the host and the nature, dose, tropism and virulence of the infectious agent are important determinants in pathogenesis. Additional factors of particular significance which influence clinical and epidemiological features in nervous disorders include the routes of entry and spread of the aetiological agents, and the anatomical areas and cell types targeted. The route of entry, whether through the bloodstream, along the nerves or through tissues by local extension, frequently determines the anatomical location of lesions and, consequently, the neurological signs observed.

Haematogenous bacterial infections

Bacterial invasion of the central nervous system (CNS) can occur via the bloodstream in generalized diseases (Table 85.1). It can also occur when there is local extension from a septic focus of infection caused by pyogenic organisms, such as staphylococci or *Arcanobacterium pyogenes*, resulting in neural abscessation. In acute, generalized bacterial diseases, widespread vascular damage is common. This vascular damage may result from replication of agents such as rickettsiae in endothelial cells, from the action of bacterial toxins, or through immune-mediated mechanisms. Irrespective of the nature of initiating mechanisms, the subsequent mural inflammatory reaction, which may be accompanied by thrombosis, leads to parenchymal degeneration and necrosis.

Cerebrospinal leptomeningitis is a common sequel to haematogenous bacterial invasion of the CNS. It is usually purulent although mononuclear cells may sometimes predominate. Pleocytosis in the cerebrospinal fluid (CSF) is an important indicator of bacterial meningitis. Moreover, circulation of CSF

contributes to the diffuse distribution of the meningeal lesions through surface spread of aetiological agents. The development of concurrent ventriculitis can lead to increased intracranial pressure. Suppurative cerebrospinal leptomeningitis, which is rare in dogs and cats, is frequently encountered in newborn farm animals. A major contributory factor to its occurrence in this group is colostrum deprivation. Lack of maternally- derived antibody allows septicaemic spread of coliform bacteria, streptococci and other opportunistic organisms from the intestines and umbilical vessels. Polyserositis and polyarthritis are additional lesions which are almost invariably present in animals with bacterial leptomeningitis.

The widespread distribution of lesions in most bacterial haematogenous infections of the CNS results in non-specific neurological signs. Although neurological disturbance may be the most prominent presenting sign, dysfunction of other organ systems is usually present.

Haematogenous viral infections

In systemic viral diseases, replication of the aetiological agents usually occurs in lymphatic tissues prior to the development of viraemia. When encephalitis develops, it is generally non-suppurative and can primarily affect either the vasculature or the neural parenchyma.

Table 85.1 Haematogenous bacterial diseases with systemic clinical signs including neurological dysfunction.

Disease	Pathogen	Susceptible species
Bastard strangles	*Streptococcus equi*	Foals
Canine monocytic ehrlichiosis	*Ehrlichia canis*	Dogs
Glasser's disease	*Haemophilus parasuis*	Pigs
Heartwater	*Ehrlichia ruminantium*	Cattle, sheep, goats
Lyme disease	*Borrelia burgdorferi* sensu lato	Dogs
Ovine ehrlichiosis	*'Ehrlichia ovina'*	Sheep, goats
Rocky Mountain spotted fever	*Rickettsia rickettsii*	Dogs
Salmonellosis	*Salmonella* Choleraesuis	Pigs (weaners)
Salmon poisoning disease	*Neorickettsia helminthoeca*	Dogs
Sporadic bovine encephalomyelitis	*Chlamydophila pecorum*	Cattle
Streptococcal meningitis	*Streptococcus suis*	Pigs (young)
Thrombotic meningoencephalitis	*Histophilus somni*	Cattle
Tick pyaemia	*Staphylococcus aureus*	Sheep (lambs)

A number of different pathogenetic mechanisms may contribute to the

development of vascular lesions in multi-systemic viral diseases. The endotheliotropic viruses of swine fever and infectious canine hepatitis produce necrosis of endothelial cells resulting in capillary damage, haemorrhage and lymphocytic infiltration of vessel walls. In contrast, the immunopathological mechanisms involved in the pathogenesis of feline infectious peritonitis result in fibrinoid degeneration in vessel walls and a mixed cell reaction which includes neutrophils. Fibrinoid degenerative vasculitis is also found in malignant catarrhal fever (MCF) but the contribution of immunopathological mechanisms to the pathogenesis of MCF has not been clearly established. In canine distemper and canine herpesvirus 1 infection, discrete lesions are present in the parenchyma of many organs including the CNS, with minimal evidence of vasculopathy. Non-suppurative meningoencephalitis has been reported in swine vesicular disease and in encephalomyocarditis virus infection of piglets.

Table 85.2 Viraemic diseases with generalized clinical signs including neurological dysfunction.

Disease	Virus	Susceptible species
African swine fever[a]	African swine fever virus	Pigs
Canine distemper	Canine distemper virus	Dogs
Canine herpesvirus 1 infection	Canine herpesvirus 1	Dogs (pups)
Classical swine fever[a]	Classical swine fever virus	Pigs
Encephalomyocarditis virus infection	Encephalomyocarditis virus	Pigs
Equine herpesvirus 1 infection	Equine herpesvirus 1	Horses
Feline infectious peritonitis[a]	Feline coronavirus	Cats
Infectious canine hepatitis[a]	Canine adenovirus	Dogs
Malignant catarrhal fever	Alcelaphine herpesvirus 1, ovine herpesvirus 2	Cattle

a, vascular lesions predominate.

In common with generalized bacterial diseases affecting the CNS, the diffuse distribution of the viral lesions produces non-specific clinical signs. Malfunction of other organ systems may induce signs of greater relevance for clinical diagnosis. Viraemic diseases in this category are listed in Table 85.2.

When the neural parenchyma is specifically targeted in viraemic disease, the principal clinical presentations relate to neurological dysfunction (Table 85.3). The location of lesions in grey matter in these viral diseases can vary widely. For example, the cerebral cortex is the principal location for lesions in the equine encephalitides, whereas brain stem and spinal cord are target regions in Teschen–Talfan disease. As a result, the clinical presentations range from blindness, compulsive walking and depression in horses with western equine

encephalomyelitis to spinal ataxia and paraplegia in pigs with Teschen–Talfan disease. Although obvious morphological evidence of vascular damage is not found in these conditions, perivascular mononuclear infiltration is common. Additionally, endothelial swelling and microthrombi have been described in the equine encephalitides.

Table 85.3 Viraemic diseases in which neurological dysfunction is the main clinical presentation.

Disease	Virus	Susceptible species
Borna disease	Borna disease virus	Horses, sheep rarely
Eastern equine encephalomyelitis	Eastern equine encephalitis virus	Horses, other *Equidae*
Louping ill	Louping ill virus	Sheep, cattle, horses, dogs, goats
Teschen–Talfan disease	Porcine teschovirus 1	Pigs (post-weaning)
Tick-borne encephalitis	Tick - borne encephalitis virus	Sheep
Venezuelan equine encephalomyelitis	Venezuelan equine encephalitis virus	Horses, other *Equidae*
Western equine encephalomyelitis	Western equine encephalitis virus	Horses, other *Equidae*

Table 85.4 Bacterial and viral diseases in which invasion of the central nervous system occurs via nerves.

Disease	Agent	Susceptible species
Listeriosis	*Listeria monocytogenes*	Sheep, cattle, goats, llamas
Aujeszky's disease (pseudorabies)	Porcine herpesvirus 1	Pigs and many domestic species
Rabies	Rabies virus	All domestic species especially dogs and cats
Vomiting and wasting disease	Porcine haemagglutinating encephalomyelitis virus	Pigs (suckling piglets)

Infection via peripheral nerves

Microbial pathogens, which can invade the CNS via peripheral nerves, are listed in Table 85.4. The clinical signs produced by infections with these agents are often attributable to malfunction of specific areas of the brain and associated nerves. For example, invasion by *Listeria monocytogenes* along cranial nerves from sites of infection in the oral cavity in ruminants results in characteristic microabscesses in the medulla and other parts of the brain stem. The local tissue destruction, which is often asymmetrical in distribution, can result in unilateral facial paralysis with drooping of the eyelid, lip and ear. The coronavirus which causes vomiting and wasting disease of piglets is also considered to induce a characteristic clinical response by affecting specific areas of the nervous system. After initial replication in respiratory, alimentary or pharyngeal epithelia, virus spreads in peripheral nerves to invade ganglia such as the sensory vagal nucleus in the medulla and the myenteric plexus of the gastric wall. Replication in these

sites results in vomiting and constipation.

The sites of viral replication and the intra-axonal mode of transport in rabies contribute not only to the clinical signs but also to the length of the incubation period and to the spread of the disease. Significant variation in incubation periods, observed in individual cases, has been attributed to the different distances between the point of introduction of the virus and the brain, and to the rate of viral replication in myocytes at the point of introduction. In addition, intra-axonal transport of the virus to the CNS is relatively slow and this may correlate with a prolonged incubation period. In the CNS, the virus replicates extensively in the hippocampus and other parts of the limbic system, often leading to aggressive behavioural changes which are of particular significance in carnivores. Centrifugal spread of virus from the CNS along nerves produces infection of other organs, including the salivary glands. Replication of the virus in the salivary epithelium with consequent contamination of secretions is responsible for infection of the bite wounds inflicted by the rabid animal.

Although neurotropism is less marked with porcine herpesvirus 1 than with rabies virus, infection of the CNS in susceptible pigs is usually considered to occur along the axoplasm of cranial nerves from infected oronasal epithelia. Latency has been demonstrated in pigs with Aujeszky's disease; the virus persists in ganglia and tonsillar tissues. Latent carriers shed virus intermittently in oronasal secretions and can be responsible for spread of infection in pig herds. Intense skin irritation, which can lead to self-mutilation, is a feature of pseudorabies, particularly in cattle and sheep, although it may occasionally occur in dogs and cats. Pruritus develops when virus reaches the segment of cord supplying the affected area.

Infections with lentiviruses

Neurological dysfunction has been described as an uncommon clinical feature in a number of diseases caused by lentiviruses (Table 85.5). The development of clinical signs is usually insidious and prolonged in these entities. In visna and in caprine arthritis- encephalitis (CAE), randomly distributed periventricular demyelination is the most prominent neuropathological feature in the brain. Demyelination in the spinal cord is multifocal and subpial in distribution. The pathogenesis of the demyelination is obscure. Neurological dysfunction in visna rarely affects sheep less than 2 years of age and manifests as muscular tremors and ataxia progressing slowly to paresis and paralysis. In contrast, neurological

dysfunction in CAE develops most commonly in kids at about 3 months of age, with rapid progression from spinal ataxia to paralysis within weeks. The clinical features of CNS involvement in feline immunodeficiency virus infection include dementia and other behavioural disturbances suggestive of cerebral lesions. In equine infectious anaemia, spinal ataxia is a rare clinical presentation which can be attributed to granulomatous encephalomyelitis.

Table 85.5 Diseases caused by lentiviruses in which neurological dysfunction may occur.

Disease	Main target organs	Neuropathology
Caprine arthritis-encephalitis	Joints, mammary glands, lungs	Non-suppurative encephalitis, demyelination, leukomalacia (kids)
Equine infectious anaemia	Macrophages and lymphoid cells	Granulomatous leptomeningitis and ependymitis
Feline immunodeficiency	Lymphatic tissues	Non-suppurative meningoencephalitis
Maedi / visna	Lungs (maedi, progressive pneumonia of sheep), mammary glands, joints	Non-suppurative meningitis and encephalomyelitis, demyelination (visna)

Viral infections which cause developmental anomalies

Viruses that cause developmental anomalies in the CNS of domestic animals are listed in Box 85.1. The susceptibility of the developing nervous tissues to the destructive effects of these viruses is closely related to the stage of gestation at the time of infection. Destruction of germinal cells by these viruses results in teratological defects such as cerebral cavitation and cerebellar hypoplasia. Teratological changes may follow intrauterine infection with pestiviruses such as classical swine fever virus, bovine viral diarrhoea virus and border disease virus. Hypomyelinogenesis, another developmental defect in lambs with Border disease, is attributed to delayed maturation of oli- godendrocytes. Lambs with tremors typical of the condition can recover with careful management.

Box 85.1Viruses with teratogenic effects on nervous tissues.

- Akabane virus
- Border disease virus
- Bovine herpesvirus 5
- Bovine virus diarrhoea virus

- Cache Valley virus
- Feline panleukopenia virus
- Classical swine fever virus

Transmissible spongiform encephalopathies (TSEs)

Spongiform encephalopathies are so called because of the characteristic vacuolation of the neural parenchyma seen in affected animals. These neurodegenerative diseases have been described in a number of domestic and captive animal species (see Chapter 83). They have many common features including the nature of the aetiological agent, transmissibility, and long incubation periods and clinical courses.

It is widely accepted that the aetiological agents, prions, are structurally modified forms of protein normally present on cell membranes. The structural change, initiated post-translationally in the normal protein through association with abnormal prion protein, results in accumulation of these abnormal molecules. In the abnormal protein, α-helices are largely replaced by β-sheets which resist enzymatic digestion. This conformational change allows polymerization, often demonstrable as amyloid plaques in the brain tissue of some affected species. Scrapie- associated fibrils containing the abnormal protein can be demonstrated in extracts of brain tissues from animals with spongiform encephalopathy and serve as markers of the disease.

There is strong experimental evidence in mice and sheep to support the view that, after ingestion and processing in regional lymph nodes, spread of the aetiological agent to the spinal cord is via the splanchnic nerves. Although there are differences in lesion distribution between the various spongiform encephalopathies, major vacuolar changes are found in the brain stem, particularly in the medulla, in all affected animals. In the later stages of the disease, diffuse astrogliosis may be present. Clinical signs, though somewhat variable, usually relate to loss of motor control and to behavioural changes.

Algal, bacterial and fungal neurotoxicity

Diseases caused by bacterial agents which elaborate toxins affecting neurological function are listed in Table 85.6. In the clostridial diseases,

botulism and tetanus, the toxins affect neuromuscular function. In botulism, following ingestion of preformed toxin, there is flaccid paralysis as a result of blocking of acetylcholine release at neuromuscular junctions. The tetanus toxin, elaborated by organisms in an infected wound, blocks inhibitory signals from the CNS with resultant muscular spasms. Morphological tissue changes are absent in botulism and tetanus.

Focal symmetrical encephalomalacia and oedema disease are caused by toxins elaborated in the intestinal tract by replicating *Clostridium perfringens* type D and certain strains of *Escherichia coli*, respectively. These toxins usually produce acute disease with sudden or rapid death. Degenerative lesions relating to vascular damage develop in animals that survive the acute phase and there is progressive neurological disturbance.

Ingestion of preformed neurotoxin is the main method of exposure in toxicoses of algal and fungal origin (Table 85.7). Neurological signs may follow ingestion of fungal toxins in grasses of the *Paspalum* genus on which *Claviceps paspali* is growing. Similar neurological signs follow the ingestion of lolitrems in perennial rye grass contaminated with *Neotyphodium lolii* and feed or pasture contaminated by tremorgens produced by other fungi. Affected animals have fine head tremors at rest and may show incoordination, stiffness or muscular spasms if forced to move. Mortality is low and recovery occurs shortly after removal from affected pasture or withdrawal of contaminated feed. The clinical signs in equine leukoencephalomalacia are more severe and include depression, blindness, pharyngeal paralysis and staggering. Death follows within a few days.

Table 85.6 Neurological diseases produced by bacterial toxins.

Disease	Bacterium / Method of exposure	Toxic effects	Species affected
Botulism	*Clostridium botulinum* / ingestion of preformed toxin – toxigenic form. Toxin produced in infected wound or intestine – toxicoinfectious form (rare)	Blocking of acetylcholine release at neuromuscular junctions	Many species
Focal symmetrical encephalomalacia	*Clostridium perfringens* type D / Enterotoxaemia	Vasculopathy, encephalomalacia in midbrain and basal ganglia	Sheep (lambs), goats
Oedema disease (cerebrospinal angiopathy)	Strains of *Escherichia coli* producing oedema disease / Toxin produced in intestine and absorbed into bloodstream	Vasculopathy, fibrinoid necrosis of arteriolar walls, encephalomalacia	Pigs
Tetanus	*Clostridium tetani* / Toxin produced locally in infected tissue	Blocking of presynaptic transmission of inhibitory signals from neurons in CNS	Most species, especially horses and sheep

Table 85.7 Neurological diseases caused by algal and fungal toxins.

Disease	Microbial agent / Method of exposure	Toxic effects	Species affected
Blue-green algal toxicoses	Cyanobacteria / Preformed toxins ingested with water	Mimics action of acetylcholine	Many species
Aspergillus clavatus toxicosis	*Aspergillus clavatus* / Feed containing preformed toxins	Chromatolysis in neurons of brainstem, spinal ganglia and cord, Wallerian degeneration in cord	Cattle, sheep
Equine leukoencephalomalacia	*Fusarium verticillioides* / Corn-based feed containing preformed toxins	Vasculitis, perivascular oedema, malacia	Horses
Tremorgen intoxication			
Paspalum staggers	*Claviceps paspali* / Ingestion of sclerotia on paspalum grasses	Interference with neuromuscular function causing tremors	Ruminants, horses
Penitrem staggers	*Penicillium crustosum* and other *Penicillium* species / Ingestion of contaminated pasture	Clinical effects similar to paspalum staggers	Cattle, sheep
Perennial ryegrass staggers	*Neotyphodium lolii* / Ingestion of contaminated ryegrass stubble	Clinical effects similar to paspalum staggers	Ruminants, horses

Algal and fungal infections

Algal and fungal infections which may affect the nervous system are listed in Box 85.2. Although lesions produced by the fungi are generally located in the respiratory tract, infection of the CNS may occasionally occur. A defective immune response, immunosuppressive therapy or prolonged administration of antibiotics predispose to tissue invasion by fungi. Infection with the algal agent, *Prototheca zopfii*, may occasionally spread to the CNS from a primary site in the intestines.

Box 85.2 Algal and fungal infections which may affect the nervous system.

- Aspergillosis
- Blastomycosis
- Coccidioidomycosis
- Cryptococcosis
- Histoplasmosis
- Protothecosis

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Chapter 86

Interactions of microbial pathogens with the male and female reproductive systems

Microbial agents represent a relatively small proportion of the aetiological factors which can affect reproductive performance in domestic animals (Box 86.1). Protozoal diseases, such as ovine toxoplasmosis and bovine neosporosis, can be a greater threat to foetal survival than bacterial, fungal or viral infections. When taken together, microbial and protozoal agents account for less than 30% of the diagnoses recorded in many surveys of abortion in farm animals. Chromosomal, hormonal, nutritional, toxic and physical factors may account for large numbers of unspecified embryonic and foetal deaths. Certain microbial infections are, nevertheless, important causes of reduced reproductive performance in many countries.

The development and functional integrity of the male and female reproductive systems in domestic animal species are dependent on delicately balanced hormonal interactions. In male animals, the clinical effects of microbial infections on reproductive performance relate largely to tissue destruction and associated anatomical alterations. In contrast, microbial infections of the female tract can disrupt the hormonal interactions which influence tissue and behavioural changes during oestrous cycles, and those which are essential for the maintenance of pregnancy.

Infections of the male reproductive system

Microbial infections of the male reproductive system in domestic animals may lead to the development of lesions which adversely affect fertility. In addition, venereal spread of infection by an infected male may have a serious impact on the reproductive performance of susceptible females. In some venereal infections, such as those involving bovine herpesvirus 1 (infectious pustular vulvovaginitis) and equine herpesvirus 3 (equine coital exanthema), lesions are usually confined to the mucosal surfaces of the penis, vulva and vagina. Other,

more serious venereal infections may cause metritis or abortion.

Infections which produce inflammation of the penis and prepuce (balanoposthitis) are rarely of major clinical significance. Balanoposthitis, caused by herpesvirus infections in bulls and stallions, can result in ulcerative lesions which may be extensive but resolve spontaneously within a few weeks. A diverse population of bacterial, fungal and protozoal species is present in the prepuce in many domestic animals. Some of these microorganisms are potentially pathogenic and, in a suitable microenvironment, selective overgrowth of a species can result in clinical disease. The development of ulcerative balanoposthitis due to the activity of *Corynebacterium renale* in wethers and rams on high protein diets is an example of the effect of this type of microenvironmental influence.

Primary testicular infections are usually haematogenous in origin, whereas those involving the epididymis generally originate in the urogenital tract. Infection, once established in the scrotal sac, may spread to involve both testis and epididymis. Inflammations of the testis (orchitis) and of the epididymis (epididymitis) are often concurrent. Among the bacterial pathogens which affect the male reproductive system, *Brucella* species have a particular predilection for testicular and epididymal tissues in the bull, boar, ram and dog. In bulls, infection with *B. abortus* produces an acute orchitis leading to tissue necrosis. In rams, infection with *B. melitensis* also results in orchitis, whereas epididymitis is the main effect of infection with *B. ovis*. The epididymis is also the primary target of *Actinobacillus seminis* and *Histophilus somni* infections in rams. Multifocal suppurative orchitis in boars, caused by *B. suis*, is often accompanied by lesions in the epididymis. Concurrent orchitis and epididymitis are present also in *B. canis* infection in dogs. In addition to *Brucella* species, other bacterial agents which produce orchitis and epididymitis in bulls include *Escherichia coli* and *Salmonella* serotypes. *Burkholderia mallei* can produce testicular lesions in boars and dogs. *Arcanobacterium pyogenes* is an important cause of orchitis in bulls, boars and rams. Lesions involving testicular tissues of stallions have been described in glanders and in infections with *Salmonella* Abortusequi. Viral infections in stallions in which orchitis may be a feature include equine viral arteritis and equine infectious anaemia. Orchitis and epididymitis have been reported in canine distemper virus infection in dogs.

Box 86.1Factors which may adversely affect

reproductive performance in domestic animals.

- Microbial agents
  – Bacteria
  – Fungi
  – Viruses
- Parasitic agents
  – Protozoa
- Anatomical defects
- Genetic factors and developmental defects
- Hormonal imbalance, constitutive or induced
- Nutritional deficiencies
- Physical injury
- Toxic agents including mycotoxins

In bulls, microbial infections caused by *Arcanobacterium pyogenes*, staphylococci, streptococci and *Brucella abortus* often result in seminal vesiculitis. The seminal vesicles of the bull are also considered to be a major site for the localization of *Leptospira interrogans* serovar Hardjo. In the dog, urinary pathogens such as *E. coli* and *Proteus* species can invade the prostate as part of an ascending infection through the urethra.

Infections of the non-pregnant uterus

Although the non-pregnant uterus is relatively resistant to infection, susceptibility to pathogens varies during the oestrous cycle. In early oestrus, uterine motility increases under the influence of oestrogens, contributing to mechanical expulsion of potential pathogens. In addition, neutrophils in the uterine lumen appear to be particularly active during this phase of the cycle. The uterus becomes more vulnerable to infection in dioestrus when progesterone secretion from the corpus luteum (CL) increases. During this phase, the phagocytic activity of neutrophils in the uterine lumen is reduced and immunosuppressive products are secreted into the lumen. Moreover, experimental studies have demonstrated an increased susceptibility of the progesterone-stimulated endometrium to opportunistic pathogens.

Box 86.2 Microbial pathogens which can be

transmitted by venereal contact.

- **Cattle**
  – Bovine herpesvirus 1
  – *Brucella abortus* (rare)
  – *Campylobacter fetus* subsp. *venerealis*
  – *Chlamydophila abortus*
  – *Leptospira interrogans* serovars
  – *Mycoplasma bovigenitalium*
  – *Ureaplasma diversum*
- **Horses**
  – Equine herpesvirus 3
  – Equine arteritis virus
  – *Klebsiella pneumoniae*
  – *Pseudomonas aeruginosa*
  – *Taylorella equigenitalis*
- **Sheep**
  – *Brucella ovis*
  – *Brucella melitensis* (rare)
  – *Chlamydophila abortus* (rare)
- **Pigs**
  – *Brucella suis*
  – Porcine reproductive and respiratory virus
  – Porcine herpesvirus 1
  – Porcine parvovirus
- **Dogs**
  – *Brucella canis*
  – Canine herpesvirus 1

Many of the microbial agents which invade the nonpregnant uterus can be transmitted by venereal contact (Box 86.2). The outcome of postcoital infection, even if caused by agents specifically capable of inducing uterine disease, is usually a mild short-lived endometritis. Opportunistic pathogens, such as *A. pyogenes*, *E. coli* and streptococci, are transient inhabitants of the vagina in many species and are often associated with postcoital endometritis. The mare appears to be particularly susceptible to postcoital endometritis because the marked relaxation of the cervix during oestrus facilitates the introduction of opportunistic pathogens from the vulva, the vagina or the external genitalia of

the stallion.

Box 86.3 Microbial pathogens implicated in postpartum metritis of farm animals.

- **Cattle**
  – *Arcanobacterium pyogenes*
  – *Bacteroides* species
  – *Brucella abortus*
  – *Campylobacter fetus* subsp. *venerealis*
  – *Escherichia coli*
  – *Fusobacterium necrophorum*
  – Haemolytic streptococci
  – *Proteus* species
  – *Pseudomonas aeruginosa*
  – *Staphylococcus* species
- **Horses**
  – *Bacteroides* species
  – *Clostridium* species
  – *Escherichia coli*
  – *Klebsiella* species
  – *Proteus* species
  – *Pseudomonas* species
  – *Staphylococcus aureus*
  – *Streptococcus zooepidemicus*
- **Sheep**
  – *Fusobacterium necrophorum*
  – *Salmonella* serotypes
  – *Streptococcus* species
- **Pigs**
  – Opportunistic Gram-negative bacteria
  – *Staphylococcus* species

The uterus and uterine tubes are especially vulnerable to infection immediately following parturition. Retention of the placenta and trauma resulting from difficult parturitions are important factors contributing to the development of post-partum metritis and salpingitis. Abortion, both infectious and noninfectious, is often followed by retention of the placenta and delayed uterine involution

which allow access for opportunistic pathogens through the cervix. Post-partum metritis can follow infection with a wide range of bacteria, some of which may also be responsible for placentitis and abortion (Box 86.3). These infections, which may be mixed, often resolve spontaneously. However, when severe, infection can result in death from toxaemia. Moreover, a chronic metritis may develop, characterized by persistent inflammatory exudation into the uterine lumen.

Pyometra

In cattle, post-partum bacterial metritis can progress to pyometra, the accumulation of pus in the uterus. In this condition the diseased endometrium produces insufficient prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a luteolytic factor normally responsible for regression of the corpus luteum (CL). The persistent CL continues to secrete progesterone which stimulates hyperplasia of the endometrium and increases its susceptibility to infection. In addition, myometrial activity is inhibited, the cervix remains closed, and pus and uterine secretions accumulate.

Box 86.4 Microbial pathogens implicated in canine pyometra.

- *Escherichia coli*
- Haemolytic streptococci
- *Klebsiella* species
- *Pasteurella* species
- *Proteus* species
- *Pseudomonas aeruginosa*
- *Staphylococcus* species

The sequence of events leading to pyometra in domestic carnivores is somewhat different. Canine pyometra usually occurs in unbred, mature bitches. The disease develops during dioestrus, when the oestrogen-primed endometrium comes under the influence of progesterone. In addition to copious amounts of intraluminal pus, a characteristic cystic endometrial hyperplasia is present. It has been demonstrated experimentally that cystic endometrial hyperplasia can be induced by injury to the endometrium during early dioestrus. The injury may be produced mechanically or by introducing appropriate strains of *E. coli* into the

uterus. In naturally occurring canine pyometra, *E. coli* is the organism most frequently isolated from the abnormal secretions, although other opportunistic pathogens may also be involved (Box 86.4). Endotoxaemia is often encountered in affected bitches, particularly in closed pyometra. In some cases, circulating immune complexes are deposited in renal glomeruli and the resulting glomerulonephritis can lead to renal failure. Renal function may also be compromised by the reduced cortical perfusion associated with endotoxaemic shock.

Pyometra is encountered less commonly in queens than in bitches, and its relationship to endometrial hyperplasia is less clear because evidence of endometrial hyperplasia is present in almost all mature queens.

Infections of the pregnant uterus

Maintenance of early pregnancy in farm animals and horses is largely dependent on secretion of progesterone by the CL. During the oestrous cycle, lysis of luteal tissue follows synthesis and release of $PGF_{2\alpha}$ from the endometrium. Implantation of a normal blastocyst inhibits this endometrial $PGF_{2\alpha}$ production and prevents luteolysis, thus maintaining pregnancy. Death of the embryo results in loss of this inhibitory effect with synthesis and release of $PGF_{2\alpha}$, regression of the CL and return to oestrus. The dead conceptus may be resorbed or expelled at a later stage. Although most early embryonic deaths are considered to be due to chromosomal abnormalities, some are attributable to infections such as *Campylobacter fetus* subsp. *venerealis* in cows and *Klebsiella pneumoniae* in mares. In these uniparous species, expulsion of an early embryo may not be observed, and the only indications of embryonic death may be a slight vaginal discharge and late return to oestrus.

Death of more developed foetuses during the first half of pregnancy in the cow, mare and ewe has variable effects on the persistence of the CL. In some cases, the CL persists and dehydration and mummification of the dead foetus may occur before expulsion. In other instances, lysis of the CL results in relatively rapid expulsion of the dead foetus. In contrast, death of a foetus during the second half of pregnancy in these species is often followed by immediate abortion because foetal hormone production, which is necessary for maintenance of pregnancy, ceases.

Corpora lutea persist throughout gestation in the multiparous bitch, queen and

sow. Mummification develops more often in these species than in uniparous species. In fact, a cluster of clinical features including stillbirths, mummification, embryonic death and infertility (SMEDI) is characteristic of a number of viral infections in pregnant sows. These various clinical presentations in infected sow herds reflect the different stages of development at the time of foetal infection. A similar spectrum of changes in infected foetuses, along with a history of infertility, is seen in viral diseases of other species. In some of these conditions, such as border disease of sheep and bovine viral diarrhoea virus (BVDV) infection, there is also evidence of growth retardation, developmental defects and persistent infection, all of which are related to the gestational age of the foetus at the time of infection. The development of persistent infection with BVDV in cattle, for example, can occur only if infection takes place before foetal immune competence is established.

Microbial infections of cattle and sheep which can result in abortion are indicated in Tables 86.1 and 86.2, respectively. Infections caused by a number of bacterial agents, including *Leptospira interrogans* serovars, *Salmonella* serotypes, *Chlamydophila abortus, Listeria monocytogenes* and *Bacillus licheniformis*, can result in abortion in both species. However, differences between the two ruminant species in susceptibility to a particular agent influence the outcome of infection. *Leptospira interrogans* serovars, especially serovar Hardjo, are important causes of reproductive losses in cattle. Sheep appear to be relatively more resistant to leptospiral infection than cattle, and abortion is a rare occurrence although it may be encountered in ewes in intensive management systems. In bovine leptospiral abortion, pathological changes in foetal tissues and placentae are often absent and isolation of the causal agent is difficult. Serological tests must, therefore, be used to establish the presence of leptospirosis in an infected herd because abortions tend to occur some time after the acute phase of the disease. In contrast, abortions in outbreaks of bovine and ovine salmonellosis occur during the acute phase of the disease when pyrexia and diarrhoea are clinically evident. Although many *Salmonella* serotypes infect domestic ruminants, *Salmonella* Dublin is of particular significance in bovine abortion in many countries. When ruminants abort due to infection with *Salmonella* serotypes, extensive replication of the organisms in the placenta is a major factor leading to foetal expulsion.

Chlamydophila abortus is an important cause of abortion in sheep flocks. Naive ewes, newly introduced into an infected flock, and animals pregnant for the first time are particularly susceptible to infection. Infected animals usually

remain clinically normal until invasion of the placenta in the last month of gestation leads to abortion. Lambs, which are infected shortly after birth, may remain latent carriers until they become pregnant. Placentitis is a prominent feature of infection and, occasionally, multifocal hepatic necrosis is present in infected foetuses.

Abortion caused by *Listeria monocytogenes* appears to be dependent on the ingestion of large numbers of organisms and, in cattle and sheep, is commonly associated with the feeding of poor quality silage which can contain large numbers of these bacteria. Placentitis and foetal infection are recorded in both species, and abortion occurs in the last third of gestation. Infection and retention of a dead foetus close to term can result in dystocia, with subsequent septicaemia or metritis in the dam. Aborted lambs occasionally have numerous small, pale areas of microabscessation throughout the liver.

Abortion associated with *Bacillus licheniformis* is a problem in cows and ewes in Scotland, the north of England and Ireland. The infection is associated with feeding of poor quality silage and mouldy hay. The thickened, leathery appearance of the placenta resembles that observed in mycotic abortion.

Bovine brucellosis, caused by *Brucella abortus*, is the most extensively studied reproductive disease in cattle. The disease is recorded in most parts of the world and, in regions where control measures are ineffective, is usually endemic. Reproductive performance in affected herds is severely impaired, and there is the possibility of zoonotic transfer to the human population. Sexually immature cattle are relatively refractory to infection. In susceptible mature animals, especially females, infection may persist in lymph nodes and other tissues for prolonged periods, without producing clinical disturbance. Because *B. abortus* has a predilection for endometrial and placental tissues, spread of the organism and replication in these tissues occurs during pregnancy. Abortion, the main clinical manifestation of the infection, usually occurs during the seventh or eighth month of gestation. There may be extensive necrotic lesions in cotyledons with oedema of intercotyledonary areas. Organisms can be demonstrated in smears from affected cotyledons and can be cultured from uterine fluids, cotyledons and the abomasal contents of aborted calves.

Table 86.1 Microbial pathogens implicated in bovine abortion.

Agent	Comments
Aspergillus fumigatus Mortierella wolfii	Haematogenous spread to placenta from foci in lungs and elsewhere. Abortion after 7 months' gestation. Placentitis with leathery intercotyledonary areas and necrotic cotyledons. Occasionally, circumscribed pale mycotic plaques on foetal skin

Bacillus licheniformis	Placentitis with dry leathery lesions. Oral infection from poor quality silage, mouldy hay, bedding or feed
Bovine herpesvirus 1	Abortion between 5 and 8 months of gestation. Placentitis, foetal autolysis and multifocal necrosis in foetal liver can occur
Bovine viral diarrhoea virus (BVDV)	Effects, ranging from fertilization failure to abortion and congenital defects, are largely determined by the stage of gestation at time of infection. Foetuses may be fresh, autolysed or mummified. Thymic atrophy, cerebellar hypoplasia. Live calves immunotolerant to BVDV if infected before day 100 of gestation
Brucella abortus	Abortion occurs after mid-gestation. Necrotic placentitis, diffuse endometritis and foetal oedema. Organisms in uterine discharges and milk. Brucellae can spread to placenta from localized chronic foci
Brucella melitensis	Sporadic abortions, excretion in milk
Campylobacter fetus subsp. *venerealis*	Occasional abortions around fifth month of gestation
Chlamydophila abortus	Abortions usually sporadic and occur after 6 months of gestation. Infection usually acquired from sheep. Severe placentitis and endometritis. Foetal lesions include petechiae in thymus and on mucosae and serosae, enlarged liver and ascites. Faecal–oral transmission
Leptospira interrogans serovars	Most abortions occur after 6 months of gestation. Aborted foetuses often autolysed. Calves may be born weak. Organisms spread to placenta and foetus via bloodstream. Transmission through contaminated environment or venereal contact
Listeria monocytogenes	Sporadic abortions during last third of gestation. Retained placenta is common. Oral infection from poor-quality silage
Salmonella Dublin and other serotypes	Sporadic or epidemic abortions. Placentitis. Foetal autolysis and putrefaction. There may be concurrent enteritis
Ureaplasma diversum	Abortions in last third of gestation. Premature birth of weak or dead calves. Retained placenta, placentitis with haemorrhages and necrosis

Of the microbial pathogens capable of causing abortion in sows, viruses are particularly significant (Table 86.3). Viral infections spread readily under intensive husbandry conditions operating in breeding herds and often result in serious economic loss from reproductive failure. The spectrum of clinical presentations has already been mentioned. The SMEDI syndrome of stillbirths, mummification, embryonic death and infertility is a major indicator of viral infection in affected herds. Latency or subclinical infection may allow viral persistence in a herd.

Microbial causes of equine abortion are listed in Table 86.4. Equine herpesvirus 1 (EHV-1) is important worldwide as a cause of abortion, sometimes resulting in abortion storms. A related virus, equine herpesvirus 4, which is mainly responsible for rhinopneumonitis, has also been isolated from aborted foals. Abortions due to EHV-1 usually occur after 7 months of gestation. Multifocal pinpoint necrotic lesions may be evident in the livers of aborted foals. Latent carriers may act as reservoirs of infection.

Table 86.2 Microbial agents implicated in ovine abortion.

Agent	**Comments**
Bacillus licheniformis	Placentitis. Oral infection from poor-quality silage or mouldy hay, bedding or feed

Border disease virus	Effects, ranging from embryonic and foetal death to congenital defects and weak newborn lambs, relate to gestational age when infected. Intrauterine growth retardation. Placentitis
Brucella melitensis	Abortion may be the only evidence of infection. Uterine discharges heavily contaminated. Successive abortions may occur in infected ewes
Brucella ovis	Venereal transmission important. Sporadic abortion. Intercotyledonary tissue thickened and oedematous. Mummification or autolysis of foetus. Epididymitis in affected rams
Campylobacter fetus subsp. *fetus*	Faecal–oral transmission. Localization in pregnant uterus from bacteraemia. Abortion late in gestation. Placentitis and mild enteritis. Umbilicated, pale, necrotic lesions in some foetal livers
Campylobacter jejuni	
Chlamydophila abortus	Enzootic abortion of ewes. Abortion commonly in last month of gestation. Placentitis. Thickening and oedema in intercotyledonary tissue. Foetal livers may be swollen with pinpoint necrotic lesions
Coxiella burnetii	Rare, sporadic abortions occurring late in gestation. Diffuse placentitis. Persistent infection. Shedding of organisms in milk
Listeria monocytogenes	Infection *per os*. Silage often the source. Abortion usually sporadic. Placentitis. Multifocal hepatitis in foetus. Abortion late in gestation may be followed by metritis and septicaemia
Salmonella serotypes	Some *Salmonella* serotypes cause abortion with minor clinical disturbance in the dam. Those due to *Salmonella* Dublin and *Salmonella* Typhimurium can produce systemic signs and abortion. Abortion occurs late in gestation

Table 86.3 Microbial pathogens implicated in porcine abortion.

Agent	Comments
Brucella suis	Mainly venereal transmission. Foci of infection in male and female genitalia, in joints and in bones. Chronic metritis with multiple granulomatous nodules in mucosa. Abortion in second half of pregnancy. Stillborn and weak piglets
Classical swine fever virus	Stillbirths, mummification, embryonic death and infertility (SMEDI) syndrome in affected breeding herds. Foetal growth retardation. Congenital defects in central nervous system. Vaccine strains can produce congenital defects
Encephalomyocarditis virus	One of the main causes of SMEDI syndrome in the USA. Myocarditis in young pigs
Leptospira species (particularly serovars Pomona, Tarassovi and Bratislava)	Abortions late in gestation may be the only indication of infection in a herd. Subclinical infections. Bacteria infect uterus and foetus during leptospiraemia. Stillbirths, mummification, autolysis and weak newborn piglets
Porcine teschoviruses, porcine sapelovirus (porcine enteroviruses)	First viruses to be associated with SMEDI syndrome, but role in abortion probably minor
Porcine herpesvirus 1 (Aujeszky's disease virus)	Abortion secondary to fever and systemic disease. Some strains invade placenta and foetus. Multifocal necrosis of placenta and foetal organs. SMEDI syndrome in affected breeding herds
Porcine herpesvirus 2 (cytomegalovirus)	Subclinical infections in sows. Foetal death and mummification. Necrotizing rhinitis in neonates
Porcine parvovirus	Oral and venereal transmission. SMEDI syndrome in susceptible sows introduced into infected herds. Virus invades rapidly dividing cells in foetus
Porcine reproductive and respiratory virus	Pneumonia and reproductive wastage. SMEDI syndrome in affected breeding herds

Table 86.4 Microbial pathogens implicated in equine abortion.

Agent	Comments
Equine herpesvirus 1 (EHV1)	EHV1 most common cause of equine abortion; EHV4 causes sporadic cases. Abortion after 8 months of gestation. Foetus usually fresh, indicating recent death. Multifocal hepatitis, icterus and pulmonary oedema in foetus
Equine arteritis virus	Over half of infected mares abort or have stillborn offspring. Autolysis and excess pleural and peritoneal fluid in foetus
Leptospira interrogans serovars	Abortion is often a sequel to acute leptospirosis. Large multinucleate hepatocytes in foetal livers
Taylorella equigenitalis	Implicated in abortion at about 7 months' gestation

Table 86.5 Infectious agents which induce abortion in association with systemic disease.

Infectious agents	Hosts
Bacteria	
Coxiella burnetii	Sheep, goats
Anaplasma phagocytophilum	Sheep, cattle
Erysipelothrix rhusiopathiae	Pigs
Streptococcus suis type 2	Pigs
Viruses	
African swine fever virus	Pigs
Akabane virus	Cattle, sheep
Bluetongue virus	Sheep, cattle
Canine herpesvirus 1	Dogs
Ephemeral fever virus	Cattle
Nairobi sheep disease virus	Sheep, goats
Rift Valley fever virus	Sheep, cattle
Wesselsbron disease virus	Sheep, cattle

Brucella canis causes abortion in bitches and is the only microbial pathogen which primarily affects the male and female canine reproductive systems.

Abortions may occur incidentally during many generalized bacterial and viral infections as a consequence of the direct effects of viraemia, septicaemia or toxaemia on maternal and foetal tissues and, indirectly, on the hormonal regulation of pregnancy. Those microorganisms which may induce abortion during generalized infections are listed in Table 86.5.

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Chapter 87

The role of microbial pathogens in intestinal disease

The digestive system is constantly bombarded by organisms derived from environmental sources. In the anterior part of the tract, members of all of the major groups of microorganisms are capable of colonizing the oral and pharyngeal mucosae and, in some instances, can produce defined clinical conditions. Some of these conditions, such as canine oral papillomatosis, are caused by particular infectious agents; others like acute necrotizing ulcerative gingivitis are associated with overgrowth of some members of the resident microbial flora, often as a result of immuno- suppression. Other parts of the upper digestive system are less susceptible to microbial colonization. The stratified squamous epithelium of the oesophageal mucosa and the acidity of the secretion from gastric mucosal glands, along with the layer of mucus covering both oesophageal and gastric or abomasal mucosae, are inimical to microbial colonization. In contrast, the environment of the intestinal tract is particularly suitable for microbial colonization and many enteric organisms have evolved superficial structures which allow attachment to the surface of enterocytes.

Intestinal structure and function

The intestinal tract is the portion of the digestive system which is largely responsible for digestion of food and for absorption of nutrients, water and electrolytes. Although there are considerable species differences in the length and anatomical positioning of the tract, its general structure and function are similar in all domestic animals. The two main parts of the tract, the small and large intestines, differ functionally with regard to digestion and absorption; these functional differences are reflected in structural differences in their mucosal surfaces. In the small intestine, where most digestion and absorption of organic compounds take place, the mucosal surface area is greatly increased by folds and villus formation. In the large intestine, mucosal folding is less prominent, villi

are absent and microvilli are less numerous. In carnivores, the functions of the large intestine are mainly confined to the absorption of water and electrolytes. In herbivores, the metabolic activities of members of the normal microbial flora of the caecum and colon produce nutrients which are absorbed along with the water and electrolytes. Goblet cells, which are present in the mucosal epithelium throughout the intestine, are particularly numerous in the large intestine where mucus secretion is important for faecal lubrication. In all species, after a storage period of 24 to 36 hours, the solid or semisolid contents of the rectum are expelled as faeces.

The integrity of the mucosal epithelium is maintained by replication of undifferentiated cells in the glandular crypts. The immature enterocytes differentiate as they migrate on to mucosal surfaces, replacing effete epithelial cells which are constantly shed from the tips of villi in the small intestine and from the surface of the large intestine.

In addition to participation in the digestion and absorption of nutrients, enterocytes have an important role in controlling water and electrolyte transfer between the intestinal lumen and the lamina propria. The end result of this activity is the absorption of water from the lumen and the production of formed faecal material. Disturbance of the mechanisms involved in this process is a major factor in diseases produced by some enteropathogenic microorganisms.

In the small intestine, absorption of sodium and chloride ions and non-electrolytes, such as glucose and amino acids, occurs mainly by transcellular transport. Ionic balance in the cells is maintained by loss of intracellular hydrogen and bicarbonate ions. Transfer of water from the intestinal lumen into intercellular spaces occurs through the tight junctions between enterocytes. This paracellular transport is induced by an osmotic gradient between intestinal contents and the fluid in the intercellular space. The gradient is produced by energy-dependent transfer of sodium ions into the space through the basolateral parts of enterocyte plasma membranes. Because tight junctions in the small intestine are relatively permeable, especially in the duodenum and jejunum, back-flow occurs. The overall balance is in favour of absorption with diffusion into the capillaries of the lamina propria. Because the tight junctions in the large intestine are more impermeable than those in the small intestine, a high osmotic pressure is maintained in intercellular spaces and in the lamina propria, contributing to water absorption. Moreover, absorption of volatile fatty acids in the colon results in further water absorption.

Solute movement across enterocyte plasma membranes is controlled by

peptide hormones acting through the intracellular secondary messengers adenyl cyclase and guanyl cyclase. Activation of these enzymes produces increased cellular levels of cyclic AMP and cyclic GMP, which depress sodium absorption and promote chloride secretion by crypt cells.

Normal flora

The intestinal tract is bacteriologically sterile at birth. Within hours, the tract is colonized by a range of bacteria including *Lactobacillus* species, *Escherichia coli* and strict anaerobes such as *Clostridium* species, various species of anaerobic Gram-positive cocci and *Fusobacterium necrophorum*. Because the ingesta in the anterior small intestine tends to retain the acidity derived from the secretion of gastric acid, these bacterial populations usually establish in the terminal part of the small intestine and in the large intestine where they persist throughout the life of the host. In ruminants, the resident microflora of the rumen, caecum and colon, which includes yeasts and protozoa along with bacteria, is responsible for the degradation of cellulose and for metabolic processes involving other carbohydrates and nitrogenous compounds. Similar digestive functions are performed by the normal microflora of the caecum and colon in monogastric herbivores, thereby contributing to their nutritional requirements.

Following establishment of a resident microflora, antigenic stimulation promotes expansion of the gut- associated lymphoid tissue (GALT), allowing local production of immunoglobulins, an important factor in preventing colonization by pathogenic microorganisms. Components of GALT include intraepithelial lymphocytes and Peyer’s patches, localized aggregates of lymphocytes and plasma cells in the mucosa and submucosa of the small intestine. The epithelial cells (M cells) covering Peyer’s patches are actively pinocytotic and appear to be able to sample, process and present antigen to the underlying lymphocytes. The immunoglobulins produced by GALT are predominantly IgA. They are secreted on to the surface of the intestinal epithelium where they protect against pathogen adhesion to enterocytes.

Short chain fatty acids produced by certain members of the resident flora inhibit growth of exogenous bacteria. Moreover, competition between bacterial species for energy-producing nutrients and for receptors on enterocytes influences the composition of the intestinal microbial population.

Pathogenetic mechanisms in enteritis

The specific intestinal niches occupied by the various resident bacteria are determined by the affinity of surface structures on the microorganisms for specific receptors on the enterocytes. Changes in the normal microflora may allow access of pathogenic microorganisms to epithelial cell receptors leading to the establishment of infection. Factors that contribute to changes in the normal microflora include antimicrobial drug therapy and stress related to changes in feeding or management practices. Moreover, animals are particularly susceptible to infection with pathogenic microorganisms during the neonatal period before the resident microflora becomes fully established. The significant pathogenic microorganisms associated with intestinal disease in large animals are listed in Boxes 87.1 to 87.4.

Pathogenic microorganisms utilize a number of mechanisms to produce the metabolic and structural changes in the intestinal epithelium which lead to diarrhoea and to dysentery. Because of the complex microenvironment in the intestine and the possibility of synergism between pathogens, categorization of the functional and structural changes produced by individual pathogens is not always possible. Nevertheless, certain pathological alterations, including hypersecretion, villous atrophy, mucosal distortion and necrosis, may result from infection with particular enteric pathogens.

Box 87.1 Some microbial pathogens associated with intestinal disease in cattle.

- *Escherichia coli*
- *Salmonella* serotypes
- *Mycobacterium avium* subsp. *paratuberculosis*
- *Clostridium perfringens* types B and C
- Rotavirus
- Bovine coronavirus
- Bovine viral diarrhoea virus
- Rinderpest virus

Box 87.2 Some microbial pathogens associated with intestinal disease in sheep and goats.

- *Escherichia coli*

- *Clostridium perfringens* types B and C
- *Salmonella* serotypes
- Rotavirus
- Peste-des-petits-ruminants virus

Box 87.3 Some microbial pathogens associated with intestinal disease in pigs.

- *Escherichia coli*
- *Clostridium perfringens* types A and C
- *Brachyspira hyodysenteriae*
- *Lawsonia intracellularis*
- *Salmonella* serotypes
- Rotavirus
- Transmissible gastroenteritis virus
- Porcine epidemic diarrhoea virus
- Classical swine fever virus
- African swine fever virus

Box 87.4 Some microbial pathogens associated with intestinal disease in horses.

- *Salmonella* serotypes
- *Clostridium perfringens* types A and C
- Clostridium difficile
- Neorickettsia risticii
- Rhodococcus equi
- Actinobacillus equuli
- *Escherichia coli* (role unclear)
- Rotavirus

Hypersecretion

Functional disturbance of intestinal epithelial cells is exemplified by infection with enterotoxigenic strains of *E. coli*, a common aetiological agent of diarrhoea in neonatal calves, pigs and lambs. The toxic mechanisms involved in this type of enteric infection are detailed in Chapter 24. These enterotoxigenic strains of *E. coli* possess fimbrial adhesins which allow attachment to enterocytes in the small intestine. The hypersecretion induced by the enterotoxins relates to the

activation of adenylate cyclase or guanylate cyclase in enterocytes. Hypersecretory diarrhoea results from a combination of increased chloride and water secretion and inhibition of sodium and water absorption. The excess fluid entering the large intestine overloads its absorptive capacity. Morphological and inflammatory changes in the mucosa of the small intestine are absent or negligible.

Villous atrophy

Destruction of the epithelial cells on the surface of villi or in the crypts of the small intestine results in changes in the size and shape of the villi and enterocytes. The villi, which become stunted and often fuse, are covered by cuboidal epithelium. This villous atrophy is encountered in the terminal small intestine during some infections with bacteria such as attaching-effacing *E. coli*. However, it is most commonly encountered in enteric viral infections. The degree of epithelial damage and subsequent villous change ranges from the relatively mild alterations encountered in rotavirus infections of neonatal farm animals to the marked structural disruption produced by infection with canine parvovirus. These differences relate not only to viral virulence but also to the cells targeted by the particular virus. In rotavirus infections, the mature epithelial cells near the tips of villi are affected. Replacement cells, produced from the pool of undifferentiated replicating cells in the crypts of Lieberkuhn, may be immature and cuboidal. In uncomplicated infections, epithelial replacement with clinical recovery may occur within a few days. Nevertheless, interference with digestive and absorptive processes, due to stunting of villi and incomplete differentiation of replacement epithelial cells, can result in fluid overload of the colon with consequent diarrhoea. The coronavirus of pigs, transmissible gastroenteritis virus, also targets enterocytes on villi. However, villous damage is much more extensive than that encountered in rotavirus infections and may be permanent. In affected newborn piglets, severe diarrhoea may result in rapid dehydration and high mortality.

Canine parvovirus targets actively dividing cells. In enteric infection, the virus invades and destroys the progenitor cells in the crypts of Lieberkuhn interfering with the mechanism for replacement of villous epithelium and leading to widespread villous atrophy in the jejunum and ileum. Dilation and collapse of glandular structures may produce irreparable mucosal damage. If stem cells survive, restoration of the mucosa occurs. Because rapidly dividing cells in the

germinal centres of lymphoid tissues including GALT are also targeted by the virus, secondary bacterial infection often exacerbates the condition.

Infiltrative and proliferative distortion of the mucosa

Paratuberculosis (Johne's disease), a chronic, progressive, cell-mediated immunoinflammatory disease of adult ruminants, is caused by *Mycobacterium avium* subspecies *paratuberculosis*. The condition is characterized by the recruitment of large numbers of macrophages and T lymphocytes into the lamina propria and the submucosa, mainly in the terminal part of the ileum and large intestine. The large numbers of infiltrating cells produce crypt compression and villous distortion and atrophy. As a result, the absorptive surface area in the ileum is markedly reduced and there is interference with fluid resorption in the large intestine. Lymphatic drainage from the intestinal wall may be partially impeded by granulomatous lymphadenitis and lymphangitis which are constant features of the disease. Lymphatic blockage may be a contributory factor to the protein loss, which occurs in bovine paratuberculosis. Increased permeability of vascular endothelium and of the tight junctions between enterocytes may heighten protein loss. Moreover, loss of plasma albumin into the intestine and the consequent hypoalbuminaemia can result in further loss of fluid from the circulation. The protein- losing enteropathy of paratuberculosis accounts in part for the fact that affected animals become emaciated while usually retaining their appetite.

The effects of proliferative mucosal changes are evident in the intestinal adenomatosis complex in growing pigs. The various clinicopathological syndromes within this complex are caused by *Lawsonia intracellularis*. As its name implies, this organism is present and replicates in the cytoplasm of enterocytes in the crypts, especially in the ileum. Mitosis of infected enterocytes results in glandular hyperplasia and the production of a population of enterocytes which remain undifferentiated. These undifferentiated cells are not shed and their retention results in the formation of pseudo-stratified columnar epithelium with expansion of glandular structures and thickening of the mucosa. The mechanisms involved in the stimulation of mitosis by *Lawsonia intracellularis* are unknown. Necrosis of the adenomatous tissues has been attributed to replication of anaerobic organisms colonizing the terminal ileum. Proliferative haemorrhagic enteropathy, a sporadic condition affecting young adult pigs, is part of the intestinal adenomatosis complex. It is characterized by necrosis of the

adenomatous epithelium, marked neutrophil infiltration into the mucosa and haemorrhage. These lesions are consistent with a hypersensitivity reaction to antigen derived from *Lawsonia intracellularis*.

Mucosal necrosis

Necrosis of the enteric mucosa is a feature of some bacterial infections. The severity and extent of the necrosis depend on the virulence of the infecting organism and the immune status of the host. Cytotoxins of *Clostridium perfringens* type C cause an acute necrotizing enteritis of the distal parts of the small intestine in calves, lambs, piglets and foals. The necrosis often extends to the deep tissues of the mucosa and results in haemorrhagic lesions.

Severe, extensive, mucosal erosion along with heavy neutrophil infiltration occurs in the small intestine of young domestic animals infected with some strains of *E. coli* and with *Salmonella* species. Moreover, endotoxin derived from Gram-negative organisms is responsible for thrombosis in mucosal vessels, further contributing to enteric damage through ischaemic necrosis. Salmonellosis can affect all age groups of cattle, with a fibrinohaemorrhagic enteritis involving the terminal small intestine and the large intestine. In contrast, the aetiological agent of swine dysentery, *Brachyspira hyodysenteriae*, targets the mucosa of the colon. Large numbers of *B. hyodysenteriae* in association with other organisms, including *Bacteroides* species and *Fusobacterium necrophorum*, are found in superficial erosive lesions. Hypersecretion of mucus is an important feature of the disease, and capillary thrombosis is present in the mucosa. Fluid and electrolyte transport in the small intestine are normal. Diarrhoea results largely from interference with fluid absorption from the colon.

Further reading

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Chapter 88

The role of microbial pathogens in respiratory disease

The respiratory system can be divided into the upper respiratory tract comprised of the nasal cavities, pharynx and larynx, and the lower respiratory tract comprised of the trachea, bronchi, bronchioles and lung parenchyma. Because the mucosa of the bronchus is not significantly different from that lining the trachea, larynx or pharynx, the passages that extend from the nose to the bronchi can be considered as conducting airways, whereas the bronchioles and alveoli are treated as integral parts of the lungs.

The primary function of the respiratory system is to replenish oxygen removed from arterial blood during passage through metabolically-active tissues and, concurrently, to eliminate carbon dioxide generated by tissue metabolism. The lungs provide the respiratory surface through which oxygen can diffuse down a pressure gradient from inspired air into the pulmonary capillaries and carbon dioxide can diffuse from blood into pulmonary alveoli for elimination at the next expiration. Gas exchange between air and blood takes place by diffusion across a very thin blood-gas barrier, approximately 1.0 to 2.0μm across and consisting of alveolar epithelium, endothelium of the alveolar capillaries and, between them, the fused basal laminae of the two cell types. Since this barrier offers little resistance to the diffusion of oxygen and carbon dioxide, the efficiency of gas exchange depends on how closely the flow of air in the alveoli (ventilation) matches the flow of blood in the alveolar capillaries (perfusion). A mismatch of ventilation and perfusion is the main reason for impaired gas exchange in diseases of the respiratory system; when the mismatch is large, it can cause severe respiratory distress. Gas exchange can be impaired by disease processes in the blood or in the cardiovascular system as well as by disorders of the respiratory system. Microbial pathogens are responsible for several diseases of the respiratory tract that cause a decline in ventilation and in gas exchange. Hypoxia due to ventilation/perfusion mismatch can be an important complication in pneumonic animals and, also, in animals in which ventilation is

impaired by obstructive lesions in the upper respiratory tract. The flow of air can be obstructed by mucus or inflammatory exudate within the lumen of the airway or by thickening of the mucosa of an inflamed airway.

Perfusion and ventilation provide the two main avenues through which microorganisms can enter the respiratory system. All of the venous return from the systemic circulation passes through the lungs *en route* from the right ventricle to the left ventricle. Thus, the pulmonary capillaries are frequently exposed to blood-borne pathogens including bacteria and viruses that show tropism for endothelial cells. Likewise, inhaled air exposes the respiratory tract to large numbers of microorganisms, some of which establish a complex microflora of both resident and transient microbes in the upper respiratory tract (nasal cavity, pharynx and larynx). The composition of this microflora is influenced by the host species and by the prevailing animal husbandry system. Some bacteria present in the nasal flora are potential pathogens that can cause serious respiratory disease when host defences are compromised. For instance, *Mannheimia haemolytica*, a commensal resident of the nasal cavity in cattle, can cause severe bronchopneumonia and pleurisy (‘shipping fever’) in young cattle subjected to stressful conditions, such as transportation, assembly in feedlots or close confinement.

Aerosol contamination of the environment results in exposure of the respiratory tract to potential pathogenic organisms. This is more likely to occur during epidemics of respiratory disease or when high numbers of animals share a confined air space in poorly ventilated, unhygienic conditions. Inhaled air often contains a range of inorganic particles, organic particles and noxious gases that can damage the tissues and facilitate colonization of the airways by airborne pathogens. In ordinary circumstances, the anatomy and organization of the respiratory tract provides defence mechanisms sufficiently robust to ensure that the lungs and the thoracic portions of the bronchi remain essentially free of microorganisms.

Defence mechanisms in the conducting airways

The respiratory tract can utilize a range of defensive mechanisms to limit access of inhaled pathogens to the delicate tissues of the blood-gas barrier, where an inflammatory exudate would impair gas exchange. The more important of these

mechanisms are removal of large particles during passage through the nasal cavity, mucociliary clearance in the airways and innate and acquired humoral defences.

The epithelium lining the conducting airways consists of ciliated cells, secretory goblet cells and dendritic cells. The luminal surface of these airways is covered by fluid secreted by the epithelial cells and by submucosal glands. The surface fluid consists of a viscous mucous layer that overlies a lubricating serous layer in which the cilia beat. The mucus traps and transports airborne particles, a process known as mucociliary clearance. Both layers are permeated by antimicrobial agents produced constitutively in the underlying tissues. Thus, as well as acting as an important physical barrier to airborne microorganisms, the surface fluid provides a chemical barrier to colonization by microbes that are susceptible to products such as lysozyme, lactoferrin, defensins, catheli-cidins and secretory IgA (see Chapter 3).

Structural features of the nasopharynx help to bring inhaled microorganisms into contact with the mucous blanket that covers the mucosa of the conducting airways. Projecting conchae are responsible for turbulence in the flow of inspired air through the nasal cavity. Because of the turbulence, airborne particles larger than 5μm together with associated microorganisms collide with the layer of mucus and become trapped in it. The serous fluid, the mucus and the entrapped material are propelled by the underlying cilia towards the pharynx, where all three components are swallowed or are expelled by coughing. Mucociliary clearance propels the fluid in a caudal direction from the nasal cavity to the pharynx and in a cranial direction from the bronchi and trachea to the pharynx. In addition, the conchae help to prevent desiccation of the alveoli; they warm and moisten inspired air as it passes through the nasal cavity and, in turn, they reclaim heat and moisture from the saturated vapour that is expelled during expiration.

The mucosa of the conducting airways contains a dense network of macrophages and dendritic cells, both of which serve as antigen-presenting cells (Holt *et al*., 2008). Dendritic cells are located both within the surface epithelial layer and directly underneath the epithelial basement membrane. Branching processes of intraepithelial dendritic cells extend between adjacent epithelial cells and reach the lumen of the airway without disrupting the integrity of the epithelial barrier (Vermaelen and Pauwels, 2005). Thus, the dendritic cells are able to sense, sample and process microbial antigens in inspired air. Processed antigens are displayed on major histocompatibility (MHC) molecules on the

surface of the dendritic cells. After migration to local lymph nodes, dendritic cells present the antigens to naïve lymphocytes and induce proliferation of antigen-specific T cells that target the microbial antigen at the site of entry (see Chapter 3).

Defence mechanisms in the lungs

Within the lungs, the bronchi branch many times, eventually forming small airways called bronchioles. There is a gradual decrease in the number of goblet cells and submucosal glands in the subordinate branches of the bronchial tree with the result that these secretory cells are no longer present in bronchioles. The narrow terminal branches of the bronchioles are continuous with alveolar ducts and the numerous associated alveoli that contribute the epithelial component of the blood-gas barrier. Approximately 95% of the lining of the alveolar cavity is composed of squamous epithelial cells (type I alveolar cells); the remaining 5% is composed of type II alveolar cells that secrete surfactant, which reduces the tendency of alveoli to collapse. Type I cells cannot replicate when damaged; type II cells can replicate to replace them. When there is severe damage to type I cells, extensive replacement by type II cells is described as epitheliali-zation of alveoli, a condition that decreases gas exchange in the affected alveoli. To prevent such an outcome to an encounter with a noxious agent, the host must be able to activate and control effective innate and adaptive immune responses which do not cause residual damage to pulmonary parenchyma.

Box 88.1 Soluble protective factors in alveolar lining fluid.

- Non-specific factors which affect microbial survival
  –lysozyme
  –lactoferrin
  –complement
  –surfactant
- Factors which inhibit inflammatory mediators or injurious enzymes
  –glutathione peroxidase
  –catalase
  –(α_1-antitrypsin

Microorganisms that enter the lungs by the haematogenous route may have evoked systemic defence mechanisms but they have to contend with local defence mechanisms when they enter the pulmonary tissues. Local innate defences are offered by antimicrobial peptides and immunoglobulins in alveolar fluid (Box 88.1). In the alveoli, IgG tends to be the predominant immunoglobulin whereas IgA is the more important immunoglobulin in the conducting airways. Surfactant also plays a part in protecting the blood– gas barrier from injury. As members of the collectin family, surfactant proteins A and D contribute to host defences: they opsonize bacteria, they are potent chemoattractants for monocytes and neutrophils, and they enhance the phagocytic activities of alveolar macrophages and neutrophils (Haagsman *et al*., 2008). Uncontrolled inflammation in the respiratory tract can be life-threatening to an animal (Thacker, 2006).

As the most important antigen-presenting cells in the lungs, dendritic cells are central to the integration of innate and adaptive immunity. The lungs are well supplied with immature dendritic cells and it is probable that the increase in number in infected lungs follows arrival of monocytes from the bloodstream. Cells that can react to pathogens without the aid of professional antigen-presenting cells, such as γδT cells and natural killer (NK) cells, may play a significant role in lung immunity (Nikod, 2005). These cell types respond rapidly during the early stages of pulmonary responses to bacterial, fungal and viral infection. For example, γδT cells are active in the early phase of *Mycobacterium bovis* infection in humans, mice and cattle. Data from experimentally induced sepsis indicate that laboratory animals deficient in γδ T cells show more tissue damage, delayed resolution of inflammatory infiltrates and higher mortality rates than control animals with the natural complement of γδT cells (Hirsh *et al*., 2006). These findings suggest that γδT cells at the site of inflammation act to regulate the tissue response and to protect pulmonary parenchyma from residual damage.

Macrophages are present in the interalveolar septa from where they can migrate into the lumen of the alveolus and adhere to epithelial cells. Under normal conditions, the primary role of these alveolar macrophages is to engulf redundant surfactant and to protect the blood-gas barrier from antigens or infective agents that reach the alveoli. They phagocytose bacteria and they release factors, including IL-8, that attract neutrophils. Activated neutrophils phagocytose bacteria and they can secrete factors, including defensins, TNF-α, IL-1 and IL-6, that contribute to clearance of pathogens. The priority is to

regulate the host response so that the blood-gas barrier remains intact and functional. Once the infection or the inflammatory process has been controlled, the macrophages engage in the removal of exudate and tissue debris from the alveolus.

Mariassy *et al*. (1975) reported that alveolar macrophages are seldom seen in the alveolar sac in cattle and suggested that the presence of pulmonary intravascular macrophages may compensate for the scarcity of alveolar macrophages. In cattle (and in sheep, goats, pigs, horses and cats), intravascular macrophages are attached to the endothelium of alveolar capillaries. These are highly phagocytic cells and they play an important role in the clearance of circulating bacteria from the pulmonary vessels. However, they release pro-inflammatory mediators that are capable of damaging lung tissue during inflammatory responses to blood-borne pathogens (Caswell and Williams, 2007).

Microbial diseases of the conducting airways

Although infection may sometimes be confined to one part of the conducting airways, the likelihood is that more than one of the component parts may be affected. Rhinitis, inflammation of the nasal cavity, can be a primary local condition or it may be part of a systemic disease. Causes of rhinitis include pathogenic microorganisms, allergens, irritant gases or dust. Some bacterial and viral causes of rhinitis are listed in Box 88.2. The basic reaction of the nasal cavity to infectious agents consists of vascular congestion, hyperactivity of goblet cells and mucous glands, and cellular infiltration of the mucosa. Most cases of acute rhinitis begin with a serous nasal discharge which often changes to catarrhal or mucopurulent discharge when the quantity of necrotic leucocytes and desquamated tissue increases. The severity of the tissue reaction can vary from mild catarrhal rhinitis seen in many bacterial and viral infections to rhinitis with extensive necrosis of the luminal epithelium, often with fibrinous deposits on the surface. In pseudomembranous rhinitis, the fibrinous deposits can be peeled off without loss of underlying tissue. In contrast, the fibri-nonecrotic (diphtheritic) lesions of calf diphtheria caused by *Fusobacterium necrophorum* leave an ulcerated surface when the fibrinous membrane is removed. Fungi and mycobacteria cause granulomatous lesions. In the nasal form of glanders, *Burkholderia mallei* causes pyogranulomatous nodules on the mucosa of the nasal septum.

Box 88.2 Some diseases in which rhinitis is a significant feature.

- Infectious bovine rhinotracheitis
- Kennel cough (Canine adenovirus 2)
- Bovine malignant catarrhal fever
- Mucosal disease
- Rinderpest
- Equine viral rhinopneumonitis
- Influenza
- Bluetongue
- Inclusion body rhinitis
- Atrophic rhinitis
- Glanders
- Strangles
- Feline calicivirus infection
- Feline viral rhinotracheitis

Inflammatory processes in the nasal cavity can extend to the pharynx, larynx, trachea, and bronchi, as well as to the paranasal sinuses and, in *Equidae*, to the guttural pouches. In strangles, caused by *Streptococcus equi*, suppurative lesions in the upper respiratory tract and associated lymph glands can extend to cause empyema of the guttural pouches. *Aspergillus* species can also extend along the auditory tubes to cause diphtheritic inflammation in the guttural pouches, from which location the fungi and the inflammation may extend to involve the internal carotid artery and branches of the ninth and tenth cranial nerves.

Colonization of the nasal cavity by *Pasteurella multocida* causes progressive atrophic rhinitis in young pigs. A heat-labile toxin produced by the bacterium disturbs the normal process of modelling of the conchae, leading to deformity of the nasal cavity and the snout (see Chapter 91). The consequent impairment of the filtering function of the nasal cavity predisposes the host to secondary bronchopneumonia.

The pharynx and the larynx may be involved as part of inflammatory diseases of either the upper or lower respiratory tract. Laryngitis may be part of oral necro-bacillosis (calf diphtheria) caused by *Fusobacterium necrophorum* ; however, that disease may affect the larynx in the absence of lesions elsewhere. In calves, laryngeal ulcers develop as a consequence of septic phlebitis associated with *Histophilus somni* septicaemia and pneumonia. Toxins released by certain serotypes of *Escherichia coli* cause oedema disease in pigs, in some of

which oedema of the larynx may impede ventilation and cause severe respiratory distress.

The bronchi also respond to infection with hyperactivity of mucus-secreting cells, invasion by inflammatory cells, and variable hyperplasia, metaplasia or ulceration of the surface epithelium. Loss of ciliated cells is an early feature of bronchitis, leading to impaired removal of exudate and the pathogen. In some species, bronchitis is associated with hyperplasia of bronchus-associated lymphoid tissue (BALT) located around high endothelium venules at the bifurcations of the bronchial tree (Bienenstock and McDermott, 2005; Pabst and Tschernig, 2010). The accumulation of B cells and T cells causes the overlying epithelium to bulge into the lumen of the bronchus, and that segment of epithelium contains cells specialized for uptake of antigen, analogous to M cells overlying Peyer's patches in the intestine. Hyperplasia of BALT is very evident in enzootic pneumonia in pigs caused by *Mycoplasma hyopneumoniae*, perhaps induced by superantigens in the cell membrane of the pathogen. BALT is present in pigs, sheep, cats, rabbits and chickens.

Microbial diseases of the lungs

The defence mechanisms of the conducting airways and those of the lungs can cope successfully with the vast majority of noxious agents that enter the pulmonary system so that it is not necessary for the lungs to mount an inflammatory response. Thus, the lungs are not subjected to the tissue damage that can be caused during an inflammatory response by neutrophil products released to kill microbes, such as reactive oxygen species and proteases. However, if the defences prove to be inadequate, the lungs are well equipped to respond rapidly and effectively. The pulmonary microvasculature is very extensive, intact alveolar capillaries contain a marginal pool of neutrophils, and these cells can migrate directly from the alveolar capillaries rather than from postcapillary venules as happens at most other systemic sites (Burns *et al*., 2003). Moreover, additional neutrophils can be attracted to the alveolar capillaries very rapidly in response to inflammatory mediators including TNF-α, IL-1 and IL-8.

The bronchiolar-alveolar junction is a major site of deposition of particles less than 3μm that have not been trapped by the mucociliary apparatus, and many infections of the lungs result from bronchiolitis. Respiratory viruses that cause lesions in the bronchi are likely to cause changes also in bronchioles and alveoli.

As the inflammation progresses, exudate, desquamated epithelial cells and inflammatory cells accumulate in the narrow lumen of the respiratory bronchioles, leading either to atelectasis ifthe airway obstruction is complete or to trapping of air and over-distension of alveoli when the obstruction is incomplete. In either case, gas exchange is impaired in alveoli distal to the obstruction. The blockage interferes with removal of exudate from the alveolar sac and, in species in which adjacent alveoli communicate via the pores of Cohn, there is lateral spread of inflammation According to Mariassy *et al*. (1975), pores of Cohn are small and extremely rare in cattle and, because of this and the separation of distinct lung lobules by complete septae, cattle are prone to focal pulmonary atelectasis.

Factors predisposing to pulmonary infection

In the respiratory system, the outcome of interaction between host and infectious agent depends largely on the ability of the host to prevent establishment of infection. This ability relates not only to the virulence and challenge dose of the agent but also to the effectiveness of the defence mechanisms. In many infections, the balance is tipped in favour of the infectious agent by environmental factors or by intercurrent disease in the respiratory or other organ systems. Young animals are particularly susceptible to the effects of deleterious environmental and other stress factors (Box 88.3). Cold air temperatures, uraemia or dehydration can affect mucociliary function by reducing ciliary activity, slowing the rate of clearance of foreign material. The rate is slower also in hot dry atmospheric conditions as a result of fluid evaporation from the mucus component of the clearance mechanism. Immunodeficient animals are particularly prone to pulmonary infection, an indication of the importance of local immunity as a pulmonary defence mechanism. Moreover, immunosuppression may increase susceptibility to pulmonary infection which often involves both viral and bacterial pathogens. Bacterial species commonly implicated in mixed pulmonary infections in young animals are indicated in Box 88.4.

Box 88.3 Factors predisposing to the development of pneumonia in calves.

- Close confinement at markets or shows
- Transportation and other stress factors

- Poorly ventilated and overcrowded housing conditions
- Decline of maternally-derived antibody levels
- Intercurrent infections

Box 88.4 Bacteria commonly implicated in mixed respiratory infections in young animals.

- *Escherichia coli*
- *Streptococcus species*
- *Actinobacillus species*
- *Pasteurella multocida*
- *Bordetella bronchiseptica*

Patterns of pulmonary inflammation

The significant pneumonic conditions affecting large animals are presented in Tables 88.1 to 88.4. Two main patterns of pneumonia, namely bronchopneumonia and interstitial (proliferative) pneumonia, are recognized.

Bronchopneumonia

Usually aerogenous in origin, bronchopneumonia is commonly caused by bacterial infection. Predisposing factors, including viral or mycoplasmal infection of the respiratory tract and environmental stress, can interfere with respiratory clearance mechanisms and with immune responsiveness. These factors are almost always involved in the pathogenesis of the condition. Lesions, characteristically located in the anteroventral regions of the lungs, consist of irregular areas of consolidation. Affected regions of lung, which are reddened and swollen during the acute inflammatory phase of the pneumonia, collapse as it resolves. Inflammatory lesions develop initially at the bronchiolar-alveolar junction, a location where inhaled bacteria and aerosol droplet nuclei are often deposited. Neutrophil infiltration and serofibrinous exudation extend from the original nidus to surrounding alveoli and bronchioles within affected lobules. The outcome of bronchopneumonia depends on the virulence of the causal agent and on the severity and extent of the inflammatory reaction. When alveolar basement membranes remain intact and inflammatory exudates are cleared rapidly, complete restoration of structure and function can occur. More often,

because of the extent of the original lesion, chronic suppuration and fibrosis develop. If pyogenic organisms such as *Arcanobacterium pyogenes* and *Rhodococcus equi* persist and proliferate in lesions, abscesses form. Clinical signs of respiratory involvement may be minimal in chronic bronchopneumonia, although there may be considerable economic loss due to poor productivity in affected herds and flocks. An acute fibrinonecrotic form of pneumonia occurs in which the bronchopneumonic pattern of lesion development is not readily detected. The inflammatory reaction spreads rapidly through pulmonary tissues often involving entire lobes. Pneumonias of this type are caused by infection with virulent strains of *Mannheimia haemolytica* in ruminants, *Actinobacillus pleuropneumoniae* in pigs and *Pasteurella multocida* in a number of domestic animal species. Affected pulmonary tissue is swollen and dark red and exudes blood-stained fluid from cut surfaces, on which irregular pale areas of necrosis may be detected. The interlobular septa may be distended with serofibrinous exudate, and fibrinous deposits are usually present on the overlying pleura. Septicaemia or toxaemia frequently develops and some animals may die suddenly.

Table 88.1 Important pathogens associated with pneumonia in cattle.

Pathogen	Comments
Mannheimia haemolytica type A1	Associated with acute fibrinonecrotic bronchopneumonia. Often affects beef and store cattle after transportation in overcrowded conditions. It is also encountered in housed calves. May exacerbate viral pneumonia
Histophilus somni	Produces pulmonary lesions similar to those caused by infection with *Mannheimia haemolytica*
Pasteurella multocida	Occasionally isolated from lesions of acute fibrinonecrotic bronchopneumonia in adult cattle
Mycobacterium bovis	Causes chronic granulomatous lesions in lungs. In advanced lesions, caseation relates to cell-mediated hypersensitivity
Mycoplasma mycoides subsp. mycoides (small colony type)	Causes contagious bovine pleuropneumonia, an acute fibrinonecrotic pneumonia with serofibrinous exudation in alveoli and in thickened interlobular septa. The disease is notifiable in most countries
Mycoplasma bovis	Associated with the enzootic pneumonia complex in calves. Peribronchiolar and perivascular lymphoid hyperplasia is prominent in affected lungs
Mycoplasma dispar	Associated with enzootic pneumonia complex in calves. May cause low grade bronchiolitis
Parainfluenza virus 3	Associated with enzootic pneumonia complex in calves. Consolidation of ventral portions of cranial and middle lobes of lungs. Perivascular and peribronchiolar lymphoid cuffs. Eosinophilic intracytoplasmic inclusion bodies in bronchiolar epithelial cells
Bovine respiratory syncytial virus	Associated with enzootic pneumonia complex in calves. Syncytial giant cells, present in bronchioles and alveoli, may contain intracytoplasmic inclusions
Bovine herpesvirus 1	Causes infectious bovine rhinotracheitis, mainly affecting upper respiratory tract. Direct effect of virus on pulmonary tissues not clearly shown. Severe infections result in secondary bacterial pneumonia in calves
Bovine viral diarrhoea virus	May predispose to bacterial pneumonia by causing immunosuppression

Table 88.2 Microbial pathogens associated with pneumonia in sheep and goats.

Pathogen	Comments
Mannheimia	Causes acute fibrinonecrotic pneumonia and pleurisy in lambs. Stress factors predispose to the development of the disease.

haemolytica	Parainfluenza 3 virus and *Mycoplasma ovipneumoniae* may be implicated in lesion development
Mycoplasma capricolum subsp. capripneumoniae	Causes classical contagious caprine pleuropneumonia
Mycoplasma mycoides subsp. *capri*	Subspecies of *M. mycoides* cause pleuropneumonia. Lung lesions include serofibrinous bronchopneumonia with thickening of interlobular septa due to inflammatory exudates
Maedi/visna virus	A lentivirus (retrovirus) which causes maedi (ovine progressive pneumonia, zwoegerziekte), a chronic interstitial pneumonia of adult sheep. Affected lungs are grossly enlarged and weigh considerably more than normal lungs. Thickening of alveolar walls and marked lymphoproliferative changes around vessels and bronchioles occur
Caprine arthritis-encephalitis virus	A lentivirus closely related to maedi/visna virus. Chronic interstitial pneumonia with alveolar epithelialization and intra-alveolar exudation of proteinaceous fluid
Jaagsiekte sheep retrovirus	Causes ovine pulmonary adenomatosis, a chronic proliferative pneumonia of sheep. The proliferative epithelial tissue, which occurs as multiple foci of columnar or cuboidal cells lining alveoli, has the characteristics of a low grade carcinoma; foci of these cells are occasionally present in the regional lymph nodes. There is marked fluid accumulation in the lungs

Table 88.3 Microbial pathogens associated with pneumonia in pigs.

Pathogen	**Comments**
Pasteurella multocida	Often involved as secondary invader in enzootic pneumonia of pigs caused by *Mycoplasma hyopneumoniae*. Produces acute fibrinous pneumonia
Actinobacillus pleuropneumoniae	Causes porcine contagious pleuropneumonia usually in young pigs. Haemorrhagic consolidation of dorsocaudal areas of lungs close to hilus. Necrotic foci in areas of consolidation
Mycoplasma hyopneumoniae	Causes porcine enzootic pneumonia, a non-fatal disease of young pigs. Secondary bacterial infections may cause death. Cranioventral pulmonary consolidation. Peribronchial and perivascular lymphoid accumulations and macrophage infiltration in alveolar lumen are prominent microscopic features
Influenza A virus	Classical swine influenza is caused by subtype H_1N_1. All swine influenza virus subtypes are potentially zoonotic. Cranioventral consolidation. Secondary bacterial infections often associated with fatalities
Porcine herpesvirus 1	Causes Aujeszky's disease. Some strains associated with pneumonic lesions
Porcine reproductive and respiratory syndrome virus	This arterivirus has an affinity for pulmonary macrophages. Causes pneumonia in neonatal piglets. Predisposes to infection with *Streptococcus suis, Haemophilus parasuis* and porcine respiratory coronavirus

Table 88.4 Microbial pathogens associated with pneumonia in horses.

Pathogen	**Comments**
Rhodococcus equi	Causes suppurative bronchopneumonia in foals less than 6 months old
Burkholderia mallei	Causes glanders, an important zoonosis. Pyogranulomatous nodules develop in the lungs of chronically affected animals
Streptococcus equi subsp. *equi*	Causes strangles, an upper respiratory tract infection. Systemic dissemination occurs in bastard strangles; abscessation develops in the lungs and other internal organs
Equine herpesvirus 1 and 4	Cause pneumonia in neonatal and young foals. EHV-4 usually affects foals between 2 and 12 months of age. Pulmonary disease caused by EHV-1 is less important
Influenza A virus	Equine subtypes A/equi 1, H7N7 and A/equi 2, H3N8 cause upper respiratory disease mainly in young horses. In severe disease, broncho-interstitial pneumonia may be exacerbated by secondary bacterial infection
Equine adenovirus A	Subclinical infection widespread in horses. Disease occurs in Arabian foals with severe combined immunodeficiency. Necrotizing bronchiolitis and intranuclear inclusions in hyperplastic bronchiolar epithelial cells are often present. Secondary infection with *Streptococcus zooepidemicus* may occur

Interstitial pneumonia

In contrast to the tissue reactions in bronchopneumonia, the exudative, infiltrative and proliferative reactions associated with interstitial pneumonia primarily involve alveolar walls. Although sometimes associated with the ingestion of toxic chemicals or with hypersensitivity responses, interstitial pneumonia is also a feature of a number of bacterial and viral infections. Spread of infection to the lungs is often haematogenous, particularly in acute systemic disease, resulting in a diffuse or multifocal lesion distribution with no clear-cut relationship to airways. This type of acute interstitial pneumonia occurs, for example, in canine distemper and in septicaemic salmonellosis in calves and pigs. Although transmission of canine distemper virus is generally through aerosol, lung involvement results from viraemia following viral replication in the tonsils and other lym-phoid tissues. The alveolar walls are infiltrated with lymphoid cells, and multinucleate giant cells, derived from type 2 alveolar cells, may be present in alveoli along with alveolar macrophages. In the later stages of the disease, focal areas of alveolar epithelialization may be present. In septicaemic salmonellosis, alveolar walls are thickened as a result of leukocytic infiltration. Damage to capillary and alveolar walls, presumably due to endotoxin, is followed by fibrinohaemorrhagic exudation into the alveoli. Acute septicaemic infections of this type usually occur in young animals which succumb before further pathological changes can develop.

Chronic interstitial tissue changes occur in ovine progressive pneumonia caused by maedi/visna virus. This lentivirus is transmitted to adult sheep by aerosols and to lambs in the milk of infected dams. The virus, which targets monocytes and macrophages, persists and can replicate in the presence of the immune response of the host. Affected sheep may be clinically normal for several years after infection. Gradual loss of condition and hyperpnoea may then develop. At post-mortem, the lungs do not collapse and may be up to four times the weight of normal lungs. Grey areas of consolidation can be detected on cut surfaces. The condition is characterized microscopically by infiltration of macrophages and lymphocytes into the alveolar walls and proliferative lymphoid nodules around bronchioles and blood vessels.

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Chapter 89

Interactions of microbial pathogens with the renal system

The urinary system eliminates waste solutes brought to the kidneys by the circulating blood. The kidneys transfer these waste products from the bloodstream to a multitude of blind-ended epithelial tubes (renal tubules). The waste products are finally passed through a series of muscle-invested tubes that transport the urine to the exterior (Fig. 89.1). Normally, the kidney and the ureters do not have a microbial flora. However, pathogenic microorganisms can reach these organs via the bloodstream (the haematogenous route) or by ascending the excretory pathway. The pathogenesis of ascending infection of the urinary tract differs considerably from that of haematogenous infection of renal tissues. Valuable insights into the pathogenesis and pathology of urinary infections can be gained from a focused review of the microanatomy of the kidney and of the outflow channel, with particular emphasis on how structural adaptations influence dynamic host–pathogen interactions within the urinary system.

The first part of this chapter is devoted to the interaction of pathogens with the excretory passages distal to the kidneys, while the second part deals with infectious conditions affecting the parenchyma of the kidneys and those parts of the excretory passages within the kidneys.

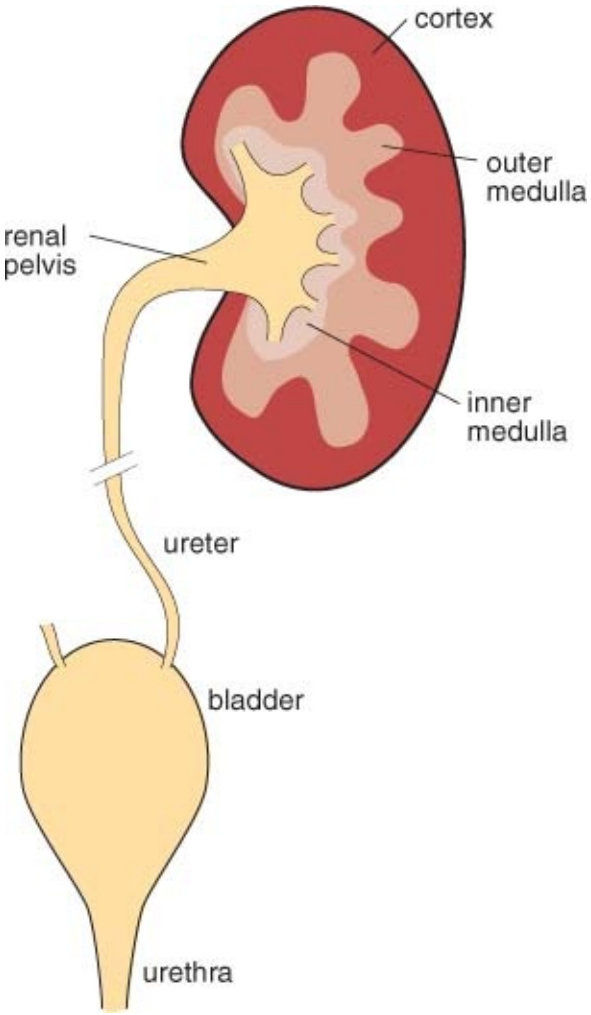
Ascending infection of the excretory pathway

Evidence from domestic animals and from human patients supports the hypothesis that most ascending infections of the urinary tract are caused by members of the normal flora of the large intestine of the host, predominantly strains of *E. coli* that possess virulence factors that enable them to survive in the urinary tract. Female animals are more prone to ascending infection than are male animals, largely because of the relatively short female urethra and the closer proximity of the urinary and intestinal orifices; other contributory factors

include more frequent exposure to infection from the genital tract, and the greater probability of the introduction of exogenous microbes during mating, artificial insemination, intrauterine procedures or catheterization of the urinary bladder. Ascending bacterial infection may be localized in the urinary bladder (cystitis), the kidneys (pyelonephritis), or the prostate gland (prostatitis).

In females, the distal end of the excretory passage is frequently colonized by bacteria from the anus, skin, and vagina. It is thought that these bacteria often gain entry into the urinary bladder but most of these organisms do not form firm attachments to the lining epithelium and they are flushed out by the next voiding of urine. Many of these microorganisms grow poorly in urine and the hosts remain asymptomatic. Moreover, the presence of potential pathogens in urine is not necessarily indicative of active infection of the urinary tract. On the other hand, the urinary system is vulnerable to attack by a range of microorganisms (Table 89.1) that possess ligands by which they bind to complementary receptors on the host epithelium, inducing inflammatory reactions and clinical symptoms. For example, *Actinobaculum suis* is a normal inhabitant of the prepuce of boars that can be transmitted to sows at mating. This organism causes cystitis and pyelonephritis in the sows, often with a fatal outcome due to renal failure.

Figure 89.1 Schematic representation of the gross anatomy of the urinary system, showing a kidney in sagittal section.



Structure and function of the excretory passages

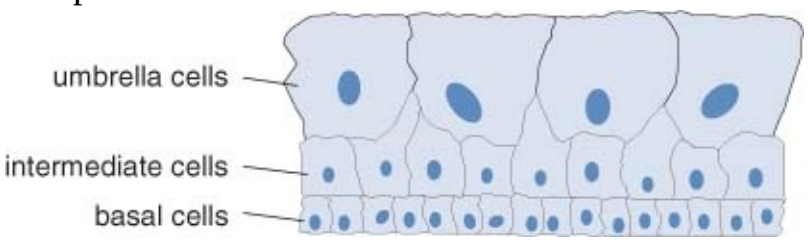
The kidneys begin the excretory process by non- selectively filtering about 20% of the renal blood flow into the tubules as protein-free fluid devoid of cells. The epithelial cells of the tubules modify the composition of the filtrate by selective reabsorption of many solutes and water and by selective secretion of other substances. As a result of these adjustments, approximately 1% of the filtrate is delivered to the ureters as hypertonic urine which remains virtually without change as it is propelled by peristalsis along the excretory pathway to the exterior. The pathway is lined by transitional epithelium, which protects the underlying tissues from damage by the hypertonic fluid. This specialized uroepithelium consists of basal cells and undifferentiated intermediate cells

underlying the differentiated umbrella cells at the luminal surface (Fig. 89.2). The stratified epithelium is sufficiently distensible to permit storage of urine in the bladder and sufficiently 'tight' to maintain a permeability barrier between the hypertonic urine and the subepithelial tissues, including the draining blood vessels. Uroepithelium is impermeable to urinary solutes and also prevents the passage of water from the underlying tissues into the hypertonic urine (Lewis, 2000; Apodaca, 2004). Thus, uroepithelium prevents significant changes in the composition of urine as it is transported, stored and eliminated through the excretory passage. It also acts as a physical barrier to invasion by bacteria.

Table 89.1 Bacteria frequently isolated from the urinary tract of domestic animals.

Bacterial species	Comments
Escherichia coli	The most frequent isolate in host species
Corynebacterium species	*C. renale*, *C. pilosum* and *C. cystitidis* cause cystitis and pyelonephritis in cows
Klebsiella pneumoniae, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*	Cystitis, pyelonephritis in dogs and horses; formation of struvite and apatite uroliths
Staphylococcus pseudintermedius	Cystitis in dogs; formation of struvite uroliths
Actinobaculum suis	Important cause of cystitis, pyelonephritis in sows; venereal transmission, often fatal

Figure 89.2 Urinary bladder: schematic representation of the three layers of uroepithelium.



Scanning electron microscopy has shown that the apical membrane of the differentiated umbrella cells is covered almost entirely by scallop-shaped plaques each of which contains several hundred transmembrane proteins called uroplakins. The carbohydrate moieties of the uroplakins act as specific receptors for several pathogens that colonize the urinary tract (uropathogens). There are no cilia on the umbrella cells, nor do they have the protection of a continuous layer of secreted glycosaminoglycans (such as mucus, mucin, polysaccharide or mucoprotein) that could impede attachment by bacteria (N'Dow *et al.*, 2005). To prevent pathogens from establishing a foothold on the uroepithelial surface, the urinary system has to rely on the flushing effects of micturition and on the antiadhesive role of the soluble Tamm-Horsfall protein, a glycoprotein released

into the urine by the epithelial cells of the thick ascending limb of the loop of Henle. Mannose residues in Tamm-Horsfall protein can bind uropathogens, thus competitively blocking attachment to uroepithelial receptors and leading to the elimination of the bacteria in urine. Members of the two main families of antimicrobial peptides, the defensins and the cathelicidins, are secreted continuously by uroepithelial cells and tubular epithelial cells of the nephron (Valore *et al*., 1998; Chromek *et al*., 2006). The constitutive expression of these peptides is low, but on contact with bacteria the epithelial cells promptly increase the rate of release of the peptides, thus creating an antimicrobial barrier on the luminal surface of the epithelium. When inflammatory cells respond to the presence of pathogens, the infiltrating cells act as an additional source of antimicrobial peptides.

The vesico-ureteral valve is the principal barrier to ascending infection entering the ureters. This barrier function can fail either due to a developmental defect of the valve mechanism or to high pressure as a result of obstruction to the outflow of urine, whether by uroliths, tumours, hypertrophy of the prostate, or malformations of the bladder or the urethra. Irrespective of the primary causes, the result is reflux of urine into the ureters and urinary stasis, making the excretory pathway a fluid-filled continuum which greatly increases the risk that pathogens may spread to the kidneys and cause pyelonephritis.

Virulence factors of uropathogens

Although many strains of *E. coli* do not have the ability to survive within the urinary tract, the uropathogenic strains (UPEC) express virulence factors that enable them to adhere to the uroepithelial cells, to evade or resist the innate immune response of the host, to compete with host tissues for essential nutrients and to proliferate in the new location. The more important virulence factors expressed by UPEC and by opportunist invaders of the urinary tract, such as *Klebsiella*, *Pseudomonas* and *Proteus*, are adhesins, toxins and iron-uptake systems. Uropathogenic *E. coli* can be regarded as the prototype uropathogen, whose virulence factors define the interplay between the bacteria and the host tissues in the pathogenesis and pathological changes of urinary tract infections. The opportunist invaders of the urinary tract have similar virulence factors but less is known about the molecular basis of their activities.

Virulence factors of uropathogenic *E. coli*

Adhesins

Uropathogenic strains of *E. coli* express adhesins, bacterial proteins that bind to carbohydrate moieties in glycolipids or glycoproteins on the luminal surface of umbrella cells. Some adhesins are anchored within the bacterial cell membrane (afimbrial or non-fimbrial adhesins), others are present in the flexible tips of fimbriae (or pili), filamentous structures that project from the cell membrane. Typically, several hundred fimbriae are evenly distributed over the bacterial cell surface. A single bacterium may have genes for a number of different adhesins but it does not always express them: expression of each of the genes may be subject to random phase variation or, to more specific purpose, they may be switched on or off as environmental conditions change. Thus, the bacteria seek to adapt to different locations and to move between them. Uropathogenic *E. coli* make good use of phase variation as they use Type 1 fimbriae to adhere to the urinary bladder and P fimbriae to attach to the kidneys. In Type 1 fimbriae, the adhesin protein is FimH, which interacts directly with the luminal surface of the bladder by binding to terminal mannose residues in the integral glycoprotein, uroplakin 1a. Regardless of their source, most *E. coli* isolates carry the *fimH* gene although it is not always expressed. In P fimbriae, the adhesin protein is PapG; it binds to uroepithelial cells, to tubular epithelium and to renal vascular endothelium by attachment to a digalactose moiety of a cell- surface glycolipid receptor.

Toxins

Uropathogenic *E. coli* produce α-haemolysin and cytotoxic necrotizing factor 1. α-Haemolysin is a poreforming toxin that is cytotoxic for a variety of cells, including erythrocytes, leukocytes, endothelial cells, fibroblasts and uroepithelial cells. It forms transmembrane pores that result in the loss of the normal ionic gradients, without the loss of intracellular proteins; the disturbed osmotic forces draw water into the cell and death ensues by osmotic lysis. At sublethal levels, α-haemolysin is a potent stimulus for the release of interleukin-1β (IL-1β), which induces fever and the release of acute phase proteins. IL-1 is reputed to enhance the proliferation of virulent strains of *E. coli*; thus, the elevation of IL-1 levels within the host may accelerate the growth rate of the pathogens and increase the exposure of the host to their virulence factors.

In uropathogenic *E. coli*, the gene for cytotoxic necrotizing factor 1 (CNF1) is closely associated with the gene for haemolysin. Of the strains that produce CNF1, only a small proportion are non-haemolytic. CNF1 causes rearrangement of the cytoskeleton of host epithelial cells, facilitating engulfment of non-invasive bacteria. Also, it has been shown to reduce the migration of neutrophils across epithelial layers and to decrease their phagocytic activity. It is capable of killing cultured uroepithelial cells by apoptosis, which has led to the suggestion that it may be involved in the exfoliation of infected uroepithelial cells from the bladder.

Lipopolysaccharide

Lipopolysaccharide interacts with cells that have the toll-like receptor 4 (TLR-4), such as macrophages, neutrophils, dendritic cells and B lymphocytes. At low concentration, LPS is a pathogen-associated molecular pattern (PAMP) that provides early warning signals to activate the innate immune response. At high concentrations, LPS activates complement, Factor XII and macrophages, resulting in the release of interleukin-1 (IL-1), TNF-α, nitric oxide and other mediators that induce fever, inflammation, disseminated intravascular coagulation and hypotensive shock.

Iron-uptake systems

The availability of iron is low within the urinary tract. For *E. coli* to survive in those conditions, the organism must be able to acquire iron from the iron-binding proteins of the host. For this purpose, *E. coli* produces iron-binding siderophores. The uropathogenic strains produce more of these iron chelators than do faecal strains. Inadequate uptake of iron weakens the ability of the organism to infect the kidney; hence, iron- uptake systems are listed amongst the virulence factors of uropathogenic *E. coli*.

Virulence factors of opportunistic uropathogens

Like uropathogenic *E. coli*, opportunistic bacteria require multiple virulence determinants. Opportunistic bacteria have a range of adhesins, fimbriate and afimbriate. The practical significance of many of these adhesins has yet to be elucidated but it is known that some of the fimbriae that are important for colonization are subject to phase-variable expression. For instance, *Proteus mirabilis* and *P. vulgaris* are opportunistic pathogens that cause lesions in the

urinary tract in dogs and horses. Both can express genes for a number of adhesins that bind specifically at different locations within the urinary tract. Phase variation is a factor in the strong predilection of *P. mirabilis* for the upper urinary tract where it causes pyelonephritis. It is believed that the pathogen uses one fimbrial type, *P. mirabilis* fimbriae (PMF), to become established in the bladder. Then some of the bacteria differentiate into the heavily flagellated swarming phenotype and proceed up the ureters to the kidneys, where they use another fimbrial type, mannose-resistant/*Proteus*-like (MR/P) fimbria, to adhere to epithelial cells of the kidneys.

Klebsiella pneumoniae is another opportunistic invader of the urinary tract that can express genes for a number of adhesins. Again, it is not known what role most of the adhesins play in the pathogenesis of urinary infection. Type 1 fimbriae are responsible for attachment of *K. pneumoniae* to uroepithelium in the bladder, initiating cystitis. They have been shown to bind to the cells of the proximal tubule in the kidney, and it is thought that they may be involved in the pathogenesis of pyelonephritis. However, when the bacterium invades the underlying tissues, it switches off the expression of type 1 fimbriae (phase variation) because the fimbriae trigger destruction of the bacterium by phagocytes.

In cattle and sheep, bacteria belonging to the *Corynebacterium renale* group of organisms (*C. renale, C. pilosum* and *C. cystitidis*) may move from the reproductive tract to the urinary tract. *Corynebacterium cystitidis* causes mild cystitis, while *C. renale and C. pilosum* cause severe haemorrhagic cystitis which may progress to ureteritis and pyelonephritis. This group of organisms and some other bacterial invaders of the kidneys, such as *Proteus* species, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Actinobaculum suis* (*Eubacterium suis*), produce urease (Box 89.1) which hydrolyses urea to ammonia and carbon dioxide and raises the local pH sufficiently to cause precipitation of magnesium and calcium salts from the urine as struvite and apatite uroliths. The increased alkalinity enhances bacterial proliferation, the ammonia damages the uroepithelium and the precipitates increase the inflammatory changes in the mucosa. In addition, the precipitates provide a nidus for bacterial growth in which the pathogens are protected from host defence mechanisms and from therapeutic agents. The stones can obstruct the flow of urine, diminishing the flushing out of bacteria. *Escherichia coli* does not produce urease and it is not directly associated with urolithiasis.

Box 89.1 Urease-producing bacteria.

- *Proteus* species
- *Klebsiella* species
- *Pseudomonas* species
- *Ureaplasma urealyticum*
- *Corynebacterium renale*
- *Staphylococcus pseudintermedius*
- *Actinobaculum suis (Eubacterium suis)*

A virulence factor for *Proteus mirabilis* is its polysaccharide capsule which facilitates adherence to the luminal surface of the uroepithelial cells. The adherent bacteria divide and form a biofilm. Calcium and magnesium ions may be precipitated within the biofilms, providing the bacteria with protection from host defences and interfering with the efficacy of antimicrobial therapy.

Responses of the host to ascending bacterial infection of the excretory pathway

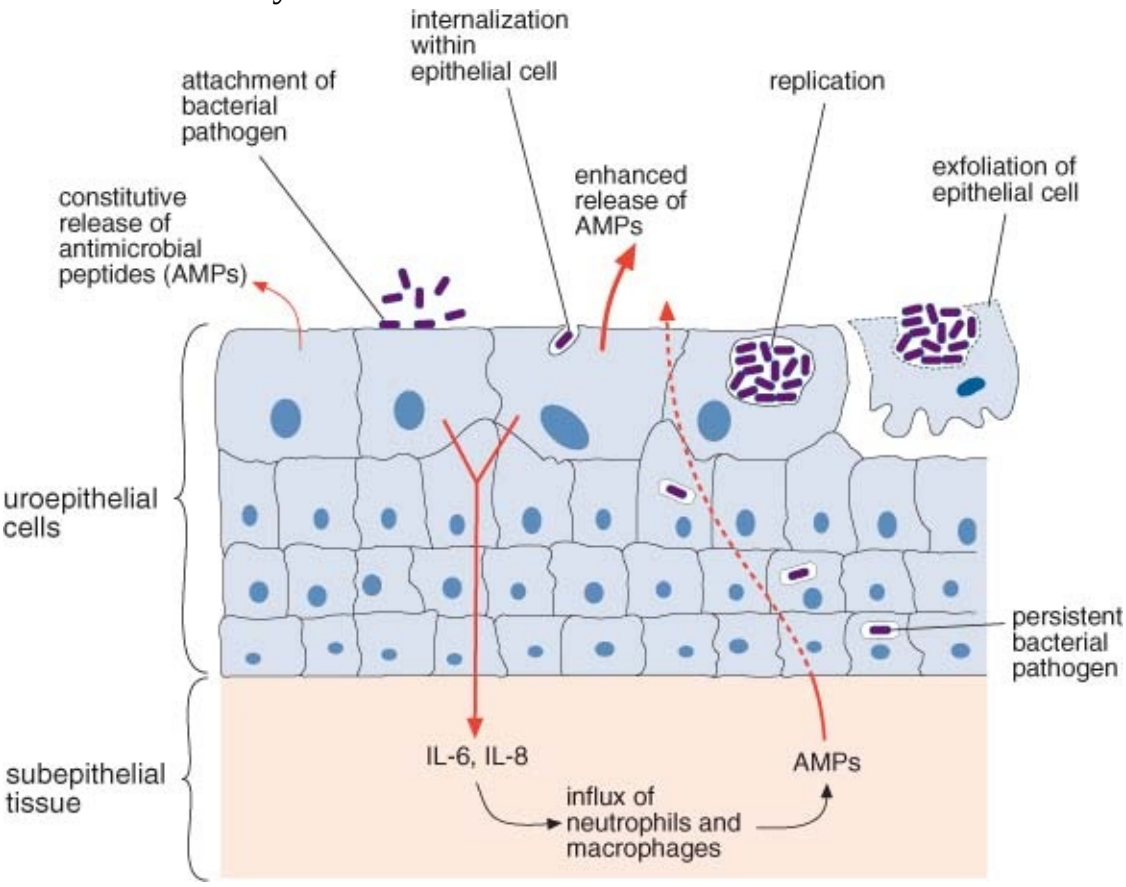
Although uropathogens are highly adapted for survival and proliferation in the urinary tract, they have to contend with an array of host factors that tend to prevent infection. Many uropathogenic *E. coli* are eliminated from the lower parts of the excretory pathway by the passage of urine: some floating free, some bound to secretory immunoglobulin A (sIgA) and adherent to (or within) neutrophils and others bound by the FimH adhesin to Tamm-Horsfall glycoprotein. The invading *E. coli* that become established within the bladder use their FimH adhesin to bind to mannose moieties of uroplakin on the luminal surface of the umbrella cells, an event that initiates a complex train of responses that determines the outcome of the infectious process. In the urinary bladder, uropathogenic *E. coli* cause cystitis; if they reach the kidneys they may cause pyelonephritis.

Cystitis

Pathogens may ascend to the urinary bladder from a distal location in the urogenital tract or, less frequently, they may descend from active lesions in the upper urinary tract. Attachment and internalization of the pathogens are essential

steps in the initiation of cystitis. Attachment activates the uroepithelium. The cell membrane of the umbrella cell expresses toll-like receptor 4 (TLR-4) which recognizes bacterial LPS and activates an innate immune response, triggering a cascade of signals (Fig. 89.3). This culminates in an inflammatory response, engulfment of the bacteria by the uroepithelial cells, exfoliation of umbrella cells and formation of intracellular bacterial communities with the possible survival of a dormant reservoir of uropathogenic *E. coli* within the undifferentiated layers of the uroepithelium. Such dormant reservoirs of *E. coli* may seed recurrent bouts of cystitis.

Figure 89.3 Cystitis: interactions of invading pathogens with the uroepithelial cells of the urinary bladder.



The activated uroepithelial cells secrete the cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) that mediate the early phases of the inflammatory reaction. In cystitis, IL-6 is found primarily in urine but in pyelonephritis it reaches measurable concentrations in both urine and serum. Systemic IL-6 induces fever and stimulates the liver to produce acute-phase proteins. It evokes both humoral and cell-mediated immune responses. It promotes the secretion of

immunoglobulins, including secretory IgA, and the proliferation of T lymphocytes. The local release of IL-8 induces neutrophils to leave the bloodstream and to mount an attack on the pathogens. The phagocytes migrate through the tissues and cross the uroepithelial barrier to interact with bacteria in urine. The pathogens release products such as α-haemolysin, LPS and CNF1, which may decrease chemotactic responses.

Although the host–pathogen interactions mediated by FimH activate a cascade of innate host defences, they also trigger a process by which the bacteria can induce their own transfer into the umbrella cells where they are less accessible to phagocytes, antibodies, T cell activity and antimicrobial therapy, and where the supply of nutrients and the conditions for growth are more favourable than in urine. Engulfment of the adherent bacteria by the umbrella cells is a consequence of FimH-induced rearrangements in the cytoskeleton of the umbrella cells and extension of the cell membrane around the bacteria. The engulfed bacteria grow and divide, establishing biofilm-like communities within the cytoplasm of the umbrella cells. The counter-response of the host to bacterial invasion is activated by FimH: the bacteria-laden umbrella cells show apoptotic-like changes and are shed into the urine, thus voiding both attached and intracellular pathogens. However, some of the pathogens are able to escape from the dying cells before the process of exfoliation is completed and they may gain entry into new umbrella cells or, perhaps more significantly, into the freshly exposed undifferentiated intermediate cells and basal cells. Bacteria that enter the less mature cells appear to enter a quiescent state, in which they can persist as an intracellular reservoir of pathogens from which they can be reactivated and trigger further episodes of cystitis.

Infectious diseases of the kidneys

Pathogens can reach the kidneys via the bloodstream or, more commonly, by ascending infection. Blood- borne pathogens target the renal cortex, whereas the ascending pathogens seek to establish a foothold in the renal medulla. The cortex and the medulla have different structures and functions and the invading pathogens have to contend with the consequences of these differences. To a large extent, the early interactions between the pathogens and the renal tissues in each of the two regions are determined by the structural adaptations of the local vasculature and of the associated tubules.

Structural features of the renal cortex

The architecture of the renal cortex is dominated by glomeruli, urinary tubules and capillaries packed in an inconspicuous interstitium. Within the kidney, blood is supplied to the renal cortex by afferent arterioles, each of which supplies two sets of capillary beds in series with an efferent arteriole as the bridging vessel. The first capillary network is the glomerulus. The second capillary network includes the peritubular capillaries in the cortex and the vasa recta in the medulla, both of which supply oxygen and important nutrients to the cells of the urinary tubules, deliver substances for secretion by those cells, and return reabsorbed water and solutes to the circulatory system. Up to 90% of the renal blood flow passes through the cortical glomeruli before entering the peritubular capillaries in the cortex; the other 10 to 15% passes through the juxtamedullary glomeruli on the way to the vasa rectae in the medulla.

The efferent arteriole, the second resistance vessel in the portal system, is a major determinant of blood pressure in both capillary beds. It provides the glomerulus with the high blood pressure (up to 60 mmHg) required to deliver an ultrafiltrate of the blood to the urinary tubules, and it ensures that the blood pressure in the second capillary bed (less than 20 mmHg) is sufficiently below the colloid osmotic pressure in the capillaries to facilitate the uptake of the water and solutes that have been reabsorbed from the filtrate by the tubular cells. The blood pressure and rate of flow are lower in the post-glomerular circulation than in other capillaries throughout the body, an important determinant in the responses of renal tissue to pathogens.

Structural features of the renal medulla

The interstitium is much more extensive in the medulla than in the cortex. When the tubular and the vascular elements enter the medulla, they both adopt a similar hairpin-like configuration before returning to the cortex. The descending and ascending limbs of the loop of Henle and those of the vasa rectae lie parallel to each other in the medullary interstitium. That configuration results in the filtrate flowing in opposite directions in the two limbs of the loop of Henle and, because of the permeability and specific ion transport properties of the two limbs, it operates as a counter-current multiplier to establish a large osmotic gradient in the peritubular fluid between the corticomedullary junction (iso-osmotic) and the inner medulla (hyperosmotic). The hyperosmolality is attributable in approximately equal measure to the accumulation of sodium chloride and of urea

in the interstitial fluid of the inner medulla. The countercurrent flow of blood in the hairpin-like vasa rectae plays a significant role in maintaining the osmotic gradient in the interstitium. The vessels are highly permeable to both water and solutes, which permits the blood they channel through the medulla to achieve osmotic balance with the surrounding interstitial fluid. Thus, the vasa rectae serve as countercurrent exchangers that remove excess water and solute that would dissipate the medullary gradient. The countercurrent exchange of water considerably increases the viscosity of blood in the vasa rectae; accordingly, for the countercurrent exchange to operate, it is essential that the haematocrit value of blood entering the vasa rectae should be low. Most measurements of the packed cell volume entering the vasa rectae give values of about 10% (Lote *et al*., 1996).

The medulla receives about 10 to 15 % of the total renal blood flow at low blood pressure and at a flow rate of less than one-fourth of the flow rate in the cortex. Because the number of blood cells per unit volume within the vasa rectae is less than in the systemic circulation and because the countercurrent flow facilitates diffusion of oxygen between the descending and ascending parts of the vasa recta, there is a considerable gradient in oxygen tension from the renal cortex to the medulla, to the extent that the inner medulla may be ‘living on the brink of anoxia’. These local conditions limit the antimicrobial activities of the medulla.

The responses of renal tissues to ascending infection

Vesico-urethral reflux facilitates the extension of infection from the urinary bladder to the renal pelvis. The urogenous pathogens cause pyelonephritis, a tubulointerstitial disease that results in destructive lesions in the renal pelvis and the underlying renal parenchyma.

Pyelonephritis

Pyelonephritis may be caused by specific urinary pathogens such as *Corynebacterium renale, C. cystitidis* and *C. pilosum* in cattle and *Actinobaculum suis* (*Eubacterium suis*) in pigs, or by opportunistic invaders such as *E. coli*, *Proteus* species, *Klebsiella pneumoniae* or *Morganella morganii* in many species of animals. Many of these pathogens use different types of adhesins in different regions of the renal system. For instance, most of the *E. coli* associated with pyelonephritis exercise phase variation when they reach the renal

pelvis, expressing P fimbriae rather than type 1 fimbriae by which, in the earlier phase, they attach to the lining of the bladder. Similarly, *Proteus* species use PMF fim-briae to colonize the urinary bladder and MR/P fimbriae to bind to the kidneys. These interactions are responsible for exposure of the renal tissues to bacterial toxins such as lipopolysaccharide (LPS), haemolysin, and CNF1; they also trigger intracellular activity of second messenger molecules that activate epithelial cells to release cytokines and chemokines. Although the primary objective of the host responses is to defend the structures and functions of the renal tissues, some of the responses contribute to pathological sequelae. Recruitment of neutrophils by IL-8 into infected tissue is essential for clearance of the pathogens but the lytic enzymes of the phagocyte may lead to local tissue damage.

Pyelonephritis begins as inflammation and necrosis of the renal papillae before it extends to the subepithelial tissues. Pathogens that penetrate the epithelial barrier in the renal medulla enter an environment that has structural and physiological features that favour bacterial survival and proliferation. The interstitial matrix is extensive and loose, which favours infiltration by pathogens and extension of the lesions they induce. Adjacent discrete lesions may coalesce. The defence mechanisms of the host are not optimal in the renal medulla. In the vasa rectae, the blood pressure is very low, the flow is slow, and the number of cells per unit volume of blood is low. Thus, phagocytes arrive slowly and in inadequate numbers during the early acute phase of pyelonephritis. Furthermore, the activities of those cells that migrate into the inner medulla are retarded by the very low oxygen tension and by the very high osmolality that prevail there. Some pathogens, such as *Proteus* species, *Klebsiella* species, *Pseudomonas* species and *Corynebacterium renale*, produce urease which hydrolyses urea to ammonia and carbon dioxide. High concentrations of ammonia can benefit pathogens in two ways: first, ammonia inhibits the activation of complement; secondly, it predisposes to the formation of uroliths in which the pathogens are protected from the defences of the host. The susceptibility of the renal papillae to infection provides pathogenic bacteria with a foothold in the kidney from which they migrate outwards through the inner and outer medulla to the cortex, typically inducing a wedge-shaped suppurative lesion in which there are intratubular and interstitial infiltrates, largely of neutrophils.

Responses of the kidneys to blood-borne challenges

Some of the more serious bacterial and viral diseases of the urinary system, notably tuberculosis, leptospirosis and infectious canine hepatitis, are blood-borne. In addition, blood delivered by the renal arteries may expose the kidneys to other challenges generated by bacteria such as bacterial toxins, circulating immune complexes, or antimicrobial antibodies that may cross- react with antigenic components of the glomerulus.

The distribution of the causal agents within the renal parenchyma is determined to a considerable extent by the unique adaptations of the renal vasculature. Immune complexes tend to lodge on the basement membrane of the glomerular filter, evoking inflammatory lesions in that location. Similarly, anti-streptococcal antibodies interact with epitopes in the filtration membrane and induce a proliferative glomerulonephritis. Approximately 90% of the post-glomerular blood flow remains in the renal cortex and as a result tubercular and leptospiral lesions are more frequent in the cortex than in the medulla.

Bacteraemia/pyaemia

Blood-borne clumps of bacteria or septic emboli are prone to lodge in the renal vasculature, predominantly in glomerular or peritubular capillaries within the cortex; less frequently they lodge in capillaries within the medulla. Typically, this is seen as multiple small abscesses in the renal cortex, a feature of several bacterial diseases, notably those in which emboli break away from umbilical lesions or from vegetative lesions on valvular endocardium.

Haematogenous dissemination of *Mycobacterium bovis* is likely to result in a number of small granulomatous lesions in the cortex of both kidneys. The initial entrapment of these acid-fast bacteria occurs in capillaries within or just distal to the cortical glomeruli. The large throughput and high oxygen tension of arterial blood within the glomerulus encourages the bacilli to proliferate, and on occasion this can lead to rupture of some of the capillary loops and the discharge of the organisms and cellular debris into the proximal tubule. In turn, passage of this infected material is likely to stall at the nadir of the loop of Henle from where the bacteria can break out to initiate lesions in the medulla. Enlargement and confluence of the lesions in this location are facilitated by the physical structure of the medullary interstitium, by local impairment of antibacterial activities due to the slow flow and low oxygen tension of the blood supply and by the hypertonicity of the interstitial fluid. Lesions in the medulla can erode the wall of the renal pelvis, enabling bacteria to gain access to urine and cause

descending infection of the excretory channels.

Renal damage by pathogenic leptospires

The urinary system plays a very significant role in the epidemiology of leptospirosis. New hosts acquire pathogenic leptospires by direct or indirect exposure to the urine of infected animals. The spirochaetes invade the host through cuts, abrasions, water- softened skin, or intact mucous membranes, and they pass, via lymphatics, to the bloodstream. Avirulent leptospires are cleared rapidly by phagocytosis whereas virulent organisms evade phagocytosis. The pathogens can survive engulfment by macrophages, in which they induce apoptosis. It has been suggested that the organisms use the phagocytic cells as a means of transport to preferred tissue sites (Merien *et al*., 1997). The leptospiraemic phase elicits an adaptive immune response which, typically, clears the bacteria from the bloodstream within 10 days. Less frequently, the leptospires proliferate rapidly and cause an acute illness that may culminate in death. In maintenance hosts, leptospires can persist in niches to which immunoglobulins do not have ready access, such as the proximal renal tubule, the aqueous humour of the eye, the cerebrospinal fluid and, in the case of some serovars, the female reproductive tract. The renal niche is of particular importance: the leptospires attach to the luminal surface of the epithelial cells of the proximal tubule and from there they can be shed into the urine, the principal vehicle for transmission of spirochaete infection.

Remarkably, prolonged persistence of leptospires within the renal tubule of the maintenance host appears to have little effect on the histology or function of the kidney. In contrast, haematogenous dissemination of leptospires in accidental hosts may give rise to systemic pathological changes and functional derangements of varying severity. Depending upon the severity and extent of the lesions in the kidney, the host may develop acute renal failure, chronic renal failure, or only mild and transient functional disturbances.

The basic lesion in the various organs and tissues is injury to the endothelium of small blood vessels. Blood and bacteria escape into the tissues where the disruption of normal vascular perfusion reduces oxygen tension and attenuates the innate antimicrobial responses, allowing the bacteria and their products to cause significant damage to the tissues and organs. In the kidney, the leptospires can escape from glomerular capillaries or from peritubular capillaries. Leptospires break out of the glomerulus and pass via Bowman’s space into the

proximal tubule. Spirochaetes that leave the peritubular capillaries enter the interstitial space in the renal cortex, where they induce lesions of tubulo-interstitial nephritis, manifested by necrosis of the epithelium and rupture of the basement membrane, oedema of the interstitium, and a mixed infiltrate of lymphocytes, monocytes and plasma cells, with occasional neutrophils. From the interstitial space, the leptospires move into the lumen of the proximal tubule where they reside as clumps of bacteria attached to the luminal surface of the epithelium and from whence they can be excreted in urine.

Systemic infection by pathogenic leptospires can give rise to a wide spectrum of responses including an acute life-threatening disease, a subacute moderately severe disease, a chronic disease, or a subclinical infection detected only by serology. Virulence of the leptospires is a function of components of the outer membrane that are exposed on the surface of the bacterium and are expressed during infection. The infected mammalian host responds promptly with an innate immune response followed by an adaptive immune response to constituents of the outer membrane. The major surface components are lipopolysaccharide (LPS), lipoproteins, and glyco-lipoprotein (GLP). Leptospiral LPS is at least tenfold less toxic than is LPS of other Gram-negative bacteria but, following recognition by CD14 and TLR-2, it activates renal epithelial cells, macrophages and B cells and, thus, it is central to both the innate and the adaptive immune responses. LPS is the target for circulating antibodies that opsonize leptospires for phagocytosis by macrophages and neutrophils. The specific epitopes that induce protective antibodies reside in the polysaccharide side-chains of LPS. Thus, the induced antibodies protect against serovars that possess the specific epitopes but do not protect against serovars that lack these antigenic determinants. A number of surface lipoproteins also activate an innate immune response, after interaction with CD14 and TLR-2 receptors. Two surface proteins have been identified as adhesins that attach to components of the extracellular matrix: one mediates attachment to laminin, the other to fibronectin. The adhesin responsible for attachment to the epithelium of the proximal tubule has not been identified but that function may be exercised by surface-exposed proteins recently identified and referred to as Lig (leptospiral Ig-like) proteins. Many pathogenic leptospires release haemolysins. For instance, *L*. Pomona releases a haemolysin that induces haemolytic anaemia in young calves. An outer membrane GLP is cytotoxic and, because it inhibits the activity of sodium pumps in epithelial cells of renal tubules, it predisposes to hypokalaemia.

Damage by microbial toxins

Several bacterial species release toxins that damage the tubular epithelium. The epsilon toxin of *Clostridium perfringens* type D causes degenerative changes in the epithelial cells of both the proximal convoluted tubule and the distal convoluted tubule. The damage is significant in both regions despite the fact that the luminal surface of the proximal tubule does not have the specific receptors for the toxin that are present on the distal tubule of a number of species, including sheep, cattle and humans. The pulpy kidney found at necropsy of lambs which have died from acute type D enterotoxaemia is a product of both the tubular degeneration and the very rapid onset of autolysis that is characteristic of the disease. In humans, some strains of *E. coli*, such as O157:H7, release a shigatoxin that damages endothelial cells in glomeruli and peritubular capillaries and has a direct toxic effect on tubule cells in both cortex and medulla

Immune-mediated diseases

Immune-mediated diseases of the kidneys fall into two categories: those that begin when antigen– antibody complexes, preformed in the bloodstream, are trapped at the glomerular filtration membrane (immune-complex glomerulonephritis), and those in which the pathological processes begin when antimicrobial antibodies bind to constituents of the glomerular basement membrane, thus creating novel complexes *in situ* (anti-GBM disease). The antibody component of a preformed complex may have been elicited by a microbial antigen or by a novel antigen that had been expressed when a pathogen had damaged host cells or tissues. Immune-complex glomerulonephritis has been described in canine pyometra and in diseases of viral origin, such as infectious canine hepatitis, feline leukaemia, equine infectious anaemia, feline infectious peritonitis, classical swine fever, and African swine fever.

In anti-GBM disease, circulating antibodies to a microbial antigen bind to either a homologous epitope that is a native constituent of the glomerular basement membrane or to a non- glomerular antigen that has interacted with components of the glomerulus and has been incorporated (‘planted’) within the membrane.

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Chapter 90

Microbial diseases of the cardiovascular system

The cardiovascular system consists of the heart, the pulmonary circulation and the systemic circulation. In this system, blood flow is maintained by the activity of the two adjacent pumping chambers of the heart (Fig. 90.1). The right ventricle pumps blood through the pulmonary circulation, a low-pressure system that replenishes the supply of oxygen in exchange for carbon dioxide. Simultaneously, the left ventricle pumps blood through the systemic circulation, a higher pressure system that transports and distributes oxygen and other essential substances to the tissues and removes by-products of tissue metabolism, including non-volatile substances. Blood returns to the right atrium via the venous limb of the systemic circulation and is then pumped through the pulmonary circulation before it returns to the arterial limb of the systemic circulation. The indispensable distributive functions of this closed circulatory system can be damaged by microbial diseases that compromise the pumping activities of the heart or restrict the flow of blood through the larger blood vessels.

The heart: structural and functional relationships

The pumping function of the heart can be disturbed by microbial diseases that affect the pericardium, the myocardium, or the endocardium.

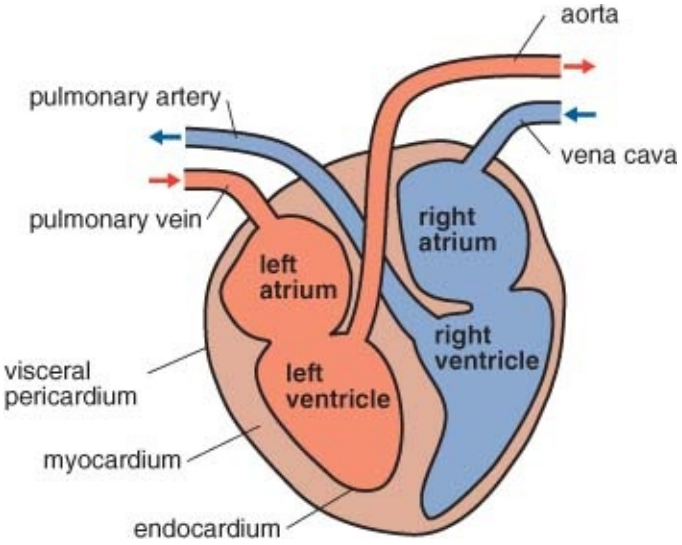
Pericardium

The pericardium is a fibrous sac within which the heart is suspended from the great vessels of the systemic and pulmonary circulations. The sac, which anchors the heart within the mediastinum, is composed of two layers: a thick outer fibrous layer and a thin inner serous layer. The fibrous layer is composed mostly of collagen; it is stiff and relatively inelastic. Because of its low compliance, the

fibrous layer prevents overextension of the myocardium during acute increases in intracardiac volume, as it does during administration of large blood transfusions. It can expand to accommodate gradual increases in intra-pericardial pressure, as it does when the heart enlarges during prolonged training of human athletes, greyhounds and racehorses. However, an acute increase in intrapericardial pressure causes the pericardium to stiffen rather than to expand. This can have a significant impact on the work of the heart.

The inner layer of the pericardium consists of a sheet of mesothelial cells that covers the outer surface of the heart, as the visceral pericardium or epicardium, and then is reflected on to the inner surface of the fibrous pericardium as the parietal pericardium. Thus, the parietal and visceral portions of the serous pericardium surround a closed space, the pericardial cavity, into which the mesothelial cells secrete pericardial fluid, a lubricant that minimizes friction during cardiac movement. Under physiological conditions, the pericardial cavity contains a small volume of fluid (less than 10 ml in the dog, and about 20ml in the horse). It has a modest reserve capacity, provided by the normal slight slackness of the fibrous sac and by anatomical recesses and sinuses of the serous layer. Because of the low compliance of the stiff fibrous pericardium on its outer boundary, any increase beyond the reserve capacity of the pericardial cavity results in a steep increment in intrapericardial pressure and interference with the pumping activity of the heart.

Figure 90.1 Schematic representation of the mammalian heart showing two adjacent pumping chambers. The chamber on the right delivers blood at low pressure to the pulmonary circulation. The chamber on the left delivers blood at high pressure to the systemic circulation.



Myocardium

The energy for pumping blood around the circulatory system is generated by the myocardium, which is composed of striated cardiac muscle cells (cardiomyocytes) that fall into two broad classes: those that contract and those that conduct the action potentials that excite the contractile elements. The efficiency of the pumping activity of the heart depends on the functional integrity of its various parts and on an ordered sequence of events initiated by the electrical pacemaker in the sino-atrial node. From the pacemaker the cardiac impulse spreads over the atrial wall to reach the atrioventricular node. It then passes to the ventricles via the bundle of His and its ramifications Myocardial lesions that involve the pacemaker or the conduction system can disturb the cardiac rhythm and interfere with the function of the cardiac pump.

Blood is pumped during contraction of the ventricles (systole); the heart refills during the phase of myocardial relaxation (diastole). In diastole, the walls of the atria and of the ventricles are relaxed and the atrio-ventricular valves are open, allowing the heart to act as if it has a single chamber on either side; blood from the great veins passes freely through the atria and the open valves into the relaxing ventricles. During the initial phase, filling of the ventricles is very rapid because the atria are maximally filled just prior to the opening of the atrioventricular valves. In the final filling phase, the atria contract (atrial systole) forcing more blood into the ventricles. Thus, ventricular filling is mostly passive and depends on the venous return. An increase in volume or speed of venous return increases the quantity of blood ejected by a single contraction, the stroke volume; decreased venous return causes a reduction in stroke volume. An example of this physiological principle is seen in constrictive pericarditis. As the disease progresses, the pressure exerted on the relaxed myocardium by the fibrotic pericardium can grow to exceed the normally low diastolic pressure within the right atrium, thus impeding filling of that chamber and, consequently, reducing stroke volume.

Endocardium

The endocardium lines the chambers of the heart, including the heart valves. It consists of connective tissue underlying a layer of endothelial cells that is continuous with the endothelium of the vessels entering and leaving the heart. The endothelial surface has a negative charge which repels circulating blood cells, also negatively charged. The intact endothelium does not interact with

normal platelets. In contrast, constituents of the subendothelial layer are highly attractive for blood cells and, when breaks in the continuity of the endothelium expose them to blood, platelet plugs initiate thrombus formation at the sites of injury. Injury to the endothelial surface occurs most frequently near the free ends of the cardiac valves where the cusps are apposed as the valves close. These endothelial cells are particularly vulnerable to injury because of repeated physical contact at closure and because the vessels supplying blood to the cardiac valves do not extend beyond the proximal third of the cusp (Leask *et al*., 2003). Infection of thrombi by bacteria or fungi leads to the development of vegetative lesions that result in stenosis or incompetence of affected valves. Incompetence or stenosis of a valve can result in a high-velocity jet stream that causes turbulent blood flow and damages the mural endothe-lium (‘jet lesion’). Deposition of platelets and fibrin at the site of the jet lesion results in the formation of a sterile mural thrombus, to which blood-borne bacteria may adhere.

Infections of the heart

Pathogens may invade cardiac tissues by the haema-togenous route, by extension from a focus of infection elsewhere in the thoracic cavity, or as a result of traumatic penetration by a foreign body. Blood-borne viruses or bacteria may invade cardiac tissues in the course of a systemic disease, as occurs with foot-and-mouth disease in neonatal calves or with *Staphylococcusaureus* septicaemia in lambs and calves. The heart may be colonized by blood-borne bacteria derived from ongoing septic lesions in the udder, uterus or feet or as a result of an episode of transient bacteraemia arising from endogenous flora. Mammals are hosts to many microorganisms, the vast majority of which inhabit the skin and mucous membranes as commensals (‘normal flora’), while a few may be responsible for local inflammatory reactions such as impetigo or gingivitis. Periodically, small numbers of bacteria from these sources gain entry into the bloodstream, where they encounter the bactericidal activity of plasma and the phagocytic activity of neutrophils. Thus, the bacteraemia tends to be transient; however, it may result in colonization of the myocardium. Normal vascular endothelium is an efficient barrier that serves to protect host tissues and organs from invasion by blood-borne bacteria but if the endocardium is damaged, even a transient bacteraemia may result in infection of the heart.

When pathogenic viruses or bacteria invade cardiac tissues, they trigger an

innate immune response. Host cells, both parenchymal cells and cells of the immune system, secrete chemokines, nitric oxide and the proinflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ. Macrophages and natural killer (NK) cells are mobilized and activated and enter the affected areas in response to microbial invasion.

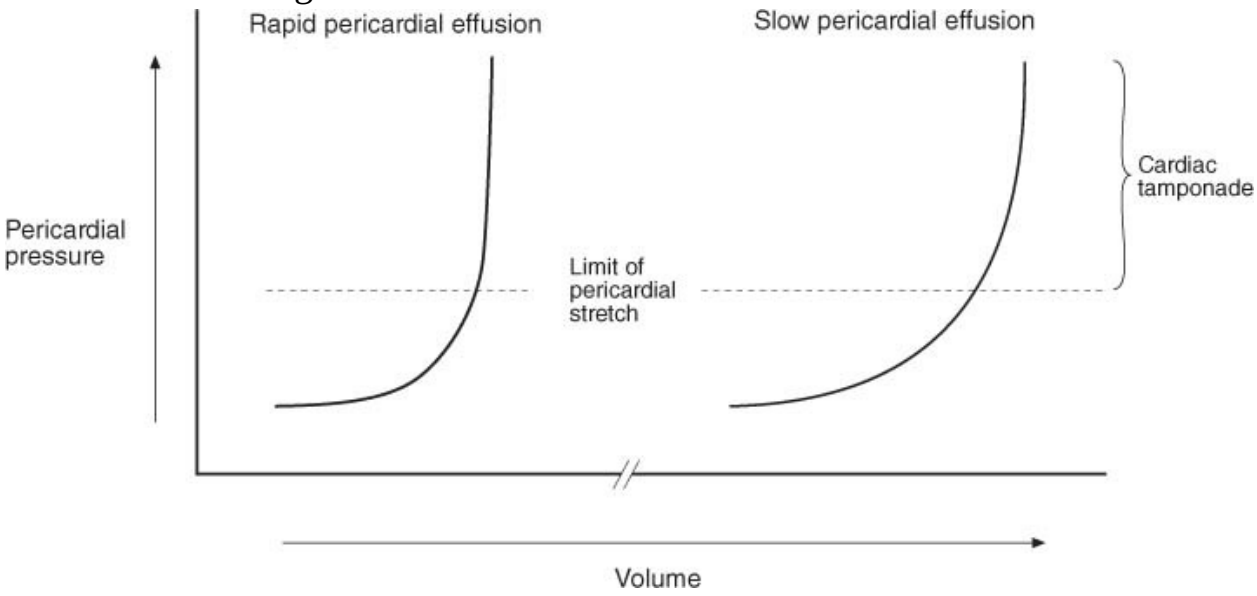
Pericarditis

Although the pericardium may be involved in a variety of microbial diseases, many pericardial lesions are not the prime cause of morbidity and mortality. Nevertheless, pericardial lesions are not insignificant: they cause haemodynamic disturbances that contribute significantly to systemic pathophysiology, even in acute septicaemic conditions. Depending on the pathogen involved, the pericardium mounts an inflammatory response that may be fibrinous, serofibrinous, fibrinohaemorrhagic or purulent in nature. The presence of fibrin in the exudate predisposes to formation of adhesions between the parietal layer of the pericardial cavity and its visceral layer (the epicardium), while the accumulation of fluid in the pericardial cavity raises intrapericardial pressure which may affect cardiac function as a result of compression.

The effects of pericardial effusions on cardiac function are influenced by the rate at which the fluid accumulates (Fig. 90.2). A rapid increase in content of the pericardial cavity soon exceeds the reserve capacity. The fibrous pericardium stiffens with the result that the heart has to compete with excess fluid for space within the pericardium. Further acute increases in the volume of pericardial fluid result in steep increments in intrapericardial pressure, leading to compression of the heart, particularly of the thin-walled right atrium and right ventricle. Compression (tamponade) interferes with filling of the right atrium during diastole, resulting in increased venous pressure and systemic venous congestion, with distension of the jugular veins, prominent jugular pulse, and ventral oedema. In turn, decreased venous return leads to a decrease in ventricular output. Thus, at an advanced stage, the condition represents right-sided, low-output heart failure. In contrast, when an effusion accumulates slowly, the fibrous pericardium can stretch gradually to accommodate a volume that may expand to be several times the reserve capacity, sometimes without clinical evidence of right-sided heart failure. In the horse, the stretched pericardium can accommodate several litres of pericardial fluid, as it does in the subacute cardiac form of African horse sickness. The volume of suppurative exudate in traumatic

pericarditis in cattle may be up to 4.5 litres or more.

Figure 90.2 Changes in pericardial pressure associated with cardiac tamponade. Pericardial pressure curve on the left shows the steep rise in pressure when the volume of pericardial fluid quickly exceeds the limit to which the parietal pericardium can stretch. The curve on the right represents a slower rate of filling of the pericardial sac, which gives the parietal pericardium more time to accommodate a larger volume of fluid.



Suppurative pericarditis in cattle is usually a sequel to penetration of the wall of the reticulum by a sharp foreign body, such as a nail or a piece of wire which then perforates the diaphragm and the pericardium. The result is a fistulous tract that can introduce a range of pathogens, such as *Arcanobacterium pyogenes, Pseudomonas aeruginosa* and *Fusobacterium necro-phorum,* into the pericardial cavity. The pericardial exudate is copious, fibropurulent and putrid. The toxic effects of the pathogens and of the putrid exudate are responsible for the systemic responses: fever, depression and leukocytosis. The volume of the sequestered exudate and the pericardial adhesions that develop over time impair cardiac function, leading to tamponade and right-heart failure. In animals that survive the systemic illness, the pericardial lesions undergo resolution or organization. In non-suppurative pericarditis, the fluid is resorbed and residual lesions vary in extent from string-like adhesions between the two pericardial surfaces to diffuse fibrosis which obliterates extensive segments of the cavity and constricts the heart. In suppurative pericarditis, organization of the adhesions may be so extensive that the pericardial cavity is obliterated. This chronic constrictive pericarditis interferes with cardiac filling and cardiac output, and

leads to compensatory hypertrophy of the myocardium and, eventually, to congestive heart failure.

Myocarditis

Inflammation of the myocardium develops in the course of many infectious and toxic diseases but is an uncommon primary lesion. Myocarditis commonly occurs as a metastatic lesion in septicaemic diseases and in pyaemic infections of young animals. In other circumstances, the inflammatory reaction may be a local response to necrosis of myocardial cells. For instance, when neonatal calves, lambs, or piglets die suddenly during outbreaks of foot-and-mouth disease, the inflammatory changes in the myocardium are secondary to virus-induced necrosis of the cardiac muscle cells.

Myocarditis may result from invasion of the cardiac muscle by bacteria, viruses, protozoa or fungi. Also, it may be a consequence of circulating toxins or of infection-induced immune reactions. The inflammatory process may be focal or diffuse; the primary damage may be to the cardiomyocytes, the conducting system, the vascular elements, the autonomic nerves or the interstitium. Clinical signs depend on the number, size, or location of the lesions. Some focal lesions may not cause clinical signs. Sometimes, signs of cardiac disease may be cloaked by the primary systemic illness. Lesions that impinge on the conduction system can interfere with the electrical conductance of the heart, resulting in partial or complete heart block and associated arrhythmia. Thus, progressive myocar-dial damage can give rise to exercise intolerance, cardiac arrhythmia, or sudden death. In some cases of acute myocarditis, the lesions may resolve completely or with only insignificant residual scars; other cases may progress to dilated cardiomyopathy and chronic congestive heart failure.

Bacterial myocarditis

Bacterial myocarditis tends to be focal in distribution, often in the form of miliary lesions resulting from haematogenous dissemination of pyogenic pathogens. Foci of acute suppurative myocarditis are a feature of septicaemia due to *Listeria monocytogenes* or *Actinobacillus equuli*. Other necrotizing bacteria, such as *Arcanobacterium pyogenes, Pseudomonas aeruginosa, Rhodococcus equi, Fusobacterium necrophorum, Clostri-dium piliforme*, staphylococci and streptococci, reach the myocardium from primary foci elsewhere in the body, as in navel ill, strangles, or metritis.

Necrotizing and haemorrhagic myocardial lesions due to *Clostridium chauvoei* have been responsible for sudden deaths in calves and lambs without evidence of blackleg lesions in skeletal muscle. In cattle, the myocardial form of the *Histophilus somni* disease complex may cause sudden death in animals that have not shown previous signs of illness, or it may develop as a sequel to an episode of respiratory disease.

Depletion of the number of circulating granulocytes can predispose animals to bacterial myocarditis. Infection with certain strains of *Anaplasma phagocytophilum* that cause tick-borne fever in ruminants results in significant reductions in B and T lymphocyte and neutrophil numbers in young lambs, increasing susceptibility of these animals to *Staphylococcus aureus*, the causal agent of tick pyaemia. Staphylococci, introduced into the tissues by tick bites, enter the bloodstream and become widely disseminated within the body, forming abscesses in many internal organs including the myocardium. Severe depression in the number of platelets and neutrophils also occurs in bracken fern poisoning in ruminants. The thrombocytopaenia is responsible for multiple haemorrhages throughout the body including the intestinal mucosa, which enable members of the intestinal microflora to invade the bloodstream. The neutropaenia weakens the ability of the host to terminate the bacteraemia, and the bacterial emboli proceed to cause multiple small lesions in the myocardium.

Bacteria may enter the myocardium from primary lesions in the pericardium or in the endocardium. In cattle, myocarditis may arise as an extension from traumatic pericarditis caused by an infected foreign body penetrating from the reticulum. This condition can involve a range of bacteria that may include *Arcanobacterium pyogenes*, staphylococci, streptococci, and *Histophilus somni*. Friable vegetations on the cardiac valves can be a source of infected emboli that lodge in the myocardium (Table 90.1).

Viral myocarditis

Myocarditis has been observed in several viral diseases of animals. In many of these diseases, the myocardium is not a primary target of the virus; however, extension of a systemic viral infection to the myocardium is likely to increase morbidity and mortality because virus-induced degeneration of cardiomyocytes and the associated inflammatory response cause haemodynamic disturbances that impair the homeostatic responses of the cardiovascular system to the systemic pathological changes.

Table 90.1 Bacterial infections of the myocardium which can derive from valvular lesions.

Host species	Bacterial species
Horses	*Streptococcus* species, *Rhodococcus equi*, *Pseudomonas aeruginosa* or *E. coli*
Cattle	*Arcanobacterium pyogenes*, staphylococci, streptococci, *Pseudomonas* species
Pigs and sheep	*Streptococcus* species, *Erysipelothrix rhusiopathiae*
Dogs and cats	*Streptococcus* species, *Bartonella* species

In viral myocarditis, two pathogenic mechanisms contribute to the destruction of myocardial tissue: virus-induced cytolysis and immune-mediated tissue damage. Each of these mechanisms is responsible for a distinct phase of the pathological process. Virus-induced cytolysis is the dominant feature of the initial acute phase during which viruses enter and replicate within the cardiomyocytes, causing focal areas of necrosis in the myocardium. At this early stage, only a few inflammatory cells are associated with the necrotic lesions. The advent of viruses activates an innate immune response by the myocardium. The cardiomyocytes possess toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) that recognize viral pathogens and trigger intracellular signalling cascades that result in the expression of mediators and effectors of the innate immune response, including the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ. The cytokines TNF-α and IFN-γ facilitate the generation of nitric oxide, which inhibits virus replication. Virus replication and cardiac damage are increased in animals subjected to strenuous exercise or to corticosteroid therapy. The innate immune response generates a wave of inflammatory cells, mainly natural killer (NK) cells, γδ T cells and macrophages, which enter the affected areas where the mononuclear cells provide an additional source of pro-inflammatory cytokines. Some large NK-like cells in this first wave of inflammatory cells release perforin molecules that cause lethal damage by forming pores in the cell membranes of the cardiomyocytes. In conjunction with the cytokines and nitric oxide, the mononuclear cells begin to eliminate the virus from the myocardium, a process that is enhanced when the myocardium is infiltrated by another wave of mononuclear cells which marks the onset of an adaptive immune response.

The second wave of cells is dominated by lymphocytes, largely T cells and a smaller number of B cells. Both $CD4^+$ helper T cells and $CD8^+$cytotoxic T cells are required for the development of myocarditis. It seems that $CD4^+$T cells are responsible primarily for the induction of the adaptive immune response, while $CD8^+$T cells are necessary for the production of lesions (Rose *et al*., 1993).

Both T cells and B cells play important protective roles in limiting proliferation of the viral pathogens: the cytotoxic T cells by destruction and elimination of virus-infected cardiomyocytes, the B cells by producing neutralizing antiviral antibody. On many occasions, these cellular and humoral immune responses are sufficient to clear the viral infection, often with little residual damage. Ideally, the local immune response should revert to its resting state once the viral infection has been controlled. However, spontaneous resolution of the inflammatory response does not always follow clearance of the virus. As a consequence, the host is likely to develop immune-mediated myocardial lesions. Continuous, excessive expression of pro-inflammatory cytokines and protracted activation of T cells can cause even greater damage to the cardiomyocytes than the primary virus infection. Some cardiomyocytes may become targets for the immune system because injury during the early phase of the infection allows antigen-presenting cells access to myocardial antigens such as cardiac myosin that are normally sequestered within the cell. Thus, this second phase (chronic phase) of the pathological process is an autoimmune response provoked by the initial virus infection. Other cardiomyocytes become targets because they express antigens that are structurally similar to viral epitopes to which the host has developed an immune response.

Persistent activation of responses can cause extensive destruction of cardiomyocytes and, since the myocardium lacks the ability to produce a new generation of muscle cells to replace them, the loss of contractile elements is permanent. The surviving muscle cells hypertrophy to maintain cardiac output. However, this compensatory response may not be sufficient to offset further deterioration in cardiac function. Loss of muscle cells is followed by the deposition and accumulation of collagen, a relatively inelastic material in the extracellular spaces. Progressive accumulation of this fibrillar collagen stiffens the myocardium and, in conjunction with progressive hypertrophy of muscle fibres, it changes the size and shape of the cardiac chambers. Stroke volume is reduced because the collagen interferes with relaxation of the myocardium during diastole and with contractility during systole. Excess collagen within the interstitium may encase muscle fibres, disrupt orderly transmission of force between contracting myocytes, and thereby impair the pumping activity of hypertro-phied hearts. Ultimately, the progression of these changes in structure and function leads to the development of cardiomyopathy and cardiac failure.

In general, viral myocarditis occurs more often and with a higher mortality rate in young animals than in adult animals. For some viruses, such as canine

parvovirus, susceptibility of young hosts to infection may be associated with the presence of mitotically active cells in the myocardium of the neonatal animal. For other viruses, age-dependent susceptibility is determined by changes in the expression of specific receptors. For instance, mouse models of myocarditis induced by coxsackievirus B have revealed that susceptibility to the virus declines in parallel with a spontaneous decline in expression of the coxsackievirus-adenovirus receptor on myocardial cells as the host progresses from foetal life to adulthood. The patterns of acute viral disease in adult animals reflect the replicating tissues available when the virus first infects its host and are often different from those induced by the same virus in the foetus or neonatal animal.

Inflammation of the foetal myocardium is a feature in a number of viral diseases that cause reproductive failure, manifest by late abortions, stillbirths, or the birth of premature, weak offspring of poor viability. Susceptibility of young animals to myocarditis is a feature of encephalomyocarditis virus disease in pigs. This cardiovirus is primarily a pathogen of rodents but it is transmissible to pigs. Although the infection tends to remain subclinical in many pigs, the virus can induce acute myocarditis that gives rise to two distinct patterns of disease. In gilts and sows, the cardiac lesions cause stillbirths and mummified foetuses. In unweaned piglets and fattening pigs, the myocarditis is responsible for sudden deaths. Porcine circovirus 2 (PCV2) has also been shown to be a cause of myocardial lesions. In herds where post-weaning multisys-temic wasting syndrome is present, pregnant sows and gilts often transmit PCV2 transplacentally during the third trimester. This leads to the delivery of stillborn foetuses and weak piglets; many of these offspring have focal areas of myocarditis in which large amounts of PCV2 antigen can be demonstrated. Opriessnig *et al*. (2006) attributed the sudden deaths of three previously healthy pigs, aged between 4 and 7 weeks, to myocarditis due to natural infection with PCV2.

Foot-and-mouth disease (FMD) is a vesicular disease with a low mortality rate in adult cattle and sheep, but in calves and lambs FMD virus frequently causes acute myocarditis and sudden death. The affected myocardium shows gross whitish lesions that give the heart a streaky appearance, described as ‘tiger heart’. Sudden death and streaky myocardial lesions are seen also in canine parvovirus infection. In 1978, when parvovirus infection was identified as a significant cause of disease in dogs, it was recognized that the small intestine was a target organ for the virus in dogs of any age but that the virus had a

predilection for the myocardium in pups up to 8 weeks of age. Typically, in these animals the virus-induced diffuse lesions consisting of foci of mononuclear cells associated with degenerating cardiomyocytes, some of which had basophilic intranuclear inclusion bodies. At that time, acute viral myocarditis was responsible for a high mortality rate in neonatal pups. In the intervening years, natural exposure and vaccination have raised immunity to the virus in the adult dog population and, consequently, there has been a substantial decline in the susceptibility of neonatal pups to the cardiac form of parvoviral disease. The viruses of canine distemper and infectious canine hepatitis also show a predilection for the myocardium in neonatal and unweaned pups.

Endocarditis

Endocarditis is usually restricted to the cardiac valves but it may extend to the mural endocardium lining the chambers. In all species, single or multiple lesions may develop on any of the valves but in some species there is variation in relative frequency among the valves. In cattle, infective endocarditis occurs most frequently on the tricuspid valve; in other species it is more common on the mitral valve. The lesions are found near the free ends of the valves on the atrial surfaces of mitral and tricuspid valves and on the ventricular surfaces of semilunar valves. Although most cases of infective endocarditis are due to bacterial infection, some are caused by fungi (Table 90.2). The bacteria that infect the cardiac valves may enter the bloodstream during the acute phase of a systemic infection or they may arise from an existing subacute or chronic focal lesion in a peripheral organ. It is recognized in human medicine that infective endocarditis is often due to transient bacteraemia that is either spontaneous or a sequel to mechanical manipulation of mucosal surfaces as in dental, endoscopy or biopsy procedures. Although the bacteraemia is often of low grade (less than 10 colony-forming units per ml) and it is cleared within 15 to 20 minutes (Seifert and Wisplinghoff, 2005), it can result in colonization of the endocardium.

Table 90.2 Microbial pathogens which have been isolated from lesions of endocarditis in animals.

Host species	**Microbial isolates**
Horses	*Actinobacillus equuli, Streptococcus equi, Streptococcus zooepidemicus, Pseudomonas aeruginosa, Rhodococcus equi, Aspergillus* species
Cattle	*Arcanobacterium pyogenes,* α-haemolytic *streptococci, Staphylococcus aureus, Pseudomonas aeruginosa*
Sheep	*Enterococcus faecalis, Erysipelothrix rhusiopathiae*
Pigs	*Streptococcus species, Erysipelothrix rhusiopathiae*

Dogs	*Streptococcus* species, *Staphylococcus aureus, Escherichia coli, Bartonella* species, *Erysipelothrix tonsillarum*
Cats	*Streptococcus* species, *Bartonella henselae*

The inflammatory process can be initiated by some virulent bacteria, such as *S. aureus*, that are able to bind to undamaged endothelial cells on a cardiac valve. Attachment of *S. aureus* to the endocardium, whether to undamaged endothelial cells or to preformed thrombus on damaged endothelium, is mediated by fibrinogen-binding proteins. Bacteria that require pre-existing damage to the endothelium enter the process at a later stage. They attach to a sterile thrombus that has formed where the continuity of the endothelial cells has been broken. This infected thrombus then develops into a vegetative lesion (cauliflower-like) on the cardiac valve. The critical elements in the pathogenesis of the vegetative lesion are focal damage to the endocardium, formation of a sterile thrombus at that site, persistent or intermittent bacteraemia, colonization of the thrombus by the blood-borne bacteria, and serial additions of platelets and fibrin as the bacteria proliferate in the thrombus.

Usually, the lesion begins where damage to the endothelium of a valve has exposed the subendothelial tissues. Circulating platelets adhere to collagen in the exposed tissues, and the aggregated platelets degranulate and stimulate the deposition of fibrin. This leads to the formation of a sterile thrombus accompanied by an inflammatory response. Some blood-borne bacteria are able to adhere to components of this sterile thrombus. The bacteria proliferate, and stimulate deposition of more platelets and fibrin, thus promoting the growth of the infected thrombus within which the bacteria are protected from host defence mechanisms. The result is continued enlargement of the thrombus by serial deposition of layers of platelets and fibrin. The vegetative lesions are friable and liable to shed emboli into the circulation, leading to infarcts and metastatic foci of infection in peripheral organs, especially in the lungs when the emboli enter the pulmonary circuit and in the kidneys when the emboli are delivered by the systemic circulation. The infection may extend to the myocardium or the pericardium. In mature thrombi, organization of the deposits of fibrin to fibrous connective tissue proceeds from the base, producing irregular, nodular, verrucose (wart-like) lesions.

Infections of the vascular channels

The vascular system can be divided arbitrarily into the arterial system, the microvasculature, the venous system, and the lymphatic system (Maxie and

Robinson, 2007). The microvasculature, comprised of arterioles, capillaries and venules, is the exchange system in which fluids, gases, electrolytes, hormones, nutrients, inflammatory cells and waste products are transferred between the blood and the extravascular tissues. The endothelium and the associated basal membrane constitute the main permeability barrier across which the exchanges take place. In addition to its function as a permeability barrier, the endothelium plays important roles in the development and remodelling of the vasculature, in the maintenance of vascular tone, and in the prevention of intravascular coagulation. Remodelling of blood vessels is an important feature of host responses to tissue damage induced by pathogens.

Endothelium plays a key role in the host response to infectious agents by regulating the extravasation of leukocytes and by producing inflammatory cytokines. When inflammatory cells are not required in the tissues, the endothelium retains the leukocytes within the bloodstream. When a pathogen invades, the endothelial cells of the postcapillary venules can be activated to express adhesion molecules and to secrete chemokines that promote the emigration of leukocytes into tissues where the cells are required.

The definition of inflammation as a local reaction of vascular connective tissue to injury places the vessels of the microcirculation at the core of all inflammatory responses to pathogens. The participation of the microcirculation in host responses to pathogens is a recurring theme in the chapters that deal with infections of individual body systems. In this chapter, the focus is on the pathological effects of infectious agents on the structure and function of all categories of vascular channels, including arteries, veins and lymphatics.

Inflammation can develop independently in the three categories of large vascular channels as arteritis, phlebitis or lymphangitis. When all three types of vessels are involved simultaneously, the term vasculitis is applied to these vascular changes. The histopathological features of vasculitis include the presence of inflammatory cells within and around the wall of a blood vessel accompanied by damage to the wall in the form of deposition of fibrin, degeneration of collagen, and necrosis of endothelial cells and smooth muscle cells (Maxie and Robinson, 2007). Vasculitis is an important component of many diseases of animals.

An infected lesion in the wall of an artery may arise by inward extension from infected tissue through which the vessel travels. Alternatively, the vascular lesion may be an outward extension of a response to a blood-borne pathogen that has invaded the wall of the vessel from the lumen or from the *vasa vasorum*. A

number of bacteria, particularly rickettsiae, and some viruses, show tropism for endothelial cells. Amongst such endotheliotropic agents are the bacteria *Histophilus somni*, the rickettsiae responsible for heartwater in ruminants and for Rocky Mountain spotted fever in dogs, and the viruses of equine viral arteritis, African horse sickness, classical swine fever, and African swine fever. Some of the damage caused by these endotheliotropic pathogens can be attributed to interference with the actions of the endothelium as the permeability barrier between the bloodstream and soft tissues. For example, the widespread petechial haemorrhages in serous membranes and the leakage of fluid into body cavities and perivascular tissues are due to increased permeability of capillaries. However, the endothelium does much more than act as a passive barrier. Endothelial cell activation is an integral part of acute inflammation, a process in which the local microvascular endothelial cells are both active participants and regulators of the cellular and tissue responses (Pober and Sessa, 2007). Endothelial cells produce and release a variety of mediators that are able to influence cellular and organ function throughout the body.

The structure of capillary endothelial cells and the mediators they release vary with anatomical location. The vascular bed of an individual tissue is adapted to meet the diverse needs of the tissues it supplies and it can express molecules specific for those particular tissues. There is considerable functional heterogeneity amongst endothelial cells located in different vascular beds. The molecular characteristics of the cells vary along the vascular tree. This endothelial diversity may be a factor in the propensity of certain endotheliotropic pathogens to target specific regions of the vascular tree.

Endotheliotropic microorganisms may target the vasculature as a whole, or they may target a specific vascular bed. Equine arteritis virus does not appear to target a particular vascular bed. The virus causes panvasculitis, characterized by inflammation of veins, capillaries, lymphatics and arteries. Early lesions in veins, capillaries and lymphatics are associated with oedema of the ventral abdominal wall and the hind limbs. This is followed a few days later by necrosis and vasculitis in medium-sized and small arteries throughout the body. African horse sickness is a viral disease in which selective vascular damage may be evident. Four forms of this disease are recognized: the mild horse sickness fever, the subacute cardiac form, the mixed or cardiopulmonary form, and the peracute pulmonary form (Mellor and Hamblin, 2004). In horse sickness fever, a condition usually involving only mild to moderate fever and oedema of the supraorbital fossae, viral antigen is found predominantly in the endothelial cells

of the spleen, with lesser amounts elsewhere, whereas, in the peracute form of the disease, viral antigen is found throughout the cardiovascular and lymphatic systems (Behling-Kelly and Czuprynski, 2007).

Inflammatory lesions in veins predispose to thrombosis and subsequent emboli. Thrombi in terminal arteries give rise to ischaemia and infarction downstream, as is seen in the diamond-shaped cutaneous lesions caused by *Erysipelothrix rhusiopathiae* infection in pigs. Branches of the renal arteries are prone to obstruction by emboli released from vegetative endocardial lesions. Sterile emboli produce typical infarcts and infected emboli cause abscesses. Infected emboli that obstruct interlobular arteries or their smaller branches cause abscesses confined to the renal cortex; those that block interlobar arteries cause abscesses that occupy segments of both cortex and medulla.

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Chapter 91

Interactions of microbial pathogens with the musculoskeletal system

The musculoskeletal system has well-recognized mechanical functions: it provides support and protection for body structures and it plays essential roles in locomotion, respiration and mineral homeostasis. The mechanical functions are executed by coordinated activity of skeletal muscles, tendons, bones, cartilage, joints and ligaments. Each of these diverse tissues has evolved distinct biological properties and structural characteristics in order to meet specific mechanical demands of avian and mammalian species. Inevitably, the remarkable adaptation of structure to mechanical function in the various tissues impacts on the interactions of the host with microbial pathogens and it has created the need for novel protective and regenerative pathways to deal with the consequences of host–pathogen encounters.

Responses of muscle to microbial pathogens

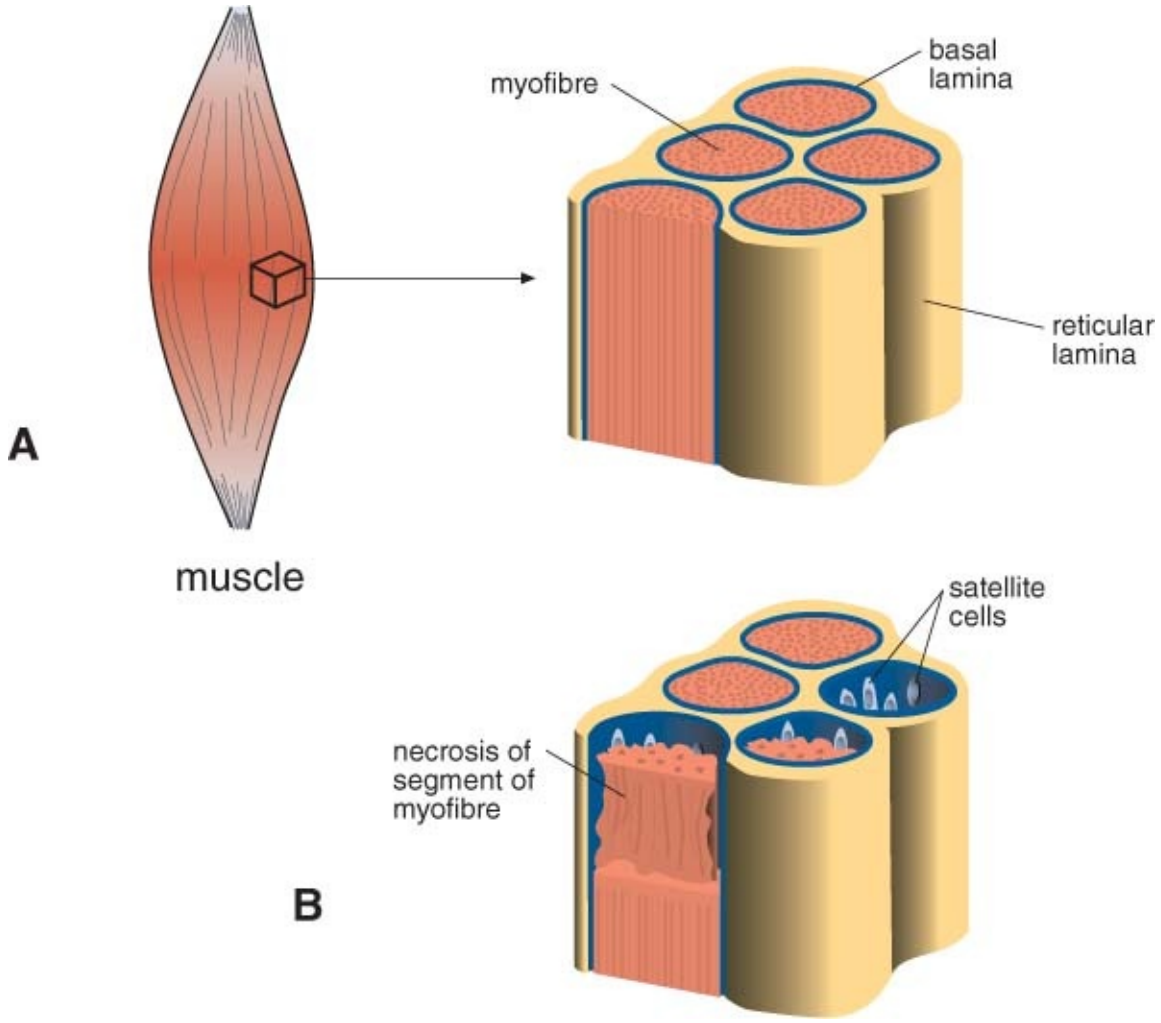
Although living muscle has been described as an inhospitable site for microorganisms (Van Vleet and Valentine, 2007), destruction of muscular tissue is a feature of many infectious diseases of domestic animals, including some that are life-threatening. Some viral infections may induce foci of necrosis in skeletal and cardiac muscle. In sheep, bluetongue virus damages endothelial cells, resulting in micro-thrombi in small blood vessels with oedema, focal haemorrhages and ischaemic necrosis of many tissues, including skeletal muscles and the myocardium. Although these virus-induced changes are defined as myositis (inflammation of muscle), they are degenerative lesions of the muscle fibres and any associated inflammatory response is secondary to muscle damage. From a clinical perspective, the more important microbial diseases of skeletal muscle are due to anaerobic spore-forming bacilli of the genus *Clostridium*. Pathogenic clostridia produce a number of toxins and enzymes that damage muscles and give rise to life-threatening toxaemia. However, the local

muscular lesion is more accurately defined as myonecrosis rather than myositis. The primary lesion is necrosis of muscle fibres. Cytokines released from the degenerating muscular tissue recruit inflammatory cells but, because of the distinctive structure of skeletal muscle, most of these cells aggregate in the interstitial tissue rather than in the muscle fibres.

Distinctive structural features of skeletal muscle

Skeletal muscles are composed of large numbers of parallel myofibres bound closely together by a network of connective tissue that forms part of the deep fascia. Each muscle is enveloped by a sheath of dense fibrous connective tissue called the epimysium. Fascia also extends into the muscles from the inner surface of the epimysium; as perimysium, it insulates muscle fibres into bundles called fascicles and as endomysium it surrounds individual fibres which it insulates from neighbouring myofibres (Fig. 91.1). Myofibres generate contractile force, while the connective tissue acts as a supportive matrix that accommodates the contractile elements and takes part in the transmission of contractile force to adjacent myofibres and to tendons (Trotter and Purslow, 1992). The intimate association between myofibres and the surrounding connective tissue is important for maintenance of the integrity and proper function of the entire muscle (Jarvinen *et al*., 2002).

Figure 91.1 Schematic representation of endomysium associated with normal and damaged myofibres. A, Normal muscle: the reticular lamina envelops the basal laminae and healthy myofibres. B, Damaged muscle: some myofibres undergoing necrotic change; basal laminae and satellite cells intact.



The epimysium enveloping an entire muscle is in contact with the sheaths of fascia that surround adjacent muscles. These smooth expanses of connective tissue provide slippery surfaces that allow adjacent structures to move and pass over one another with the minimum of friction. The potential space present between two sheaths of fascia that abut upon each other is called a fascial plane. Distinct fascial planes separate muscles in the limbs, the trunk, the neck and the head. In each of these locations, the fascial planes form natural cleavage lines within the muscular masses, providing lines of least resistance to the spread of transudate, extravasated blood, exudate, or bacteria. Thus, fascial planes determine the direction in which an infection may spread and, to an appreciable degree, they set the anatomical boundaries to the resultant lesion. Pus and other exudates gravitate to the most ventral region of a fascial plane and that defines the probable location of an emerging fistula or, alternatively, the most appropriate site for elective surgical drainage.

The perimysium and endomysium constitute the interstitial tissue through

which blood vessels and nerves reach the individual muscle fibres. The endomysium has two main histological components (Fig. 91.1): discrete areas of felt-like basal lamina that completely envelop each independent myofibre, and the more diffuse, collagenous, reticular lamina that fills the extracellular spaces between all the basal laminae within the fascicle (Sanes, 2003). Small arteries and arterioles that ramify throughout the perimysium supply the capillaries in the reticular lamina. The main course of the capillaries is parallel to the myofibres, but they branch, anastomose and form an extensive mesh around the muscle cells. Thus, the endomysium is a richly vascular tissue with an extensive system of anastomoses that provides the nutrients required by active muscles and is capable of supplying adequate numbers of inflammatory cells when and where they are required. Even without the intervention of pathogens, skeletal muscles require scavenger cells to remove cellular debris when myofibres undergo degeneration following blunt trauma, sudden forceful contractions, or unaccustomed vigorous activity. In these circumstances, recruited monocytes become macrophages, enter the necrotic myofibres and remove the cellular debris. At the same time, the intact basal lamina is a selective filter; it remains impermeable to fibroblasts and to satellite cells and their progeny. The substantial mechanical strength of the endomysium serves to protect the myofibres from contraction-induced injury. Powerful forces generated by the contractile elements are capable of injuring and rupturing the lipid bilayer of the plasma membranes of the myofibres, particularly during maximal workload. Transmembrane receptors firmly attach the plasma membrane to the closed envelope of the basal lamina, and the tight physical attachment to the basal lamina helps the cell membrane to retain its integrity and to protect the myofibre from contraction-induced injury.

Each mature myofibre is a long, cylindrical, multi-nucleated cell that is packed with contractile elements (myofibrils); approximately 80% of its volume is occupied by several hundred myofibrils (Purslow and Duance, 1990). The muscle progenitor cells are called myoblasts. During the prenatal phase, myoblasts take one of two developmental pathways: they can differentiate into mature myofibres or they can remain as quiescent satellite cells that occupy positions on the surfaces of the myofibres, between the plasma membrane and the overlying basal lamina (McGeady *et al*., 2006). As the nuclei of mature myofibres do not replicate, activated satellite cells are the main source of myoblasts required for maintenance, hypertrophy, repair or replacement of mature myofibres.

The elongated myofibre is formed by end-to-end fusion of many myoblasts, each of which contributes a post-mitotic nucleus and a segment of cell membrane to the creation of a tubular muscle cell. Subsequent development within the myotubule involves the synthesis of specific contractile proteins and the orderly array of the contractile elements (actin and myosin) in myofilaments and myofibrils that is responsible for the striations of skeletal muscle. In the sarcoplasm of a mature myofibre, the nuclei are distributed along the entire length of the fibre and each nucleus is responsible for gene expression in a segment of sarcoplasm (nuclear domain) in its immediate vicinity (Gundersen and Bruusgaard, 2008). As a consequence of this functional arrangement, segments of a fibre can act independently of other segments; commonly, when a portion of a fibre becomes necrotic, adjacent viable segments within that fibre continue to function normally. Segmental distribution of structural damage is a distinctive feature of injury to skeletal muscle.

Responses of muscle to injury

The major histopathological responses of skeletal muscle to injury are necrosis of myofibres, infiltration by inflammatory cells, regeneration of myofibres, and activation of fibroblasts in the intramuscular fascia. These processes often extend into three overlapping phases: a phase of degeneration, a phase of regeneration and a remodelling phase.

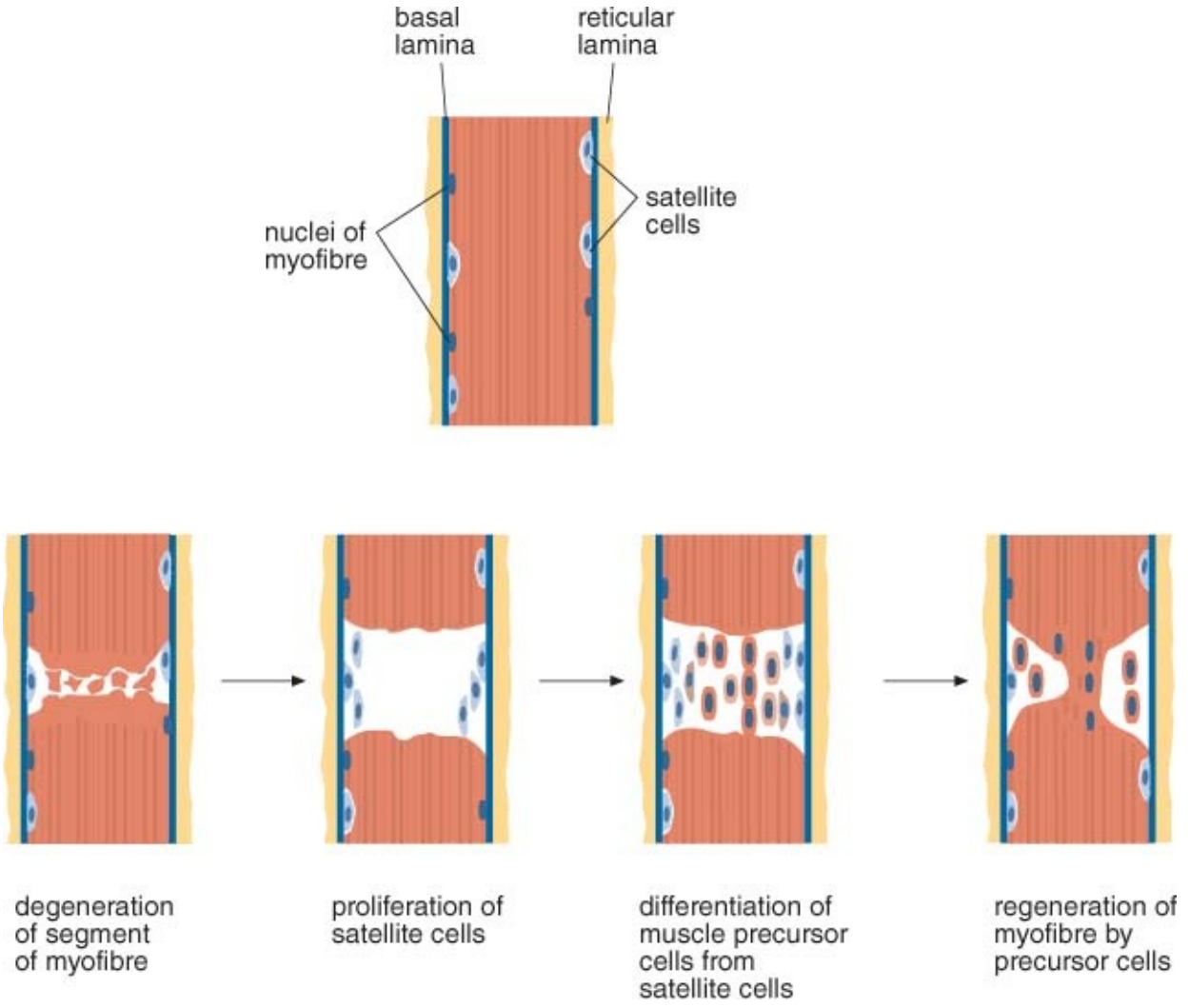
The earliest evidence of the degeneration phase is necrosis of myofibres. Most frequently, necrosis is segmental, involving a number of contiguous sarcomeres within each affected myofibre. The necrotic lesions may be induced by exogenous factors, such as microbial toxins, or by activation of endogenous proteases that degrade myofibrillar and cytoskeletal proteins. The enzymes are activated by a high concentration of intracellular calcium ions, whether due to an influx of extracellular calcium through a damaged plasma membrane or to an inability of damaged sarcoplasmic reticulum and mitochondria to regulate intracellular transfer of ions.

Inflammatory cells are attracted to the site of injury by proinflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α. These migrating cells invade the injured tissue from the extensive mesh of small blood vessels that encircles each affected muscle cell. The basal lamina does not block the entry of the phago-cytic cells into the myofibre. The initial response is an influx of neutrophils followed by monocytes that promptly become macrophages engaged

in the removal of the necrotic material, including remnants of the plasma membrane that adhere to the inner surface of the basal lamina. Macrophages specifically target the necrotic material without damaging the surrounding basal lamina or associated satellite cells. When the necrotic debris has been removed, the viable segments of myofibre proximal and distal to the lesion are linked by a hollow tube of basal lamina that has not been degraded. This empty part of the tube acts as a scaffold within which orderly regeneration of the damaged myofibre can occur.

Since mature myofibres cannot replicate, the key cell involved in regeneration of myofibres is the satellite cell. These progenitor cells are present, in quiescent mode, on the surface of mature myofibres between the plasma membrane and the basal lamina. In necrotic myofibres, satellite cells survive inside the hollow tube, where they are activated when exposed to signals emanating from injured muscle (Fig. 91.2). On activation, satellite cells proliferate within the hollow tube and differentiate into multinucleated myotubes that fuse with existing myofibres to repair damaged regions or fuse with one another to form new myofibres. The outcome can differ greatly depending on whether or not the basal lamina is intact. When the tube is ruptured, cells move in and out of it: satellite cells that escape into interstitial tissue form independent non-functional muscle giant cells, while fibroblastic cells that enter the tube lay down fibrous tissue that impedes or completely restricts regeneration of myofibres within the tube. When the hollow tube is intact, the activated satellite cells use it as a scaffold to ensure that the new segments intercalate with the proximal and distal viable segments, reinstating the structural integrity and the functional continuity of the myofibre. The new segments of myofibre secrete their own basal lamina; the corresponding stretches of the old tube are removed during the remodelling phase.

Figure 91.2 Regeneration of injured myofibre by satellite cells. See text for description.



Outside the tube, in the extracellular matrix, fibroblasts proliferate in response to some of the growth factors released by inflammatory cells and by myogenic cells. Normally, the components of the granulation tissue laid down by the fibroblasts during the early regeneration phase are degraded during the remodelling phase. Ifthis control system fails due to over-abundant production or inadequate degradation of the extracellular matrix, a fibrous scar results. Fibrosis of the intramuscular fascia leads to a loss of strength and flexibility and predisposes to recurring injury.

Responses of muscle to bacterial infection

Bacterial infection of skeletal muscle can be introduced by the haematogenous route; for example, focal embolic polymyositis is a feature of *Histophilus somni* infection in lambs and of *Actinobacillus equuli* infection in foals. More

commonly, bacterial pathogens enter the muscles via penetrating wounds or intramuscular injections, or by direct extension from suppurative lesions in adjacent structures such as tendon sheaths, joints, bones, or deep fascia. The tissue response of muscle to bacterial pathogens may be suppurative, granulomatous or gangrenous.

Suppurative myositis

The bacteria commonly isolated from pyogenic lesions in muscles include *Arcanobacterium pyogenes, Corynebacterium pseudotuberculosis, Staphylococcus aureus* and Streptococcus equi. When pyogenic bacteria are introduced into a muscle, they cause localized suppuration and necrosis of muscle fibres. This initial lesion, an ill-defined cellulitis, may undergo resolution with minor residual scarring; alternatively, it may expand along fascial planes or it may be encapsulated as an organized abscess. Spread along the fascial planes can give rise to extensive damage and may result in one or more fistulae emerging on the body surface, sometimes a considerable distance from the original lesion. An abscess in a muscle may heal slowly, or it may expand and fistulate, in which case it either proceeds to heal by fibrosis or it persists as a chronic granulomatous lesion and source of continuous or intermittent discharge. In cats, *Pasteurella multocida*-infected bite wounds often result in expanding cellulitis that leads to extensive destruction of muscle fibres with accumulation of subcutaneous exudates, especially below the level of the skin penetration The skin puncture heals readily and, in the absence of adequate drainage, abscesses and fistulae may form at the site.

In horses, *Corynebacterium pseudotuberculosis* may cause abscesses in various tissues and organs throughout the body. The pyogenic responses of horses to this pathogen are seen in three recognized clinical patterns: ulcerative lymphangitis, external abscesses, and internal abscesses. Intramuscular abscesses fall into the external category. In individual horses, the abscesses may be in a single site or in multiple sites. They are frequently located in the pectoral region and along the ventral midline of the abdomen. The well-encapsulated abscesses may reach a diameter of 15 to 20cm. The disease has been called pigeon fever because the swollen pectoral muscles look like a puffed-out pigeon breast. This disease is particularly prevalent in the southern states of the USA. In the past, this condition tended to occur as sporadic cases, often only a single horse on a farm. In recent years, thousands of horses have been affected in ‘epidemics’ that

have extended the geographical regions in which the disease is endemic (Spier, 2008). It is suspected that the pathogen may be transmitted by arthropods since the incidence of the disease fluctuates from year to year, greatly influenced by higher than normal seasonal rainfall and other conditions that favour high populations of insects.

The pathogenicity of *C. pseudotuberculosis* has been linked to the lipid coat that lies outside its cell wall and to the production of an exotoxin, phospholipase D (PLD). Each of these bacterial products facilitates the survival of the pathogens at the initial site of infection There, the early innate inflammatory response is dominated by neutrophils until macrophages take over as the more significant phagocytic cells. The neutrophils that phagocytose the pathogens at entry do not eliminate the infectious agent because cytotoxic mycolic acid in the lipid coat of *C. pseudotuberculosis* causes degenerative changes in neutrophils. In addition the PLD toxin activates complement in the tissues surrounding the invading bacteria, depleting complement locally and reducing opsonization of the bacteria (Yozwiak and Songer, 1993). When macrophages engulf *C. pseudotuberculosis*, the lipid coat protects the bacteria from the activity of the lysosomal enzymes of the macrophages; the bacteria survive and replicate as facultative intracellular pathogens. There is continued expression of PLD by the intracellular *C. pseudotuberculosis*, and this enzyme has been shown to cause a small but significant reduction in the viability of the host macrophages (McKean *et al*., 2007). When an infected macrophage dies, it releases cellular debris and viable corynebacteria, which act as a signal for another cycle of phagocytosis, intracellular replication of *C. pseudotuberculosis*, and death of phagocytes;the formation of an abscess is set in train.

PLD toxin does not elicit any systemic sign of intoxication rather, its activities are predominantly local. The primary enzymatic function of PLD is to catalyse the dissociation of sphingomyelin into ceramide phosphate and choline. Hydrolysis of sphingomyelin in host cell membranes results in leakage of aqueous contents and death of the cell. In endothelial cells PLD increases vascular permeability, leading to leakage of plasma from blood vessels into the surrounding tissues and, from there, into the lymphatic channels. Thus, PLD acts as a permeability factor that facilitates flow of fluid and the spread of pathogen-laden macrophages to secondary sites of infection, including skeletal muscles. Formation of an intramuscular abscess that is palpable externally is a prolonged process in which the cycle of phagocytosis, intracellular replication of *C. pseudotuberculosis*, and death of phagocytes is repeated many times.

Granulomatous lesions in skeletal muscle

Infectious granulomas in skeletal muscle occur in actinobacillosis, actinomycosis, botryomycosis and tuberculosis. Nowadays, the prevalence of tuberculous lesions in the musculature of cattle and pigs is extremely low. In cattle, *Actinobacillus lignieresii* is a common cause of pyogranulomatous lesions in the tongue. Timber tongue (or wooden tongue) is a chronic pyogranulomatous condition in which the skeletal muscle fibres are displaced by an expanding mass of fibrous tissue that contains a number of small loci of pyogenic infection. Within the inflammatory locus, the Gram-negative organisms are present as discrete colonies in sulphur granules where they are surrounded by radiating clubs, suspended in purulent debris, and encapsulated by the fibrous tissue. The enlarged and rigid tongue interferes with prehension and mastication *Actinomyces bovis*, the bacterium primarily responsible for mandibular osteomyelitis (lumpy jaw) in cattle, is an occasional cause of similar granulomatous lesions in the tongue. On microscopy, a distinguishing feature is that the mass of bacteria at the centre of the sulphur granule stains Gram-positive in contrast to the Gram-negative reaction of actinobacilli. Lesions due to *Actinomyces bovis* occur in the masseter muscle and adjacent muscles as a consequence of invasion from contiguous osteomyelitis.

In the horse, granulomatous lesions of botryomycosis may develop in the muscles of the neck and pectoral region as an extension from a primary granulomatous dermatitis caused by a persistent low-grade infection by *Staphylococcus aureus*. Draft horses seem to be particularly susceptible, presumably because the staphylococci invade cutaneous abrasions caused by harness. The tissue reaction to invasion by the staphylococci progresses to a hard nodular mass of fibrous tissue that encloses a number of small abscesses which may intercommunicate by sinuses and may drain to the surface of the skin as fistulae.

Necrotizing lesions in skeletal muscle

Some members of the genus *Clostridium* have a predilection for skeletal muscle, in which they may produce toxins and enzymes that damage the muscles and give rise to clinical signs of toxaemia. The bacteria involved are the histotoxic species *Clostridium chauvoei, C. septi-cum, C. perfringens, C. novyi and C. sordellii*. These bacteria are widely distributed in nature, in the soil, and in the intestinal tracts of humans and animals. Histotoxic clostridia produce a battery of

exotoxins, most of which act as extracellular enzymes that sustain the bacteria during saprophytic life in the soil and during parasitic life in animal hosts. In skeletal muscles, the clostridial exotoxins cause necrosis, oedema, haemorrhage and gas formation. The major toxins of each clostridial species have been assigned letters of the Greek alphabet, with the letter α usually designating the most significant toxin produced by that species. Because of this convention, a given Greek designation is not intended to indicate that toxins released by different species share the same structure or mode of action. For instance, the two pathogens isolated most often from myonecrotic lesions express dissimilar α toxins. There is no structural or functional relationship between the pore-forming α toxin of *C. septicum* and the α toxin of *C. perfringens*, a cytolysin that hydrolyses phospholipid components of host cell membranes.

The usual habitat of most of the clostridia is the soil from which mammals acquire the pathogenic species by the oral route or through traumatic injury. In either case, disease is not initiated merely by the entry of spores or vegetative forms of the pathogens. The growth of the anaerobes and the production of their toxins and enzymes are inhibited by the high oxidation-reduction potential of normal tissues. In the absence of an anaerobic environment, clostridial spores remain dormant. There is evidence that some spores acquired by the oral route may be disseminated by macrophages and persist as dormant spores in liver, spleen or skeletal muscle. These spores can be a source of endogenous infection when changes in local conditions allow them to germinate, multiply and produce toxins. Dormant clostridial spores have been found in healthy skeletal muscles in horses (Vengust *et al*., 2003), and germination of these spores has been advanced as a probable explanation for the fact that many horses that develop clostridial myonecrosis have a history of recent intramuscular injection or inadvertent perivascular injection of sterile medicinal products in the region of the lesion (Peek *et al*., 2003). Dormant spores may be activated also by non-penetrative trauma: for instance, the initiating event in the pathogenesis of blackleg in young unvaccinated cattle is muscle bruising and the associated hypoxia that activates spores of *C. chauvoei* already present in the muscles.

Dormancy also plays a role in the fate of clostridia acquired from exogenous sources. It is probable that the vast majority of traumatic wounds in domestic animals are contaminated by a mixed flora that includes one or more histotoxic clostridial species, but anaerobic wound infection develops in only a very small proportion of injured animals. Tissue anoxia is the critical factor that allows the anaerobes to grow in the wound. Hence, there are three possible outcomes to the

entry of histotoxic clostridia. In the absence of the required anaerobic conditions, the clostridia remain dormant, without clinical evidence of their presence in the wound, until they are eliminated by the defence mechanisms of the host. When anoxic conditions prevail, the contamination rapidly progresses to infection, tissue damage and toxaemia. The microorganisms may proliferate and give rise to clostridial cellulitis (malignant oedema), a toxaemic disease of the integument in which lesions are limited to local skin and subcutis without involvement of muscle, or they may induce clostridial myonecrosis, a condition in which there is progressive destruction of local muscle mass in association with systemic signs of severe toxaemia. Clostridial cellulitis and clostridial myonecrosis are diseases of ruminants, horses and pigs; they rarely occur in carnivores. Clinical reports often use the term gas gangrene to describe either clostridial cellulitis or clostridial myonecrosis.

Clostridial cellulitis

Typically, clostridial cellulitis develops in a superficial wound in which there is a modest amount of devitalized tissue that has been contaminated with a polymicrobial flora consisting of some histotoxic clostridial species together with some aerobes and facultative anaerobes, most of which are saprophytes with proteolytic and putrefactive properties. Transition from contamination to infection is facilitated by synergistic activities of the saprophytes and the pathogens: in particular, proliferation of the toxigenic anaerobes is promoted by the ability of the facultative organisms to reduce the oxidation-reduction potential within the wound. Much of the energy required by the proliferating clostridia is made available by the degradation of fascial proteins by the proteolytic enzymes of the saprophytes. The anaerobes can diminish the efficacy of the host’s phagocytic cells, which facilitates the proliferation of the non-toxigenic bacteria. Thus, the accompanying saprophytes play a significant role in creating the local conditions that modulate the release of toxins by the histotoxic clostridia. The quantity and potency of toxins expressed by the pathogens can vary between affected animals. The characteristic features of clostridial cellulitis are severe oedema, crepitation due to formation of gas, coldness of the affected part and signs of toxaemia. The accumulation of gas and the serosanguineous oedema fluid spread along fascial planes but, in many cases, muscles are not damaged significantly, even in fulminating toxigenic infections. However, if the blood supply to a muscle is significantly impaired by the initial trauma, or subsequently as a result of toxic injury to the blood vessels, the cellulitis may

develop into myonecrosis (Van Vleet and Valentine, 2007).

Clostridial myonecrosis

Clostridial myonecrosis arises most frequently in deep traumatic wounds in which blood flow is compromised by physical damage and by vasoconstriction of blood vessels that supply the surrounding tissues, including muscles. Wounds that can predispose to clostridial myonecrosis include castration, shearing, penetrating stake wounds and injuries to the female genitalia during parturition. Contamination of such deep wounds is likely to introduce a polymicrobial flora that includes histotoxic clostridia. The traumatized tissues within the wound provide a ready source of nutrients required for bacterial growth and the compromised blood supply creates an anaerobic environment that favours proliferation of histotoxic clostridia and the release of necrotizing toxins. The distinguishing feature of myonecrosis is that these clostridial toxins destroy healthy, living tissues. The initial bacterial growth within the devitalized tissues of the wound generates the population of aggressive pathogens that are capable of invading and destroying adjacent healthy tissue. As a consequence of reduced blood flow, hypoxic muscles contiguous to the wound switch to anaerobic metabolism, produce lactic acid and reduce the local pH, thus creating conditions favourable for colonization by clostridia from the primary wound. In those circumstances, proliferation of the clostridia proceeds apace, triggering changes in the immediate environment that are conducive to progressive extension of tissue damage. Degradation of tissue is promoted by bacterial proteases, by enzymes released from tissue lysosomes and by clostridial exotoxins Once the destruction of muscle begins, the necrotic lesion progresses along the muscle with great speed, sometimes at the rate of several centimetres per hour.

Clostridium septicum and *C. perfringens* type A, acting alone or in combination with other species, are the pathogens isolated most often from myonecrotic lesions. *C. perfringens* occurs more frequently but *C septicum* causes a more rapidly fulminating disease from which few animals recover even after intensive veterinary treatment. The tissue damage caused by these two organisms is attributed to the α toxin of *C. septicum* (Kennedy *et al*., 2005, 2009) and to the synergistic actions of the α and θ toxins of *C. perfringens* type A (Awad *et al*., 2001). Although the toxins expressed by the several histotoxic species are very different in structure and in mode of action, they cause similar histopathological lesions. However, the histological features of full-blown

clostridial myonecrosis are markedly different from the characteristic inflammatory lesions induced in softtissues by bacteria such as staphylococci or streptococci. Typically, the local tissue response to bacterial infection is dilatation of the blood vessels and increased blood flow through the site of infection, with exudation and emigration of leukocytes. Remarkably, as clostridial myonecrosis develops, the flow of blood through affected tissues undergoes an irreversible decline as does the number of leukocytes within the site of infection. When histotoxic clostridia invade a fresh wound, there is a transient phase during which the host mounts a typical inflammatory response; phagocytes engulf the bacteria but it has been shown that engulfed *C perfringens* can escape from the phagosomes and can survive within the cytoplasm of macrophages, even under aerobic conditions (O'Brien and Melville, 2000, 2004). This attribute may be a decisive factor in the survival of contaminating anaerobes when pathogen numbers are low in fresh wounds. When anaerobic conditions suitable for clostridial growth develop, the bacteria begin to multiply and to secrete toxins responsible for progressive destruction of adjacent muscle. The toxins induce changes in the local vasculature that block the flow of blood into the lesion; when incised, the affected muscle does not bleed. Although pressure exerted by gas accumulating within the muscular compartments enclosed tightly by fascia can compromise blood flow through the smaller blood vessels, interference with blood flow is due principally to the formation of occlusive aggregations of platelets, leukocytes and fibrin within the blood vessels. This leads to ischaemic necrosis of healthy muscle fibres distal to the occlusions, resulting in reduced oxidation-reduction potential and enhanced conditions for the rapid extension of the disease process. The host' s ability to limit the spread of the infection is impaired also by the toxin-induced absence of inflammatory cells from the myonecrotic lesion. The toxins can impede the egress of leukocytes from the incoming blood vessels and they can destroy the inflammatory cells that enter the site of infection. As a result, leukocytes accumulate within the blood vessels at the boundary between healthy and dead tissues. Consequently, despite extensive necrosis of muscular tissues and of the fascia and skin that overlie them, leukocytes do not infiltrate the area of myonecrosis. Thus, the histopathology of the affected muscles is distinctive: necrosis and fragmentation of muscle fibres, separation of degenerating fibres by copious quantities of fluid and gas, absence of leukocytes within the myonecrotic tissue, presence of leukocytes within fascial planes and accumulation of leukocytes within blood vessels at the boundary between the

healthy tissues and the myonecrotic tissues.

Responses of bone to microbial pathogens

Pathogens can invade bone by the haematogenous route, by extension from adjacent soft tissue lesions or following accidental or surgical trauma. The patho-genesis of bone infection can often be related to trauma. Research biologists have reported that it is not easy to infect healthy bone: they find that it is often necessary to damage bone by physical or chemical means in order to reliably induce pathological changes in an animal model. In intact healthy bone, the mineralized component protects the underlying soft tissues from colonization by pathogens. Open fractures expose the soft tissues of bone to environmental contamination with soil, grit and pathogens. When an animal is bacteraemic, blunt trauma increases the risk of bone infection, in the absence of fracture or obvious wound. Presumably, blood-borne pathogens preferentially colonize bone where trauma has made binding sites on the osteoid matrix more accessible to complementary bacterial receptors. Certain bacteria express receptors for components of osteoid matrix, such as collagen, fibronectin, laminin and sialoglycoprotein. Bacteria that infect bone must bind firmly to the host tissue, avoid host defences and proliferate *in situ*. For example, *Staphylococcus aureus* has receptors by which it can bind to collagen and sialoglycoproteins and, once bound, it secretes a mucopolysaccharide glycocalyx to create a biofilm which enhances adhesion, shields the pathogen from the host defence mechanisms, and allows it to proliferate. Host tissue reaction to proliferating pathogens is greatly influenced by some structural features, including the ongoing renewal of bone.

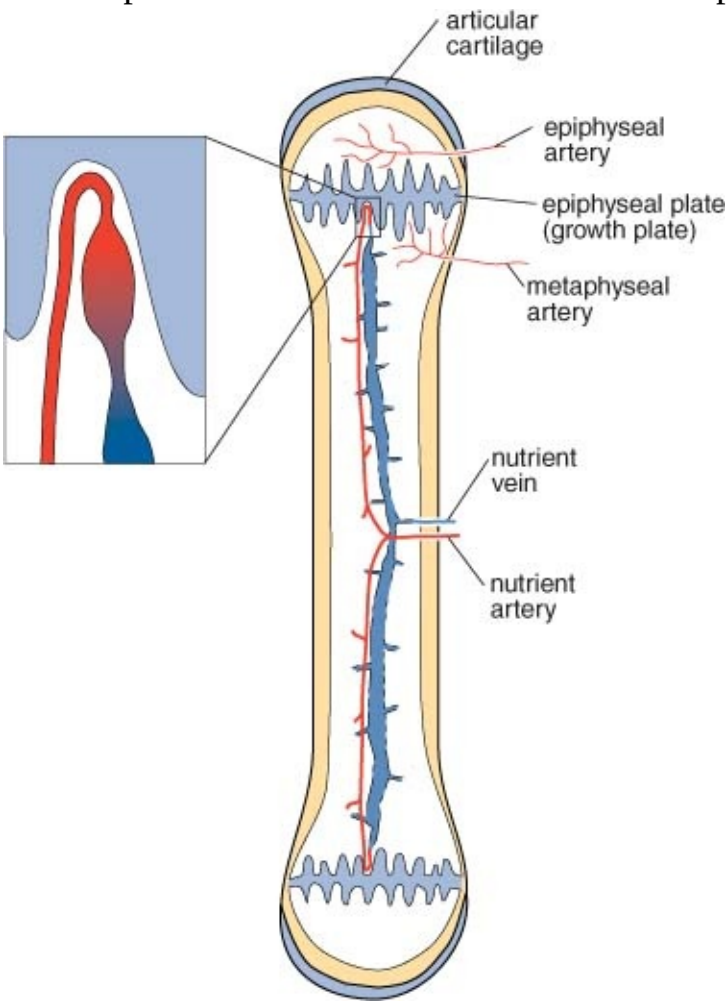
Anatomical considerations

Bone is a dynamic tissue in which structural renewal is a constant feature: the biomechanical and metabolic competence of the skeleton is dependent on its ability to replace old and damaged bone by new bone as required. The physiological turnover of old bone that occurs periodically throughout life is described as remodelling. In physiological circumstances, remodelling occurs when osteoclasts resorb small packets of old bone and then osteoblasts fill the resulting space with new bone. In this way, there is internal replacement of the bone without alteration in gross form or function. Significantly, this process is regulated by proinflammatory mediators that also play a role when pathogens

invade bones and joints.

A structural feature that distinguishes bone from other forms of connective tissue is the calcified matrix that occupies the intercellular spaces. In the presence of this hard extracellular material, the local vasculature passes through designated channels within the osseous tissue. In the cortex of long bones, there is a lattice of vascular channels consisting of haversian canals running approximately parallel to the long axis of the bone and transverse Volkmann canals that interlink with the haversian canals and also communicate with the periosteum and with the marrow cavity. Afferent blood vessels enter Volkmann canals at both periosteal and endosteal surfaces.

Figure 91.3 Vascular patterns in a long bone before closure of the growth plate. Inset depicts the sinusoidal vessels in the metaphysis.



Arterial blood is delivered to long bones by three systems of vessels: periosteal arterioles, nutrient arteries and metaphyseal-epiphyseal arteries (Fig. 91.3).

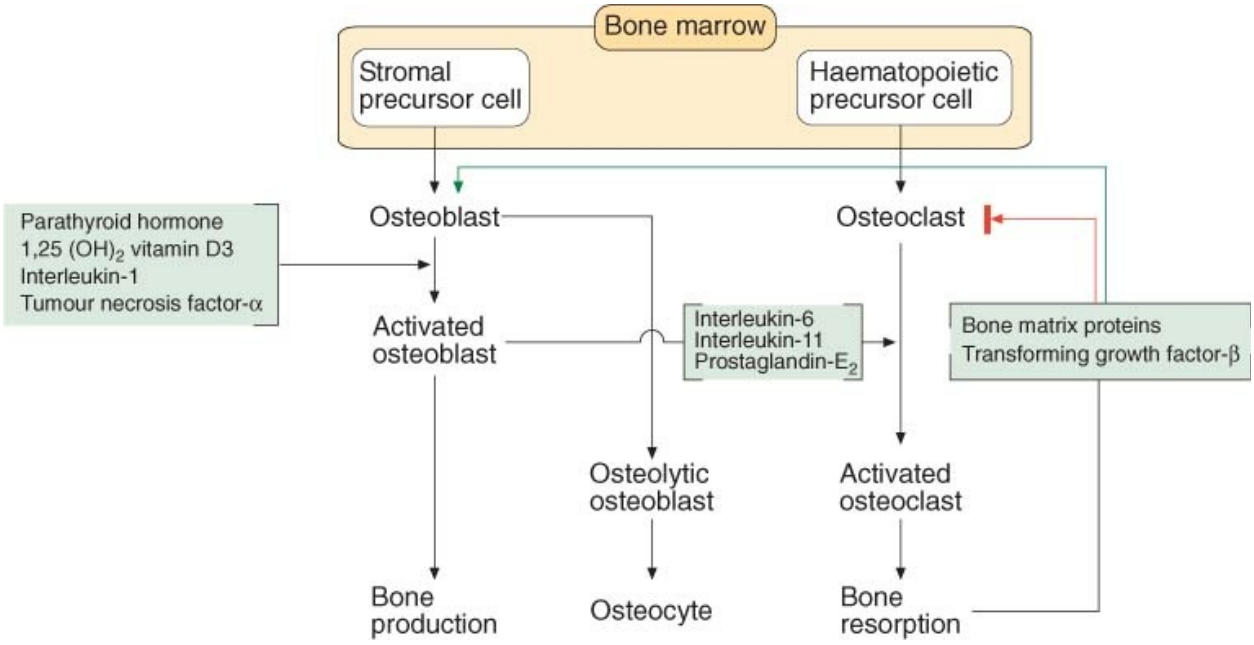
Terminal branches of periosteal arterioles enter Volkmann canals in subperiosteal cortical bone, taking a centripetal route to supply the outer regions of the cortex. In contrast, nutrient arteries, metaphyseal arteries and epiphyseal arteries enter the medullary cavity through foramina that perforate cortical bone. Within the medullary cavity, these afferent vessels give off branches, some to supply tissues in the medullary cavity, others to produce thin-walled vessels that enter portals of Volkmann canals on the endosteal surface of the bone and travel centrifugally towards the outer cortex.

Although the blood vessels present in the vascular channels of compact bone are interposed between arteries and veins, they are unable to behave as typical capillaries. In soft tissues, capillaries are embedded in loose connective tissue which allows the vessels to dilate to accommodate an increased flow of blood when the host mounts a local inflammatory response. Hyperaemia alters the transmural pressure gradients so that the increased intravascular pressure is alleviated by transfer of fluid to interstitial spaces within the surrounding connective tissue. In cortical bone, the thin-walled vessels are encircled by only a slender sheath of supportive connective tissue within the rigid canals and, as a consequence, the vessels are incapable of effective dilatation when blood flow is increased. Likewise, the small extravascular space between the basement membrane of the endothelial cells and the wall of the bony canal has only a limited capacity for extravasated fluid. When cortical bone is inflamed, these circulatory constraints give rise to vascular engorgement and turbulent blood flow which, in turn, predispose to thrombosis and associated local infarction. These pathological consequences tend to be more pronounced when the infective agent releases a toxin that damages the local endothelial cells.

The architecture of the vascular system in the growth areas of the long bones has a significant impact on the localization of lesions in haematogenous osteomyelitis. In immature animals, the disease begins in the metaphysis, the most vascular area of a growing bone; in adult animals, it commonly begins in the epiphysis. In growing animals, the metaphysis is separated from the epiphysis by the growth plate, a thick plate of cartilage that separates the vasculature of the two regions. The nutrient artery is the principal source of blood to the metaphyses. When a nutrient artery enters the medullary cavity, it divides into an ascending branch and a descending branch. After each of these medullary arterioles has given off a series of small lateral branches that ramify in the vascular channels of the cortical bone, it continues into the metaphysis, providing an extensive network of smaller arterioles that do not anastomose.

These end-arterioles terminate in fenestrated capillaries that allow blood-borne pathogens to escape into the bone marrow. As the capillaries approach the growth plate (Fig. 91.3), they form sharp hairpin bends and then expand into large sinusoidal vessels that ramify in the metaphy-seal area before draining into the nutrient vein. Blood in the vascular loops and terminal vessels has a low oxygen tension and the rate of flow through the sinusoids is sluggish and turbulent, which favours the formation of small intravascular thrombi within which bacteria are sequestered from circulating phagocytes. This enables pathogens to survive, proliferate and induce inflammation. By the time growth of a long bone has been completed, the cartilage has been replaced by bone, a process known as closure of the epiphysis. After closure, the metaphyseal and epiphy-seal vessels communicate freely through the closed growth plate and, consequently, blood-borne pathogens commonly induce primary lesions in the epiphyses of adult animals.

Figure 91.4 Origins and interactions of bone cells and the mediators that modulate their activities.



The histology of the periosteum helps to explain how bacterial infection may lead to simultaneous occurrence of degenerative and regenerative processes in an infected bone. The periosteum consists of an outer fibrous layer and an inner cellular layer. In long bones, the outer layer provides bundles of collagen fibres that bind periosteum to bone and it supplies periosteal arterioles to the outer regions of the cortex. The population of cells in the inner layer includes

quiescent osteoprogenitor cells that are capable of becoming osteoblasts when activated by appropriate stimuli. Trauma or other forms of injury which displace the periosteum from the surface of the bone activate the quiescent osteoprogenitor cells, leading to local formation of new bone. Because the attachment of periosteum to bone is looser in growing animals than in adults, this process of reactive osteogenesis is most evident following infection in young animals. The most common stimulus for this phenomenon is accumulation of exudate beneath the periosteum, a process that may occlude some of the periosteal arterioles and result in segmental areas of ischaemic necrosis in outer regions of cortical bone.

Reactions of bone to pathogens

In domestic animals, some bone infections may be caused by fungal or viral pathogens but, in immature farm animals, the majority are due to haematogenous spread of bacteria from primary lesions in the umbilicus, the intestines or the respiratory tract. In these animals, the common causal organisms are *Arcanobacterium pyogenes*, staphylococci, streptococci, *Salmonella* species and *Escherichia coli*. The responses of skeletal tissues to the pathogens are mediated by a number of cytokines, some of which are continuously engaged in regulating the activities of osteoclasts and osteoblasts in remodelling mature bone (Fig. 91.4). In physiological circumstances, osteoblasts are activated by IL-1 and TNF-α; in turn, the activated cells secrete IL-6, IL-11 and prostaglandin E_2 (PGE_2) which activate osteoclasts. Osteoclasts degrade bone matrix, releasing bone matrix proteins and TGF-β which inhibit osteoclasts and stimulate production of osteoblasts (Fig. 91.4). Then, the osteoblasts invade the resorption site and begin the process of forming new bone by secreting osteoid, the matrix in which minerals are deposited. This process of bone turnover must be carefully regulated to retain the structural features and the biomechanical properties of the bone. In infected bone, the reactive release of proinflammatory mediators, such as TNF-α, IL-1 and PGE_2, by inflammatory cells can uncouple the balance between resorption and formation of bone. Thus, bacterial infection of bone may lead to local destructive lesions, to local proliferative lesions, or to a combination of both.

Proliferation of pathogens may trigger a local acute inflammatory reaction in the soft tissues of bone, that is, in the periosteum, in the connective tissue in the vascular canals or in the bone marrow. The inflammation may begin in the

medullary cavity as osteomyelitis or it may begin in the periosteum as periostitis. However, the extensive connections between the blood vessels in the periosteum, the vascular canals and the bone marrow provide routes by which progressive inflammation can extend to involve all three sites. In each of these locations, there is little room for expansion to accommodate inflammatory exudate. In the medullary cavity and the vascular canals, exudate is confined by rigid bone. The presence of the fluid and the cellular components of the inflammatory exudate within the marrow cavity increases intramedullary pressure, which can compress branches of the nutrient arteries, resulting in thrombosis and infarction of haematopoietic marrow and of trabecular bone. As the pressure rises, it can force exudate from the medullary cavity into the vascular channels and through the cortex to the external surface of the bone. Exudate percolating along the vascular channels impedes the flow of blood to cortical bone and, in spite of numerous anastomoses, the small vessels in the rigid channels do not provide sufficient collateral circulation to prevent segmental ischaemic necrosis within the mineralized compact bone. On emergence at the external surface of the bone, exudate can undermine the attachment of periosteum to bone. If the exudate is trapped between the bone and the periosteum, a subperiosteal abscess develops; if the periosteum ruptures, inflammation spreads to the surrounding soft tissues and, in due course, the exudate may be discharged at the body surface via sinus tracts.

Thus, the main features of acute inflammation in bone are trapping of exudate in restricted spaces and the associated pressure effects on local vasculature that predispose to infarcts both in the medullary cavity and in mineralized bone. These factors, combined with the actions of bacterial toxins and enzymes, lead to necrosis of avascular fragments of bone. When necrosis is extensive, a segment of dead bone may separate from the living bone as a sequestrum, a bony corpse in a pool of purulent exudate. Later, the host may attempt to wall off the sequestrum in a sheath of granulation tissue, and in turn this is transformed into a sheath of new bone known as an involucrum. Small foci of necrotic bone may be resorbed slowly over time but larger infected segments persist as sequestra, foci of chronic osteomyelitis from which the pathogens cannot be eliminated by the host defence mechanisms or by antibiotics. Even when pathogens are inactive, the necrotic bone acts as a foreign body that interferes with the healing process. Pathogens may persist as inactive residents. For instance, some strains of *Staphylococcus aureus* can survive for long periods within osteoblasts and endothelial cells before they re-emerge as active pathogens. Some of these

strains have the characteristics of small colony variants (SCVs) that grow very slowly on culture and yield small, non-pigmented, non-haemolytic colonies. *In vivo*, SCVs display a very slow metabolic rate, an enhanced ability to persist within host endothelial cells, and strong resistance to antibiotics. In human patients, SCVs have been associated with chronic osteomyelitis that was resistant to prolonged antimicrobial therapy, and also with chronic relapsing osteomyelitis, often with long periods of quiescence between episodes.

Atrophic rhinitis

Bacterial toxins can damage turbinate bones (conchae) in the nasal cavities of young pigs. A heat-labile cytotoxin released by specific strains of *Bordetella bron-chiseptica* is capable of inducing non-progressive, moderately severe atrophic lesions of the conchae. The clinical signs are mild and the host is able to spontaneously regenerate the damaged bones. However, it is believed that the strains of *B. bronchiseptica* that produce the cytotoxin may predispose to colonization of the nasal cavity by the bacterium that causes progressive atrophic rhinitis, a disease in which there is severe, progressive and irreversible damage to the conchae. The cause of the lesions of progressive atrophic rhinitis has been identified as a heat-labile protein produced by capsulated strains of *P. multocida*, predominantly strains of serotype D, less commonly strains of serotype A (Chanter *et al*., 1986). Clinical signs include sneezing, nasal discharge, deformity of the snout, and failure to thrive. The pathognomonic sign is a shortening or twisting of the snout due to disruption of the normal processes of remodelling of the turbinate bones. The activities of osteoblasts and osteoclasts are uncoupled: the formation of bone by osteoblasts is inhibited and the resorption of bone by osteoclasts is increased. It is suggested that *Pasteurella multocida* toxin does not directly affect the osteoclasts; rather, it stimulates osteoblasts to release soluble mediators (cytokines and prostaglandins) that regulate the activities of the osteoclasts (Dominick and Rimler, 1988). In severe cases, the conchal bones may be destroyed completely.

Actinomycosis

Actinomycosis is a chronic granulomatous condition most commonly seen as ‘lumpy jaw’ in cattle. The causal agent, *Actinomyces bovis*, is a normal inhabitant of the oropharynx and the digestive tract. The bacterium enters the deeper tissues of the jaw through wounds of the buccal mucosa caused by sharp

pieces of food or by foreign bodies. Infection may also occur by direct extension from the gums through dental alveoli, particularly in young animals in which teeth are erupting. In the majority of cases the disease affects the mandible; it occurs in the maxilla less frequently. In the mandible, the disease expands progressively from an initial small swelling of the bone, often located at the level of the central molar teeth. In the bone, *Actinomyces bovis* invades the medullary cavity where it elicits a pyogranulomatous inflammatory reaction: the cavity is filled with granulation tissue in which there are many purulent foci and suppurative tracts. Colonies of the microorganism are present in 'sulphur granules' in the pus and embedded in the granulation tissue. In actinomycosis, the 'sulphur granule' consists of a mass of tangled, Gram-positive filaments surrounded by club-shaped red-staining bodies. The structure of the affected bone undergoes extensive changes as is evident when a macerated preparation of the swollen segment is examined. The gross swelling is due to the excess trabecular bone laid down when osteoprogenitor cells in the periosteum are activated. This new bone and the adjacent old bone are perforated by cavities created by advancing suppurative tracts. As a result, the bony lesion has a sponge-like structure. Thus, the lesion caused by *Actinomyces bovis* is a rarefying form of osteomyelitis. The infection may extend from bone to adjacent soft tissue, spread along fascial planes, and discharge pus containing sulphur granules through the mucous membrane or through the skin. Teeth in the affected segment of the jaw become loose, malaligned or are lost, and consequently the animal has difficulty with prehension and with mastication.

Vertebral osteomyelitis

Inflammation of vertebral bodies can develop from penetrating wounds or from infections in adjacent tissues, including intervertebral discs, but it is likely that most cases result from haematogenous dissemination of bacteria as septic emboli from primary lesions in the umbilicus or in any of several organ systems. Vertebral osteomyelitis may be secondary to navel-ill in calves and foals, to metritis, mastitis, and traumatic peritonitis in cattle, to tail-biting in piglets, and to tail-docking in pups. The disease is seen most commonly in neonatal animals, presumably in those with inadequate passive immunity. *Arcanobacterium pyogenes* is a common causative agent in several species but a range of other bacteria may be involved. In one study, *Salmonella* Dublin was isolated from the vertebral lesion in eight of 10 calves that had presented with cervico-thoracic osteomyelitis (Healy *et al*., 1997). In pigs, discospondylitis occurs in association

with polyarthritis caused by *Erysipelothrix rhusiopathiae* Vertebral osteomyelitis often leads to destruction and collapse of the affected vertebra, compression of the spinal cord and sudden onset of neurological signs.

Responses of joints to microbial pathogens

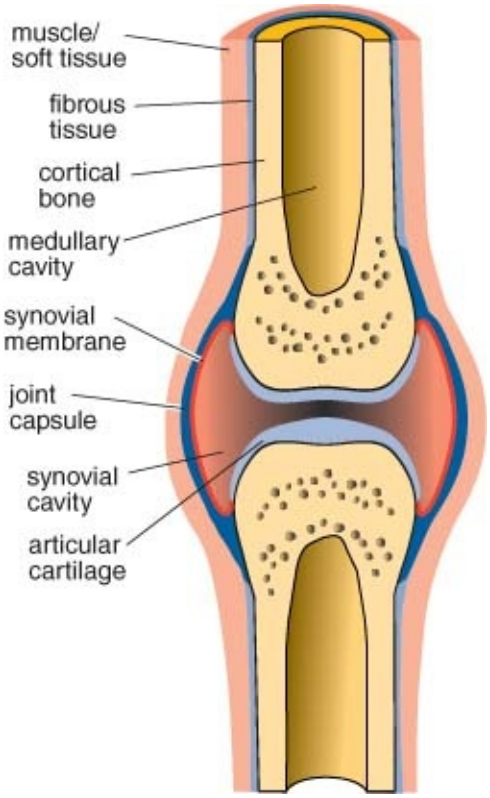
Many bacteria, viruses and fungi have been isolated from infected joints of domestic animals. Infectious arthritis is common in horses and in farm animals, often as a sequel to neonatal bacteraemia in young animals that have not absorbed an adequate quantity of colostral immunoglobulins. In very young animals, bacteria that gain entry to the bloodstream from primary infections in the nasopharynx, in the intestines, or in the umbilicus frequently settle in richly vascular synovial membranes. The resultant arthritis may be part of a systemic disease that causes lesions in several other organs and tissues. In other instances, the secondary lesions may appear to be restricted to the skeletal system, commonly in more than one joint; it is not always evident whether this is because the joints are the only secondary sites affected or because the disease has persisted in joints after it has been cleared from other sites (Thompson, 2007). In general, arthritis occurs in young animals as an acute disease involving several joints simultaneously, whereas in mature animals it tends to be a chronic degenerative disease, commonly in a single joint. In both situations, the development of the articular lesions reflects the interrelationships between the several structural components of the joint and, also, the distribution of forces between the joint and the subchondral tissue of the articulating bones.

Anatomical considerations

In a typical synovial joint, the ends of two opposing bones are covered by hyaline cartilage, separated by a thin layer of synovial fluid, and held in position by a cuff of dense fibrous tissue, the joint capsule (Fig. 91.5). The capsule is lined by a synovial membrane which is attached at the margins of the articular cartilage but does not encroach on the surface of the cartilage. In some recesses of the joint, this membrane has many very small villous projections. The membrane consists of two layers which are not separated by a basement membrane: a thin intimal layer of synovial cells (synoviocytes), and a subintimal layer of richly vascular connective tissue. The intimal layer consists of two populations of synoviocytes: type A phagocytic cells, which can engulf and

remove cell debris and waste material from the joint cavity, and type B cells, which release several products into the synovial fluid, including the surface-active phospholipid which is the principal lubricant in healthy joints at low loads. Type B cells also produce collagen and other constituents of the intimal interstitium. Both cell types produce a number of mediators, including TNF-α and interleukin-1.

Figure 91.5 A typical diarthroidal synovial joint.



Under normal physiological conditions, the articular cartilage provides an almost frictionless and wearresistant surface for the transmission and distribution of mechanical loads from one bone to the other. Collagen fibres bind the articular cartilage firmly to subchondral bone. The subchondral plate to which the cartilage is bound is many times more deformable than cortical bone, thus allowing more even distribution of the mechanical load between the cartilage and the bone at times of peak mechanical loads (Thompson, 2007). Structurally, the articular cartilage is a matrix composed of collagen fibres in a proteoglycan gel that contains a widely dispersed population of isolated chondrocytes, cells that are responsible for the production of the matrix. Chondrocytes regulate their activities in response to mechanical signals and to molecular signals, such as growth factors, chemokines and cytokines (Goldring and Marcu, 2009).

Chondrocytes are able to produce many of these mediators, which then act as autocrine or paracrine messengers. The matrix does not contain blood vessels, lymphatic vessels or nerves. Chondrocytes depend on the diffusion of nutrients from synovial fluid through the matrix. The extracellular matrix is approximately 70% to 80% water, which enables it to act as a resilient cushion at the weight-bearing surface of the joint. At high mechanical loads, water is forced out of the matrix into the synovial fluid, forming a layer of hydrodynamic lubrication between the opposing cartilages. When the pressure is released, water returns into the cartilage by osmotic traction within the matrix.

In contrast to ongoing turnover of bone, remodelling of articular cartilage occurs at a very slow rate. In mature animals, natural remodelling of articular cartilage is based on enzymatic turnover of the proteogly-can components of the matrix rather than on renewal of chondrocytes or collagen fibres. The majority of the degradative enzymes are secreted by chondrocytes as inactive precursors that are activated when cleaved by other enzymes in the matrix. Also in the matrix there are specific enzyme inhibitors which help to regulate the activities of the degradative enzymes. In healthy articular cartilage, turnover of proteoglycan is very slow and strictly regulated to maintain a balance between enzymatic degradation and replacement. The pace of remodelling is increased in response to mechanical injury, to changes in mechanical loading, and to molecular signals, including chemokines and cytokines released in infected joints.

Responses of joints to bacterial pathogens

Pathogens can enter joints by the haematogenous route, through wounds, by extension from adjacent bone or by extension from periarticular soft tissues. Invasion of the joint evokes an inflammatory response: increased blood flow, increased permeability of capillaries to fluid and cells, and recruitment of inflammatory cells, initially neutrophils, later monocytes and lymphocytes. Inflammation restricted to the synovium is described as synovitis. The term arthritis implies that the capsule and articular cartilage are inflamed; osteoarthritis is the appropriate term when the inflammation has extended to involve bone. Damage to intra-articular structures may be due to toxins released by the pathogens, to the inflammatory process, to proteolytic enzymes released from cartilage or synovial tissues, to persistence of microbial antigens in the joint, or to the failure of degenerating chondrocytes to maintain the proteoglycan

content of the articular cartilage.

The acutely inflamed joint is hot, swollen and painful because of the hyperaemia, the oedema of the synovial membrane and of the joint capsule and the increased volume of synovial fluid. The exudate may be fibrinous, purulent or seropurulent. As the arthritis progresses, oedema of the synovial tissues becomes less apparent but the joint capsule and the synovial membrane become thicker due to proliferation of stromal cells and synoviocytes. Particulate matter released within the affected joint is engulfed by type A synoviocytes. The debris-laden phagocytes can persist for extended periods in the subintimal layer. When a joint is severely affected, many of these synoviocytes may accumulate within the synovial membrane and stimulate a fibrotic reaction in the joint capsule. The synovial villi enlarge and may branch extensively; they contribute to the granulation tissue that spreads across the articular cartilage as a pannus, cutting off the underlying cartilage from the nutrients in synovial fluid. This is one of several negative influences that contribute to degeneration of the articular cartilage, the major consequence of microbial invasion of a synovial joint.

Because it is an avascular structure, articular cartilage cannot mount a typical inflammatory reaction but it is affected by inflammation in the synovium, capsule or subchondral bone. Articular cartilage generally remains intact in fibrinous arthritis, except in areas where it is covered by pannus. In purulent (septic) arthritis, the cartilage may undergo extensive destruction on exposure to collagenases and other lysosomal enzymes released from degenerating neutrophils. At the molecular level, the cartilage responds to a repertoire of inflammatory mediators from intra-articular lesions and from chondrocytes located within the cartilage itself. The mediators, including those that act as autocrine and paracrine messengers, stimulate the chondrocytes to produce proteolytic enzymes that contribute to destruction of the cartilage matrix; they inhibit the synthesis of the major physiological inhibitors of these enzymes and they also inhibit synthesis of new matrix. The resultant imbalance between degradation and replacement results in a net loss of matrix. The cartilage becomes thin and develops superficial fissures; hydrodynamic lubrication becomes less efficient. Progressive erosion of the articular cartilage brings the subchondral ends of the bones into contact and, as a result, the two opposing surfaces are polished by friction, become extremely hard and acquire an ivory-like appearance (eburnation). The stiffness associated with this sclerosis of the subchondral bone may reduce the ability of the bone to absorb and redistribute mechanical loads through the joint, thus increasing the load on the damaged

cartilage and hastening its destruction. A focus of inflammation in the subchondral bone can remove the support on which the cartilage rests, leading to collapse of the articular surface and degeneration of the cartilage. This sequence of events occurs in *Mycoplasma bovis* infection in cattle, and in chronic *Erysipelothrix rhusio-pathiae* infection in sheep.

Although *Erysipelothrix rhusiopathiae* is responsible for chronic arthritis in pigs, it is not always possible to isolate the organism from affected joints. In such cases, persistence of inflammatory changes in the joints may be due to the presence of bacterial antigens in the synovial tissues. Specific antibodies to these antigens have been detected in synovial fluid of chronically arthritic joints, presumably produced by the many plasma cells present in the hypertrophic synovial villi.

In septic arthritis, development of adhesions between the surfaces of the two bones interferes with mobility of the joint and may eventually lead to obliteration of the joint cavity and fixation of the joint by connective tissue, cartilage or osseous tissue. Osseous union of the bones prevents movement as the joint becomes ankylosed.

Foot infections of cattle, sheep and pigs associated with microbial agents

Lameness, particularly foot lameness, can cause significant economic loss in farm animal production. The relative importance of foot lameness in various species differs; it is especially important in cattle and sheep. Footrot caused by infection with *Dichelobacter nodosus* and other bacterial pathogens is a major cause of lameness in sheep. Relative to sheep, infections of the foot in dairy cows are less common. Although foot lameness in farm animals is mainly attributable to bacterial infections, it is also a significant clinical feature in a number of important viral diseases in which pedal lesions develop (Table 91.1). Lesions can develop in locations other than the foot in these systemic viral diseases. In contrast, bacterial infections which induce lameness are usually limited to pedal tissues. Bacteria that affect the skin and horn of the digit in farm animals include *Fusobacterium necrophorum*, *Arcanobacterium pyogenes*, *Porphyromonas* and *Prevotella* species, organisms which are often normal inhabitants of the gastrointestinal tract. Virulent strains of *Dichelobacter nodosus*, the primary cause of footrot in sheep, are not regarded as commensals.

These pathogenic strains are maintained by clinically-affected and recovered carrier sheep. Observations on the epidemiology of digital dermatitis in cattle also suggest that the putative causative organisms are maintained by carrier animals which can introduce infection into clean herds.

The aetiology and epidemiology of infectious digital disease are complex, and definitive diagnosis is difficult Isolation of anaerobic and other bacteria from pedal lesions is demanding, and specific procedures must be followed (see Chapter 37). Moreover, contamination by opportunistic bacteria may render interpretation of the significance of isolates difficult. Infectious foot conditions of sheep and cattle and their associated aetiological agents are listed in Tables 91.2 and 91.3, respectively. The aetiological role of the bacteria commonly isolated from digital lesions is well established for some conditions but is unclear in others; the primary role of *Dichelobacter nodosus* in the aetiology of footrot in sheep is not disputed whereas its role in interdigital dermatitis is uncertain. In addition, synergism between two or more organisms is important in the aetiology of many foot conditions as illustrated by the relationship between *F. necrophorum* and *D. nodosus* in ovine footrot and between *F. necrophorum* and *Porphyromonas levii* in foul-in-the-foot (interdigital necrobacillosis) in cattle.

Table 91.1 Systemic viral diseases of cattle, sheep and pigs in which foot lameness occurs.

Disease / Species affected	Virus / Genus / Family	Nature and extent of foot lesions
Bluetongue / Sheep, cattle	Bluetongue virus / *Orbivirus / Reoviridae*	Laminitis; inflammation of the coronary band (coronitis)
Foot-and-mouth disease / Cattle, sheep, pigs	Foot-and-mouth disease virus / *Aphthovirus / Picornaviridae*	Vesicles in the interdigital skin and on the coronary band; ulceration following vesicle rupture may lead to secondary bacterial invasion.
Mucosal disease / Cattle	Bovine viral diarrhoea virus / *P estivirus / Flaviviridae*	Ulcerative lesions in the interdigital clefts; coronitis. Lesions may occur in all four feet
Swine vesicular disease / Pigs	Swine vesicular disease virus / *Enterovirus / Picornaviridae*	Vesicular or ulcerative lesions on the coronary bands may involve the entire coronet producing severe lameness
Vesicular stomatitis / Cattle, pigs, horses, rarely sheep	Vesicular stomatitis virus / *Vesiculovirus / Rhabdoviridae*	Vesicular lesions on coronary bands progressing to ulceration complicated by secondary bacterial infection

Table 91.2 Infectious foot conditions of sheep.

Clinical condition	Bacteria implicated	Comments
Ovine footrot	*Dichelobacter nodosus Fusobacterium necrophorum, Arcanobacterium pyogenes*, Spirochaetes (unclassified)	Severity of lesions determined by the virulence of *D. nodosus* ; may occur as benign and virulent forms
Ovine interdigital dermatitisi	*Fusobacterium necrophorum, Dichelobacter nodosus* (benign strains)	Superficial interdigital inflammation, caused primarily by *F necrophorum*. Mild condition; also referred to as scald
Contagious ovine digital	*Treponema* species	Ulcerative lesions develop at the coronary band and extend down the hoof wall. Affected animals may have to be culled

dermatitis		
Heel abscess	*Fusobacterium necrophorum, Arcanobacterium pyogenes* together with opportunistic anaerobic bacteria	Associated with prolonged wet seasons; usually affects adult sheep. Painful pyogenic condition which often extends to the interphalangeal joints
Erysipelas laminitis	*Erysipelothrix rhusiopathiae*	Occurs in sheep following immersion in contaminated dipping fluid. The bacteria enter through skin abrasions in the hoof region causing cellulitis and laminitis.
Strawberry footrot	*Dermatophilus congolensis*	Proliferative, inflammatory lesions affecting the coronary band and the lower limb
Lamellar suppuration	Mixed bacterial infection with: *Fusobacterium necrophorum, Arcanobacterium pyogenes, Dichelobacter nodosus, Prevotella* species and other opportunistic bacteria	Pyogenic infection located between the horn and sensitive lamina. Bacteria usually enter at the white line between the horn of the wall and the sole. Often associated with trauma. Referred to as toe abscess

It is important to realize that more than one condition may be present in a herd or flock at a particular time and thus examination of sufficient numbers of animals to establish the correct diagnosis is essential (Winter, 2008). Furthermore, the occurrence of disease may be dependent on the presence of certain environmental conditions or predisposing factors before bacterial invasion can occur. Because of the complex relationship between the causative organisms and predisposing factors, the most appropriate and effective control measures are not always evident or feasible.

Table 91.3 Infectious foot conditions of cattle.

Clinical condition	Bacteria implicated	Comments
Bovine interdigital dermatitis	*Dichelobacter nodosus, Fusobacterium necrophorum, Prevotella* species, Spirochaetes (unclassified)	Benign condition with superficial lesions confined to the interdigital skin; usually subclinical
Bovine interdigital necrobacillosis	*Fusobacterium necrophorum, Porphyromonas levii*	Severe, necrotizing condition of interdigital skin; characteristic foetid odour. May extend to deeper tissues including joints. Also called foul-in-the-foot
Digital dermatitis	*Treponema* species; other opportunistic invaders	Proliferative dermatitis affecting the bulbs of heels in cattle. Also termed verrucose dermatitis. Opportunistic, secondary bacterial infection may contribute to severity of lesions
Lamellar suppuration	Mixed bacterial infection with: *Fusobacterium necrophorum, Arcanobacterium pyogenes, Dichelobacter nodosus, Prevotella* species and other opportunistic bacteria	Pyogenic infection located between the horn and sensitive lamina. Infection usually enters at the white line between the horn of the wall and the sole. Often associated with trauma. Referred to as white line abscess in cattle

Ovine footrot

Two clinical forms of footrot, virulent and benign, apparently relate to the invasiveness of the strain of *Dichelobacter nodosus* involved. Although *D. nodosus* is the principal pathogen in ovine footrot, other organisms are commonly associated with the condition (Table 91.2). The bacterium largely responsible for the initiation of footrot is *Fusobacterium necrophorum*, which causes tissue necrosis with a subsequent inflammatory reaction. Damage to the

interdigital skin as a result of constant wetting and infection with *Arcanobacterium pyogenes* are also involved in lesion development. The local anaerobic microenvironment facilitates infection with *D. nodosus* which possesses pili, allowing adherence to the epithelium of the foot. If the strain of *D. nodosus* has poor keratolytic activity, limited separation of horn from underlying matrix may occur at the heel. This benign form of footrot manifests as slight lameness which rapidly regresses after topical treatment or with the onset of dry weather. Virulent strains of *D. nodosus* cause extensive separation of horn from underlying matrix, extending from the heel to the sole and toe, with the formation of a foul-smelling necrotic exudate. Lameness, which is severe and persistent, usually involves more than one foot. Adult sheep are more commonly affected than lambs and the Merino breed appears to be more susceptible than other breeds.

Dichelobacter nodosus, a Gram-negative anaerobic bacterium, is an obligate pathogen of the feet of clinically-affected or chronic carrier ruminants. The organism can survive in warm, wet, muddy environmental conditions for up to 7 days. In summer, survival of the pathogen is favoured by grazing on lush pastures and wet underfoot conditions enhance survival when sheep are housed in winter. Both of these environmental situations can contribute to maceration of interdigital skin. For transmission to occur, daily mean temperatures must exceed 10°C.

Diagnosis of footrot is based primarily on clinical examination. A number of scoring systems have been devised to aid in the characterization and control of the disease (Whittington and Nichols, 1995). If microbiological confirmation of the strain virulence of *D. nodosus* is required, biochemical tests for virulence attributes can be used. However, tests for elastase production and gelatin liquefaction can take from 1 to 5 weeks to complete and are, therefore, of retrospective value only. Newer diagnostic methods based on PCR and detection of specific DNA products can be used for rapid detection of virulent and benign strains (Liu and Webber, 1995). Rapid detection of strain virulence is important because clinical differentiation between benign footrot and the early manifestations of the virulent form of the disease may be difficult.

Extensive studies on the control of footrot were carried out in Australia. They included comparisons of the economic return from different control options (Egerton *et al*., 1989 ; Egerton and Raadsma, 1991). Control strategies are available including topical treatment of affected feet, identification and elimination of virulent strains of *D. nodosus*, vaccination and genetic selection

for improved resistance to footrot. The conventional method of treatment and control of footrot is paring of affected feet to remove separated horn and establish drainage. This is followed by topical application of antibacterial solutions such as 10% to 20% zinc sulphate or 5% oxytetracycline solution. This method of treatment is labour intensive and is often replaced by the use of a foot bath, usually after separation of affected sheep from the rest of the flock.

Carriers of benign strains of *D. nodosus* cannot be identified as the interdigital skin remains normal and they do not develop lameness. Footrot has been eliminated from parts of Australia where control programmes involving the culling of carrier sheep have been set up. Culling takes place during the hot, dry spring/summer period when transmission of *D. nodosus* cannot occur. Elimination of footrot from other geographical regions which do not have consistent periods of hot, dry weather does not appear to be feasible (Green and George, 2008).

Although natural infection with *D. nodosus* confers no appreciable immunity, vaccination can increase short-term resistance and is a useful adjunct to control and treatment. The antigens which evoke a protective immune response are pili. There are ten major serogroups of *D. nodosus* and immunity develops to the homologous strain only. Knowledge of the serogroups prevalent in a flock is essential when selecting a vaccine. The vaccines currently available are inactivated. Although vaccines usually contain a number of different strains from the serogroups most commonly associated with footrot, protection cannot always be assured, even when the serogroups present in a flock are matched with those in a vaccine. Transformation of one serogroup of *D. nodosus* to another, which has been shown to occur *in vitro*, is a possible explanation for apparent vaccination failure in some cases (Wani and Samanta, 2006). Vaccination can be used therapeutically to reduce the severity and duration of infection. Two injections of vaccine are necessary.

Genetic selection may be aimed at increased resistance to virulent strains of *D. nodosus*. Alternatively, strategies might be directed towards increasing responsiveness to vaccination. Although considerable information is available on the genetic basis of susceptibility to footrot in Merino sheep, the practical application of selection for either increased resistance or responsiveness to vaccination has not yet been achieved.

Ovine interdigital dermatitis

In this mild disease, inflammation is confined to the interdigital skin. *Fusobacterium necrophorum*, which is the principal pathogen, invades the epidermis following maceration of the skin due to wet conditions underfoot or following local injury. The interdigital skin is erythematous and swollen, and there may be superficial greyish discoloration. Lameness is not usually apparent, and affected animals recover when underfoot conditions improve. Ovine interdigital dermatitis is clinically indistinguishable from benign footrot.

Contagious ovine digital dermatitis

This is a newly described condition which has been recognized in only a few countries worldwide, principally in the UK (Winter, 2008). It can be distinguished from other extremely virulent forms of footrot because ulcerative lesions appear first on the coronary band and interdigital lesions are not evident. The lesion progresses down the hoof wall, causing separation of the horn from the sensitive laminae and frequently resulting in complete separation of the hoof horn, leaving a raw stump. The aetiology of the condition is uncertain but treponemes were isolated from clinically affected hooves more frequently than from healthy feet (Moore *et al*., 2005). The organisms have been identified as *T. phagedenis*-like and *T. medium* / *T. vincentii*- like (Sayers *et al*., 2009) and are similar to those isolated from digital dermatitis lesions in cattle (Evans *et al*., 2008). Strains of *D. nodosus* similar to those isolated from cases of virulent ovine footrot have also been demonstrated in the feet of sheep affected by contagious ovine digital dermatitis (Moore *et al*., 2005). Thus, the role of the bacterial organisms associated with this condition and of other factors which may contribute to disease development is currently unclear.

Opportunistic suppurative conditions of the foot

In sheep, lamellar abscessation can occur at the heel or the toe. Horn defects may allow opportunistic infection which usually includes *F. necrophorum* and *A. pyogenes*. Increased weight in late gestation predisposes to heel abscessation in ewes. Extension of infection from ovine interdigital dermatitis may predispose to infection of the second interphalangeal joint. When abscess formation occurs at the toe, infection is usually confined to the corium of the hoof without joint involvement.

In cattle, lamellar abscessation is frequently termed white line disease. Infection of defective horn by opportunistic bacteria may occur at any point on

the white line. In dairy cattle, the condition often affects the lateral claw of a hind foot. This site is particularly susceptible to a combination of mechanical stress and subclinical laminitis which may predispose to disruption of horn structure, facilitating entry of pyogenic bacteria. The subsequent suppurative process may extend along the sensitive laminae to discharge at the coronary band or at the skin/horn junction of the heel. If left untreated the inflammatory process may involve the deeper tissues of the foot leading to septic arthritis of the second interphalangeal joint.

In pigs, lamellar abscessation (bush foot) occurs when traumatic lesions of the white line or the sole become infected. Trauma, due to rough floor surfaces, produces erosions of the horn and haemorrhage in almost 100% of intensively reared piglets. These minor lesions do not cause lameness unless they become infected and there is subsequent extension to the sensitive laminae. Progression of infection may be similar to that observed in other species with discharge at the coronary band. Serious sequelae such as arthritis and tenosynovitis can also occur.

Bovine interdigital necrobacillosis

This condition, which is also known as foul-in-the-foot or footrot, is an acute or subacute necrotizing interdigital dermatitis (Table 91.3). The infection results in necrosis with fissure formation in the interdigital skin and a purulent exudate. Extension of the process to underlying soft tissues is characterized by swelling. There is considerable pain and lameness. The tissue damage is a consequence of the synergistic action of *F. necrophorum* and *Porphyromonas levii*, formerly known as *Bacteroides melaninogenicus* subspecies *levii* (Berg and Loan, 1975; Berg and Franklin, 2000). In common with other bacterial infections of the digital skin and horn, predisposing factors are considered important in the pathogenesis of interdigital necrobacillosis Trauma, maceration of the skin after prolonged wetting, and nutritional deficiencies have been suggested as important contributory factors in the development of the condition. Bovine interdigital necrobacillosis usually affects one foot and extension to the second inter-phalangeal joint may occur. A par-ticularly severe form of the disease, termed ‘super foul’, in which there is rapid development of necrosis and spread to deeper tissues, has been reported (Cook and Cutler, 1995). Response to antibiotic therapy is poor. Isolates of *F. necrophorum* from these severe lesions are extremely virulent (Berg and Franklin, 2000). A possible relationship with

digital dermatitis has been noted in herds affected with ‘super foul’. Spirochaetes were observed in lesions but their aetiological role was not clearly defined (Doherty *et al*., 1998). Systemic antimicrobial therapy is the usual treatment for interdigital necrobacillosis although lesions may resolve without treatment. Early and sustained treatment is required for ‘super foul’, including local debridement of necrotic tissue and high doses of parenteral antibiotics for 5 days.

Bovine digital dermatitis

This condition was first described in Italy in 1974. Inflammatory lesions occur in the interdigital skin and may extend to the coronet. Two forms of the disease, erosive and verrucose, may reflect different stages in its development. The degree of lameness is variable and, on palpation, lesions may be tender. The aetiology of digital dermatitis is multifactorial. A number of infectious agents and environmental factors appear to be associated with the development of the disease. The condition is frequently seen in first-calved heifers. There may be an increase in the occurrence of the disease in loose-housed herds when conditions are unhygienic. The prevalence of the disease usually decreases when animals are at pasture.

Current evidence supports the hypothesis that spirochaetes have an aetiological role despite the absence of definitive experimental proof. Many of the spirochaetes which have been implicated appear to be closely related to human oral treponemes, and several different phylotypes are frequently present in a single animal (Klitgaard *et al*., 2008). In addition to their presence on the surface of lesions, treponemes can be found deep within the dermis, supporting the suggestion that they have an aetiological role in lesion development. Affected cattle produce anti-treponemal antibody, and treponemes isolated from digital dermatitis lesions are capable of inducing abscess formation in mice (Demirkan *et al*., 1999; Elliott *et al*., 2007). Other bacteria isolated from the lesions include *F. necrophorum*, *Prevotella* species, *Porphyromonas* species and *Peptostreptococcus (Peptoniphilus) indolicus* (Döpfer, 2000). Topical applications of antibiotics are used for treatment. Herd outbreaks can be treated with antibiotic solutions, usually of lincomycin, erythromycin or oxytetracycline, in footbaths, although legislative requirements may restrict their use in many countries (Laven and Logue, 2006).

Bovine interdigital dermatitis

Dichelobacter nodosus is considered to be the principal aetiological agent in this condition. The strains involved differ from those that cause virulent footrot in sheep. In several investigations, *D. nodosus* was not isolated from the lesions; a number of anaerobes including *F. necrophorum* and *Prevotella* species were isolated and spirochaetes were demonstrated in the pedal lesion. Digital and interdigital dermatitis may be closely related. Lameness is uncommon in bovine interdigital dermatitis. Footbaths containing formalin or copper sulphate are used as part of a control programme.

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Chapter 92

The role of microbial pathogens in diseases of the integumentary system

The skin is an extensive and versatile barrier between the *milieu intérieur* and the external environment. It plays an essential role in many of the homeostatic adjustments that make it possible for mammals to survive on land. Skin has a vital role in the regulation of body temperature. The skin pigment, melanin, protects the tissues from injury by ultraviolet light. The keratinized external surface of the skin limits the loss of fluid by evaporation from the body surface. The key role played by the epidermis in conserving water and electrolytes is evident from the life-threatening losses that occur when burns denude extensive areas of the keratinized layer. Skin offers a significant mechanical and chemical barrier to the entry of microorganisms and it contributes to an effective immune response when pathogens breach that barrier. Each of these functions is dependent on adaptations of histological structure that enable the skin to make the necessary homeostatic adjustments.

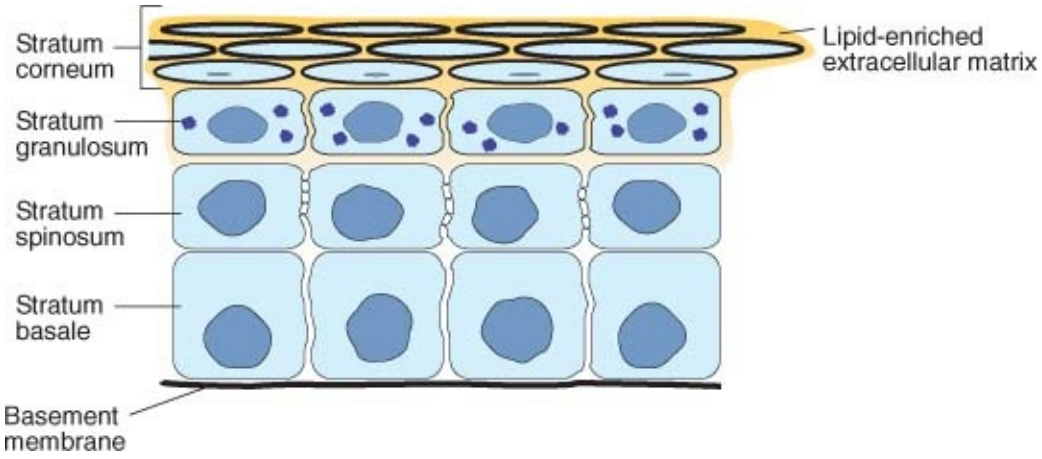
Structural and functional perspectives

The skin is composed of epithelial and connective tissue elements in two distinct but mutually dependent zones: the epidermis and the dermis. The two zones are separated by the basement membrane, a complex structure that anchors the epidermis to the underlying dermis and also serves as a semipermeable filter that selectively permits passage of fluids and cells in either direction. This is an extremely important property, because the blood vessels and lymphatics that supply nutrients to the dermis do not extend across the basement membrane. Sweat glands, hair follicles and associated sebaceous glands extend into the dermis as downgrowths of the epidermis. The dermis rests on a layer of subcutaneous adipose tissue, the *panniculus adiposus*. Beneath the fat, a sheet of fibrous connective tissue, fascia, connects the skin to the subjacent tissues and organs that it envelops.

The epidermis

The epidermis, the epithelial component of the skin, contains four distinct cell types: keratinocytes, melano-cytes, Langerhans cells and Merkel cells. The largest component of the epidermis is derived from keratino-cytes, proliferating epithelial cells that grow and differentiate into keratin-producing cells. The progenitor keratinocytes lie in the basal layer of the epidermis, where cell division occurs. The non-dividing daughter cells move outwards, enlarge, differentiate and progressively synthesize large amounts of keratin; in the process they establish a distinctive stratified squamous epithelium in which the stratum basale, the stratum spinosum, and the stratum granulosum underlie the keratinized stratum corneum (‘horny’ layer) at the surface of the skin (Fig. 92.1). The distinctive feature of the stratum spinosum is the presence of large numbers of intercellular desmosomes (‘spines’) that link adjacent keratinocytes. The granules of the stratum granulosum contain lipids that are extruded into the intercellular spaces at the junction of the stratum granulosum with the stratum corneum providing an intercellular water barrier and maintaining cohesion between the cells of the horny layer. In the stratum corneum, the cytoplasm, nuclei and organelles of the keratinocytes are degraded and are supplanted by keratin, contained within a tough insoluble sac, the cornified cell envelope. When the process of keratinization is completed, the stratum corneum consists of several layers of tightly packed, flattened, dead, horny cells (corneocytes). The association of the keratinized cells with the insoluble extracellular lipids has been compared to a wall in which the cells represent the bricks and the intercellular lipids represent the mortar. The more superficial keratinized cells are continually exfoliated as flake-like squames. The progenitor and differentiating keratinocytes express toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) by which they can sense the presence of pathogens. The keratinocytes secrete antimicrobial peptides (defensins, cathelicidins), chemok-ines and cytokines that enable these cells to participate in innate and acquired immune responses.

Figure 92.1 Schematic representation of epidermis illustrating the orderly progression of the epithelial cells from keratinocyte to corneocytes.



The other cells present in the epidermis are derived from the neural crest or from the bone marrow. Melanocytes, cells that synthesize melanin, are dendritic cells that migrated in early embryonic life from the neural crest to reside in the stratum basale. Their dendrites extend for long distances between keratino-cytes within the epidermis; each melanocyte is in contact with many keratinocytes and it can transfer melanin granules to those cells. Merkel cells are also derived from the neural crest and also reside in the stratum basale. They can detect tactile stimuli. Langerhans cells are immature dendritic cells that reside between keratinocytes, predominantly in the stratum spinosum. Their elongated dendrites extend into the interstitial spaces of the epidermis to create a cellular network that can detect infiltrating foreign antigens (Merad *et al*., 2008). They participate in both innate and adaptive immune responses. As immature dendritic cells, they recognize, capture and digest foreign antigens to peptide fragments (innate immune response) before they undergo transformation into mature dendritic cells, present the antigenic peptides to naÏve T cells in draining lymph nodes and direct the development of primary adaptive immune responses appropriate to the type of infectious agent detected (Lipscomb and Masten, 2002).

The dermis

The dermis consists of collagen and elastic tissue embedded in ground substance (a mucopolysaccharide gel) that has a high water-binding capacity. Nutrients and cells pass through the ground substance on their way from the blood vessels of the dermis to the avascular epidermis. The rich blood supply to the skin, which has three intercommunicating plexuses of vessels, terminates in the dermis where it has some direct arteriovenous shunts that play a significant role in thermoregulation. The endothelial cells of the dermal microvasculature can

express toll-l ike receptors; this may be important in sensing an infection, in the recruitment of immune cells from the bloodstream, and in healing of dermal wounds (Miller and Modlin, 2007). During inflammatory or immunological reactions, pro-inflammatory cytokines cause endothelial cells of the post-capillary venules to express greater numbers of adhesion molecules, thus promoting the passage of leukocytes into the dermis. Although the resident cell population of the dermis is scant, a variety of cell types is to be found close to the post-capillary venules: these include fibroblasts, mast cells, macrophages, lymphocytes and dermal dendritic cells, all of which express toll-like receptors and can contribute to immune responses. Mast cells contribute to inflammatory reactions: they release histamine, prostaglandins, TNF-aÏ chemotactic factors and other mediators. The dermal dendritic cells have a number of characteristics which indicate that they are not identical to Langerhans cells, although both cell lines function as antigen-presenting cells. Thus, the cellular components of the dermal immune system are located adjacent to the post-capillary venules in an intimate relationship that constitutes the dermal microvascular unit, a focal point of cutaneous reactivity to antigenic stimuli. Most resident T cells in normal uninflamed skin are located at these perivascular sites, from which they can be mobilized rapidly when a local immune response begins.

Responses of the skin to microorganisms

In health, the skin supports commensal microorganisms that locate on the surface and within the outer layers of the stratum corneum. Despite continuous desquamation of the more superficial corneocytes, some of the commensal microorganisms persist as resident microflora with which invading microbes must compete for binding sites and for nutrients. Some of the commensals act on lipids secreted by the sebaceous glands producing free fatty acids that are toxic to many bacteria. The resident flora may release antibacterial substances (bacteriocins) that inhibit the growth of other bacteria. Thus, the resident commensals help to inhibit colonization by pathogens. The intact skin provides a formidable physical barrier to invasion by pathogens. In the stratum corneum, the interlocking layers of dead keratinized corneocytes and the intercellular lipids form a ‘brick wall’ that pathogens cannot penetrate while it remains intact. The barrier function is reinforced by tight cell-to-cell adhesion within the layers of nucleated keratinocytes beneath the stratum corneum. In addition, the host has the protection of a chemical barrier created on the surface of the skin by the

constitutive release of antimicrobial lipids and antimicrobial peptides which can be released in greater amounts when the immune defences of the skin are challenged by pathogens.

Immune defences of the skin

Exposure to pathogens elicits responses from both the innate and adaptive arms of the immune system.

The innate immune arm recognizes invading pathogens, mounts immediate defensive action against them and sets the adaptive immune response in train. In turn, the adaptive immune arm is driven by dendritic cells that process microbial antigens to peptides, deliver the immunogenic peptides to draining lymph nodes, and direct the differentiation of naÏve lymphocytes into activated T cells responsive to the antigens of the invading pathogen. Thus, the immune response to cutaneous infection begins in the draining lymph nodes rather than in the skin.

Box 92.1 Key elements of skin which contribute to innate immune responses.

- Physical barrier at the stratum corneum
- Chemical barrier due to constitutive release of antimicrobial products on to surface
- Recognition of pathogens by toll-like receptors
- Induced release of antimicrobial products
- Recruitment of immune cells from the circulation

The innate immune response

The innate immune defence of the skin consists of five principal elements, of which the first two are the physical barrier to microbial invasion at the stratum corneum and the chemical barrier created by constitutive release of antimicrobial lipids and antimicrobial peptides (Box 92.1). Some of the antimicrobial lipids are synthesized and released by keratinocytes; others are delivered in secretions from sebaceous glands (Drake *et al.*, 2008). The principal antimicrobial peptides released by epidermal cells are defensins and cathelicidins. Small amounts of these cationic peptides are expressed constitutively by healthy skin. They exercise broad-spectrum activity against a diverse range of microorganisms from amongst Gram-positive and Gram-negative bacteria, fungi and enveloped

viruses. In this way, the host can prevent skin infection without generating an inflammatory response. However, when pathogens breach the physical barrier, the induced release of antimicrobial peptides by kerat-inocytes contributes to the initiation of a local inflammatory reaction.

The third element in the innate immune response is the recognition of invading pathogens by toll-like receptors. TLR-mediated responses include production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides. These molecules initiate and coordinate local and systemic inflammatory responses (Medzhitov, 2007). The local responses incorporate induced release of antimicrobial molecules (the fourth element of innate response) and recruitment of immune cells from the circulation (the fifth element). The induced antimicrobial peptides attempt to control cutaneous infection by killing the invading pathogens and by participating with the other peptides in the recruitment of neutrophils, monocytes, dendritic cells, NK cells, T cells and B cells to the sites of infection. In conjunction with several chemokines and cytokines, the antimicrobial peptides participate in a range of immunomodulatory activities in relation to the complement system, antibody production, cytokine production, phagocytosis and tissue repair (Pálffyi *et al*., 2009). Significantly, antimicrobial peptides are able to recruit and to activate dendritic cells, the antigen-presenting cells that link innate recognition of invading pathogens to the generation of appropriate types of adaptive immune responses (Lee and Iwasaki, 2007)

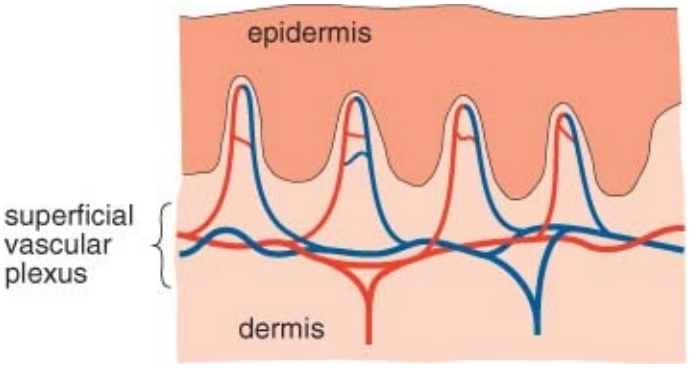
Box 92.2 Key elements of skin which contribute to adaptive immune responses.

- Invading pathogens are recognized by toll-like receptors
- TRL signals elicit release of chemokines and proinflammatory cytokines
- Immature dendritic cells engulf pathogens and present immunogenic peptides to T lymphocytes
- Dendritic cells present the peptides complexed with MHC molecules on the cell surface
- Mature dendritic cells transfer MHC–peptide complexes to draining lymph nodes
- Immunogenic peptides activate naÏve T cells present in lymph nodes
- Activated T cells travel to site of infection and initiate adaptive immune responses
- Activated B cells proliferate, differentiate into plasma cells which produce immunoglobulins

The adaptive immune response

Pathogens invading the skin are recognized by the toll-like receptors of keratinocytes, macrophages and dendritic cells (Box 92.2). In response to TLR signals, all three cell types release several chemokines and proinflammatory cytokines, principally IL-1, IL-6, and TNF-α. The chemokines and cytokines are recognized by receptors expressed on the immature dendritic cells. Thus the dendritic cells are activated by both direct and indirect signals from the TLRs. The immediate result is a sharp but transient increase in phagocytic activity by dendritic cells during which the infectious agents are engulfed and the microbial antigens are digested into immunogenic peptide fragments. After this short phase as antigen-collecting cells, the dendritic cells enter afferent lymphatics and migrate to the draining lymph node; en route, they undergo a maturation process which enables them to act as antigen-presenting cells. The main features of this transformation are loss of phagocytic capacity, expression of co-stimulatory molecules required for the activation of T cells, and expression of large numbers of MHC molecules on the cell surface.

Figure 92.2 The capillary–venular bed of the superficial plexus.



Mature dendritic cells combine the antigenic peptides with the MHC molecules, forming MHC-peptide complexes from which they can present the peptides to recirculating naïve T cells in the paracortical zones of the lymph nodes. The T cells, activated by interaction with the MHC-peptide complex, migrate from the lymph nodes to the site of infection where they undergo clonal expansion and initiate an adaptive immune response specific for the invading pathogen.

Histopathological responses of the dermis to microbial infection

The primary responsive component of the dermis is the microvasculature, specifically the capillary-venular bed of the superficial plexus (Fig. 92.2).

Proinflammatory mediators dilate these vessels, increase their permeability and activate their endothelial cells. The increase in permeability allows fluid and plasma proteins to cross from the blood to tissue spaces. Blood flows through the dilated capillaries and venules at a reduced speed, which allows the leukocytes to reach the periphery of the column of blood, in apposition to the vascular surface of the endothelial cells. Activated endothelial cells express adhesion molecules to which the marginated leukocytes attach. At the same time, some of the mediators are responsible for the expression of chemotactic factors that induce the marginated leukocytes to pass between adjacent endothelial cells into the perivascular dermis. Neutrophils, monocytes, lymphocytes, basophils and eosinophils may participate in the cellular response to cutaneous infection. The composition and the intensity of the cellular exudate can vary, depending on which mediators are released in response to the microbial challenge. The variation is attributable to the activity of mediators that modulate the expression and avidity of different adhesion molecules on the endothelial cells and of different chemotactic factors in the dermis. In most acute inflammatory reactions to bacterial pathogens, neutrophils are the first cells to invade the perivascular connective tissue and they remain the predominant cells for 6 to 24 hours. Thereafter, the infiltrate is likely to be dominated by mononuclear cells, initially by macrophages. The majority of macrophages derive from extravasated monocytes. Lymphocytes arrive at a later stage. If the bacterial infection extends into a chronic phase, the dermis is infiltrated by macrophages, lymphocytes and plasma cells. Chronic inflammatory lesions in the dermis commonly lead to the proliferation of fibroblasts and deposition of newly formed collagen fibrils. When excessive collagen forms, it becomes scar tissue.

Infection of hair follicles with accumulation of inflammatory cells within follicular lumina (luminal folliculitis) can lead to the rupture of follicular walls and discharge of the contents, including keratin, into the dermis. This condition, known as furunculosis, is caused most commonly by *Staphylococcus* species, less frequently by *Streptococcus* species and *Pseudomonas* species. Because of the discharge of keratin into the extracellular space, the dermis mounts an inflammatory reaction which includes a large number of eosinophils.

In several systemic infectious diseases, blood flow in the skin is compromised by inflammation and thrombosis in the dermal vasculature. In many instances, the consequences of the vascular lesions are evident as visible lesions in the epidermis, mainly as infarcts. Typically, the raised erythematous diamond-shaped lesions caused by *Erysipelothrix rhusiopathiae* in pigs are due to

vasculitis and thrombosis of small dermal arterioles. The visible lesion is an area of dry necrosis, an infarct, that occupies the segment of skin supplied by the occluded vessels. In several species, young animals that have recovered from septicaemic *Salmonella* infections develop dry necrotic lesions on the extremities of ears and tails due to vasculitis and thrombosis in small arterial vessels. Calves that recover from the septicaemic form of *Salmonella* Dublin infection in the first 3 months of life are liable to develop more serious dry gangrenous lesions affecting skin, underlying soft tissues and bone in the distal parts of their hind limbs. The gross lesion is similar to gangrenous ergotism caused by the alkaloids of *Claviceps purpurea* but the pathogenesis of the two conditions is different. Foci of cutaneous necrosis in the scrotum, ears, nose, nipples and limbs have been observed in dogs infected with *Rickettsia rickettsii*, the causal organism of Rocky Mountain spotted fever. This obligate intracellular pathogen replicates within host endothelial cells, destroys the cells and induces necrotizing vasculitis and thrombosis in the dermal vessels which, in turn, lead to the cutaneous infarcts.

Cutaneous necrosis is a feature of toxic shock syndrome in dogs. Many of the recorded cases have been attributed to *Streptococcus canis* but *Staphylococcus pseudintermedius* has been implicated in some dogs. Although the primary site of the infection is not always in the integument, the syndrome is often linked to necrotizing fasciitis, a deep-seated infection of the subcutaneous tissues. The pathogens may enter through a break in the epidermal barrier, perhaps a minor skin injury; they proliferate and extend along superficial and deep fascial planes. Bacterial enzymes and toxins may play a part in this expansive and extremely painful process. The streptococcal exotoxins and streptococcal superantigen cause the expression of large quantities of TNF-α, IL-1 and IL-6, setting in train a destructive process that may terminate in death. As the infected exudates travel along fascial planes, the fascia, fat and overlying dermis and epidermis become necrotic. There is thrombosis of dermal blood vessels, and extensive oedema and haemorrhage of the dermis and subcutis. Patches of the skin may be sloughed.

In oedema disease in pigs, strains of *E. coli* producing shigatoxin Stx2e proliferate in the intestine. The exotoxin is absorbed, attaches to receptors on vascular endothelial cells, increases vascular permeability and causes oedema in target tissues, including the skin of eyelids and the submandibular area.

Histopathological responses of the epidermis to

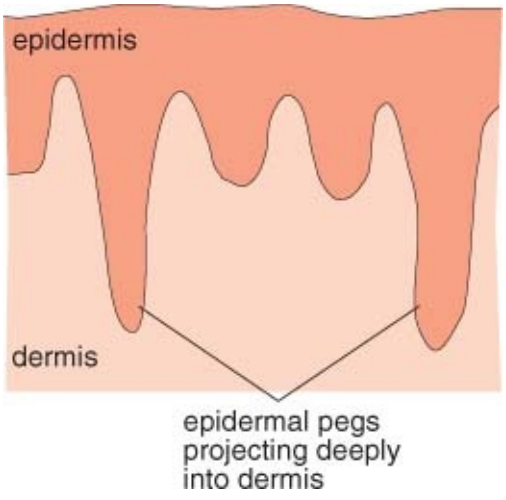
microbial infection

In normal circumstances, cell division in the stratum basale matches the loss of cornified cells from the stratum corneum. The continuity of the epidermal component of the skin is dependent on the appropriate completion of the three cardinal activities of the keratinocytes: proliferation, differentiation with outward displacement, and desquamation. Any disturbance in these processes or in the intercellular cohesion of the differentiating keratinocytes results in structural changes in the epidermis. A range of structural changes can develop during cutaneous infections.

Hyperplasia, an increase in the number of non-cornified keratinocytes, is due to an increase in cell division that is not matched by cell loss. The thickness of the epidermis is increased, usually by enlargement of the stratum spinosum, a condition described as acanthosis which is a feature of almost all chronic inflammatory skin conditions. Often, the hyperplasia results in pegs of epidermis that project down into the underlying dermis (Fig. 92.3).

Hyperkeratosis, excessive thickness of the stratum corneum, can arise as a sequel to excessive production of keratinocytes or to a reduction in desquamation. Naso-digital hyperkeratosis occurs in some cases of canine distemper (‘hardpad disease’). In some conditions, keratinization is imperfect, perhaps because the immature keratinocytes move outwards too quickly to allow the process to be completed. As a result, when the cells reach the level of the stratum corneum, they are imperfectly keratinized and they retain their nuclei, a condition known as parakeratosis. This condition is common in ringworm lesions in calves and in advanced exudative epidermitis in piglets.

Figure 92.3 Pegs of hyperplastic epidermis projecting deep into the dermis.



In the epidermis, the several layers of keratinocytes are held as a cohesive sheet of cells by the numerous desmosomes that link the plasma membranes of adjacent cells and by the intercellular cement substance. During acute inflammatory reactions in the skin, proteolytic enzymes released by neutrophils can contribute to the breakdown of desmosomes. Acantholysis, loss of cohesion between keratinocytes due to breakdown of desmosomes, results in rounded keratinocytes with clefts, vesicles, and bullae in the tissue. Clefts are slit-like spaces that do not contain fluid; vesicles, or blisters, are localized collections of tissue fluid within or beneath the epidermis; bullae are large blisters, greater than 1 cm in diameter. Cavities in the epidermis that are filled with purulent fluid are called pustules. Since the epidermis is avascular, the fluid that forms vesicles and the neutrophils that result in pustules arrive from the dermis. After they leave the bloodstream at the postcapillary venules in the outer region of the dermis, fluid and cells pass into the epidermis through the basement membrane and move along the spaces between the keratinocytes. When an influx of tissue fluid widens the intercellular spaces and accentuates the intercellular bridges, the keratinocytes appear to be elongated and the epidermis acquires a ‘spongy’ appearance. Spongiosis (intercellular oedema) is a common feature in epidermal inflammation caused by staphylococci or *Malassezia* species. Severe intercellular oedema may lead to rupture of intercellular bridges; spongiosis gives way to acantholysis. Similarly, intracellular oedema (ballooning degeneration) of keratinocytes induced by pox viruses can progress until the affected cells burst, resulting in the accumulation of fluid in multilocular vesicles whose septae are formed by the residual cell walls, a lesion described as reticular degeneration.

Acantholysis is the initial lesion in exudative epidermitis in suckling and weaned piglets (greasy pig disease). The disease is induced by epidermolysins released by *Staphylococcus hyicus* or by *S. chromogenes*. These exfoliative toxins cleave the epidermis between the stratum granulosum and the stratum corneum. The destruction of desmosomes by these exotoxins is comparable to the action of *S. aureus* epidermolysin in the scalded skin syndrome in children. The earliest microscopic lesions are subcorneal vesicles which become pustules when neutrophils move into the cavities.

Bacterial diseases of skin

The physical, chemical and immunological defences discussed above ensure that

the normal intact skin of a healthy animal is highly resistant to bacterial infection. However, bacteria can penetrate the physical barrier when the continuity of the epidermis is broken by wounds, trauma, scratches, biting insects, thorns, awns or hypodermic needles. Areas of skin that are damaged by chemical irritants, burns or freezing are liable to become infected, as are areas that are continually moist, or are covered with accumulated dirt, matted hair or exudates. Bacterial diseases of the skin occur more frequently in dogs than in other domestic animals. This is attributed to a relatively thin stratum corneum, and the small amount of intercellular lipids in the canine stratum corneum, together with the relatively high pH of canine skin and the absence of a protective lipid seal at the entrance of hair follicles. Many bacteria cause skin infections; most of the pathogens are pyogenic and the cutaneous pathological change they cause is called pyoderma. Coagulase-positive staphylococci are the most common cause of pyoderma in domestic animals. *Staphylococcus pseud-intermedius* is the predominant isolate from pyoderma in dogs and cats, while *S. aureus* is frequently isolated from pyoderma in horses, cattle and sheep, and *S. hyicus* is an important cause in pigs. Pyoderma is categorized as superficial or deep (Box 92.3). In superficial pyoderma, the lesions are confined to the epidermis and they may or may not involve the outer portion of the hair follicles. In deep pyoderma, the inflammatory lesions are located in the dermis, or the subcutis or both. They may develop fistulous tracts through which purulent exudate is released on to the surface of the skin.

Box 92.3 Classification of bacterial pyoderma.

- Superficial pyoderma
  - – skin fold pyoderma
  - – mucocutaneous pyoderma
  - – impetigo
  - – superficial spreading pyoderma
  - – exudative epidermitis of pigs
  - – folliculitis
  - – dermatophilosis
- Deep pyoderma
  - – folliculitis
  - – furunculosis
  - – cellulitis

– interdigital pyoderma complex

– panniculitis

– pyogranulomas

Superficial bacterial pyoderma

Superficial pyoderma is a generalized infection of the skin in which the gross lesions are erythema, vesicle formation, pustules, exudates, crusts, and peripheral expanding rings of flaky scales (‘epidermal collarettes’, the peeling remnants of the keratinized roof of the vesicle). The circular lesions of superficial pyoderma closely resemble ringworm lesions. The disease is commonly caused by coagulase-positive staphylococci but it is rarely associated with systemic illness and it does not leave a scar.

Impetigo is a superficial pustular dermatitis that does not involve hair follicles. In juvenile impetigo (prepubescent animals), the disease begins as subcorneal pustules that extend outwards projecting above the skin surface between the hair follicles. The pustules are composed predominantly of neutrophils; they are fragile, they rupture easily and the purulent discharge forms an adherent crust on the skin surface. In kittens, the lesions are commonly located on the dorsum of the neck and shoulders, where the queen grasps kittens in her mouth. Here, the infective agents are streptococci and *Pasteurella* species from the oral flora of the queen. Adult animals may develop a bullous (blistering) impetigo, consisting of large flaccid pustules that span several hair follicles. In dogs, this form of impetigo often occurs in conjunction with hyperadrenalism, diabetes mellitus, or hypothyroidism.

An expansion of *Staphylococcus pseudintermedius* pyoderma in dogs gives rise to a condition known as superficial spreading pyoderma in which the pustules evolve into coalescing and expanding erythematous lesions. It is thought that intense erythema indicates a hypersensitivity to staphylococci present within the pustule. Some associated lesions in the underlying dermis are interpreted as being consistent with a hypersensivity response: oedema, hyperaemia, neutrophilic vasculitis of the superficial venules and perivascular accumulations of neutrophils, eosinophils and mononuclear cells. In some affected dogs, pruritus is intense.

Staphylococcus hyicus, the cause of exudative epidermitis in neonatal and suckling piglets, produces an exfoliative exotoxin that targets cells of the stratum granulosum, destroying the desmosomal intercellular adhesion molecule that

binds those cells to the stratum corneum. The immediate result is acantholysis, cleavage of the epidermis at the interface between the two layers. The bacteria initiate subcorneal pustular dermatitis in the interfollicular epidermis which then extends to hair follicles, resulting in superficial purulent folliculitis. In neonates, the earliest lesions are on the head; they spread rapidly to thorax, abdomen and legs. Small areas of the stratum corneum peel off and the underlying tissue is quickly covered by a greasy, dark-brown exudate. The lesions rapidly coalesce and soon the whole body is covered by the exudate, giving the animal an overall greasy appearance. In the fully developed lesion, the epidermis is acanthotic, has microabscesses and has a fissured dark brown crust of keratin and exudate. In neonates, the affected skin is unable to serve its normal role in fluid and electrolyte balance: survival is compromised by dehydration and electrolyte imbalance.

Superficial exudative dermatitis is induced in a wide range of mammalian hosts by the Gram-positive actinomycete *Dermatophilus congolensis*. This bacterium expresses two distinct phenotypic forms during its life cycle: motile zoospores and filamentous hyphae. The flagellated zoospores are infective; after entry to the epidermis, they germinate into hyphae that arborize within the viable epidermis, induce inflammation and produce another generation of zoospores to complete the life cycle. In nature, the reservoir for *D. congolensis* appears to be in the skin of carrier animals, where dormant zoospores can survive for extended periods. Dry scabs on infected animals also harbour dormant zoospores for long periods. In both situations, persistent moisture is required to activate the spores before they can be transferred to new hosts by direct contact or through mechanical transfer by arthropod vectors, including ticks, flies and mosquitoes. Normally, the physical and chemical barriers at the surface of the skin are able to exclude *D. congolensis*. However, the organism can invade the epidermis when that tissue has been punctured or after it has been exposed to excess moisture over an extended period.

Dermatophilosis occurs worldwide, but most frequently in tropical and subtropical climates where the development and transmission of the disease are facilitated by the combination of high rainfall, high humidity, high temperature and the activities of biting insects that act as mechanical vectors. The proliferation of the filamentous pathogen within the epidermis elicits a strong inflammatory response that gives rise to micro-abscesses in the more superficial cell layers and a dense band of neutrophils beneath the epidermis, separating the epithelial tissue from the underlying dermis. The superficial cells cornify and

form scabs. Beneath the neutrophilic band, a new epidermis is formed by outgrowths from the hair follicles and from adjacent residual epidermis. In this way, the mass of inflammatory cells is segregated between the infected cornified epithelium and the newly formed epidermis. The new epidermis is often invaded by extension of microbial filaments from the hair follicles which initiates another cycle of bacterial invasion, inflammation and epidermal renewal. Repetition of this sequence of events results in the multilaminar purulent crusts of alternating layers of keratin and exudates that are characteristic of dermatophilosis. *Dermatophilus congolensis* is the causal agent of strawberry foot rot, a proliferative dermatitis of the lower limbs of sheep that is seen during wet summers. Small heaped-up scabs occur on the limb from the coronet to the knee or hock. Removal of the scabs reveals a bleeding mass of granulation tissue that resembles a fresh strawberry.

Deep bacterial pyoderma

Deep bacterial pyoderma may be manifest as folliculitis, furunculosis, subcutaneous abscess, cellulitis, pyogranulomatous inflammation or necrotizing fasciculitis. Staphylococci are the most common cause of these conditions in domestic animals; streptococci, *Pseudomonas* species and *Pasteurella multocida* are involved less frequently. Systemic bacterial infections can gain access to the dermis and subcutis by the haematogenous route. Blood-borne *Rhodococcus equi* can cause subcutaneous abscesses in foals, as does *Histophilus somni* in sheep. More commonly, pathogens reach the dermis through breaks in the integrity of the epidermis or by extension of an infectious process that has begun in the outer region of the hair follicle. Intense inflammation in the deep region of the follicle often leads to rupture of the follicular wall and extension of the infection into the surrounding dermis and panniculus. The local foreign-body reaction provoked by the released keratin, sebum and products of inflammation produces a furuncle from which fistulous tracts discharge purulent exudate on to the surface of the skin. Within the dermis, foci of purulent exudate may be circumscribed by granulation tissue or by mature fibrous tissue forming abscesses. Cellulitis is the term used to describe a diffuse, deep, acute inflammation that involves both dermis and subcutis. The lesion spreads underneath the skin in the panniculus and it is poorly circumscribed. Areas of the overlying skin are necrotic and may slough. Cellulitis is a common sequel to deep bite wounds in cats. In domestic animals, cellulitis has been attributed to a

wide range of bacterial infections, including staphylococci, *Pasteurella multocida*, *Corynebacterium pseudotuberculosis* and *Clostridium* species. Pyogra-nulomatous lesions, involving the dermis, the panniculus or both, occur in dogs, cats, horses and cattle. The most common isolates from these lesions are *Actinomyces viscosus* or *Nocardia asteroides*. Grossly, the lesions consist of firm nodules, abscesses, fistulous tracts, and extensive fibrosis. The exudate contains ‘sulphur granules’ Histologically, the lesion consists of a number of pyogranulomatous foci in an extensive field of fibrous tissue. Each of the abscesses contains a mass of Gram-positive bacteria clustered within an accumulation of neutrophils, which, in turn, are surrounded by epithelioid macrophages with some multinucleated giant cells. The fibrous tissue that separates and encapsulates the many abscesses is infiltrated by lymphocytes and plasma cells.

Viral diseases of skin

The intact skin is resistant to invasion by viruses, but these pathogens can gain entry through wounds or arthropod bites. Some viruses may induce skin lesions adjacent to the point of entry; for instance, the herpes-virus that causes bovine mammilitis and the papillomaviruses that induce papillomas in several species. More often, virus-induced cutaneous lesions arise during the viraemic phase of a systemic infection; this is true even for some viruses that enter percutaneously such as the virus of lumpy skin disease.

Skin lesions induced by viruses may be vesicular, proliferative or neoplastic. Vesicles are the characteristic primary lesions in foot-and-mouth disease and in swine vesicular disease. The lesions develop in skin around the mouth and on the feet, teats, and mammary gland. The viruses have a predilection for the metabolically active cells of the stratum spinosum where they cause ballooning degeneration of individual keratinocytes. The intercellular desmosomes of the affected cells are lost, the keratinocytes detach from one another, and the intercellular cavities fill with vesicular fluid. In foot-and-mouth disease, the small vesicles coalesce forming bullae. In cattle, some bullae may be up to 6 cm across and the extensive domes of epidermal cells are easily rubbed off, leaving raw ulcers that are prone to secondary bacterial infection.

Pox viruses induce both proliferative and degenerative changes in skin. A distinguishing feature of pox-virus lesions is the presence of intracytoplasmic inclusion bodies which may be seen in the epidermis, dermis or both. These

eosinophilic bodies are common in infected keratinocytes and, in the dermis, they are often seen in endothelial cells, macrophages, or fibroblasts. Inclusion bodies are prominent in the mononuclear cells that invade the dermis in sheeppox. In the epidermis, the pox lesions develop through a typical sequence: macule, papule, vesicle, pustule, crust, and scar. Histopathological changes begin with ballooning degeneration and rupture of keratinocytes, resulting in multiloculated vesicles (reticular degeneration) in the stratum spinosum. Fluid and neutrophils enter the epidermis from the dermis. Fluid distends the intercellular spaces, stretching the desmosomes (spongiosis) and progressing until the intercellular linkages are broken (acantholysis). The vesicular stage is prominent in some pox infections, such as sheeppox, but it is insignificant in others, such as contagious pustular dermatitis (‘orf’) in sheep. The infiltrating neutrophils aggregate in vesicles forming pustules. In contagious pustular dermatitis, the pustules remain flat and are covered by a thick layer of crust. In other pox diseases, the pustule has a depressed centre and raised borders. This umbilicated gross lesion is recognized as a ‘pock’. The raised borders are the result of hyperplasia of adjacent keratinocytes. The extent of epidermal hyperplasia varies among the several pox diseases; it is a marked feature of contagious pustular dermatitis in which parakeratosis and hyperkeratosis are prominent and the epidermis may reach up to four times its normal thickness. Sheeppox, the most serious pox disease of domestic animals, is responsible for marked pathological changes of the dermis as well as the typical pox lesions in the epidermis. The dermis is invaded by large numbers of virus- infected mononuclear cells with intracytoplasmic inclusion bodies (‘sheeppox cells’), severe necrotizing vasculitis develops in arterioles and post-capillary venules, and ischaemic necrosis of the dermis and overlying epidermis ensues.

Papillomaviruses induce two types of tumour-like lesions of skin: squamous papillomas and fibropapil-lomas. A typical papilloma is a wart-like mass in which there is proliferation of epithelial cells without proliferation of dermal fibroblasts. The epidermis is hyperkeratotic and hyperplastic and it is supported by thin stalks of dermis. Lesions may vary in size from small nodules to large cauliflower-like structures. In young cattle, these neoplasms regress spontaneously after some months. In fibropapillomas, the proliferating cell is the dermal fibroblast. The lesions appear as nodules or plaques covered by a variably hyperplastic and hyperkeratotic epidermis. The most common neoplasm of horses is the equine sarcoid, a fibropapilloma associated with a virus closely related to bovine papillomavirus types 1 and 2.

Fungal diseases of skin

Fungi abound in the environment but only a small proportion of the many thousands of fungal species has the ability to survive at the higher temperature in mammalian tissues. When fungi are able to cause disease in normal animals, they are regarded as true pathogens; other fungi are described as opportunistic pathogens because they induce disease only in animals in which resistance to infection is compromised by ill-health, immunosuppression, or inadequate nutrition (Box 92.4).

Based on the location of lesions, mycotic infections can be classified into three basic categories: superficial, subcutaneous, and systemic. Superficial dermatoses are infections in which the fungi are restricted to mucous membranes or to the epidermis and the keratinized structures of hair, feather, claw, and horn. These infections include candidiasis, *Malassezia* dermatitis, and dermatophytosis (ringworm). Subcutaneous dermatoses can be caused by a wide variety of saprophytic fungi that gain direct entry to the dermis and subcutis through accidental puncture of the skin by contaminated objects. The organisms are of low virulence, the disease is chronic and the lesions tend to remain localized. The lesions begin as small dermal or subcutaneous papules that enlarge gradually over months or years to become mycetomas, nodular masses of granulomatous inflammation with fibrosis and with fistulous tracts that discharge exudate on to the surface of the skin. Typically, the exudates contain grains composed of masses of fungal hyphae. Systemic mycoses are responsible for lesions in internal organs, such as the respiratory and digestive systems, but they may involve cutaneous tissues by haematogenous spread. These infections include blastomycosis and histoplasmosis.

Box 92.4 Factors that contribute to immunosuppression or render animals susceptible to infection.

- Cytotoxic chemotherapy
- Prolonged corticosteroid therapy
- Hyperadrenocorticism
- Diabetes mellitus
- Viral infections
- Neoplasia

- Mycotoxins
- Toxic plants which damage bone marrow
- Thymic atrophy related to senescence
- Exposure to ionizing radiation

Superficial dermatoses

Although *Candida albicans* is not a normal inhabitant of the skin, the yeast phase of the organism is commonly present as a commensal on the mucous membranes of the alimentary, upper respiratory and genital tracts and at mucocutaneous junctions at body orifices. Hence, most cases of candidiasis are opportunistic infections of endogenous origin. Invasion of host tissues is facilitated by the transformation of the organism from the yeast phase to the branching pseudomycelial phase. Candidiasis is mainly a disease of keratinized epithelium, most frequently in the oral cavity and in the stomach of young animals, especially piglets, calves and foals. Less frequently, when host resistance is compromised, *C. albicans* invades the keratinized epidermis. Cutaneous lesions evolve through papules, vesicles and pustules to discrete ulcers with erythematous borders and foul-smelling exudates. Special stains (such as periodic acid-Schiff or Gomori methenamine silver) can be used to demonstrate yeast cells on the surface of the lesion and pseudohyphae invading the hyperkeratotic epidermis.

Malazessia pachydermatis, a lipophilic yeast, is a member of the normal flora of the skin of dogs and cats. It is a common cause of otitis externa. On the skin, it proliferates and causes a greasy dermatitis when host defences are compromised. Clinical signs include hyperaemia, scaly plaques, hyperpigmentation, alopecia, pruritus and a rancid odour.

Dermatophytosis (‘ ringworm’) is an infection of keratin in the stratum corneum, hair, and nails. These infections are caused by a relatively small group of fungi, the dermatophytes, that are capable of degrading keratin and living on it. Keratin is an insoluble protein in which the polypeptide chains are stabilized by disulphide bonds between the abundant cysteine residues. As a result of this structural feature, keratin is very resistant to chemical degradation but reducing agents impair resistance to proteolytic enzymes. During infection, the fungi excrete large quantities of sulphite and they secrete a battery of proteolytic enzymes. The sulphite is a reducing agent that cleaves the disulphide bonds in keratin and it gives the proteases access to the reduced proteins. The proteases

degrade the polypeptides to short peptides and amino acids, which the fungi assimilate as nutrients.

Dermatophytes affecting domestic animals are members of one of two genera, *Microsporum* or *Trichophyton*. Based on their natural habitat and host preference, dermatophytes are grouped into geophilic, zoophilic and anthropophilic species. Geophilic fungi are present in the soil as free-l iving saprophytes but they can cause disease in animals and humans as opportunist pathogens, for example *Microsporum gypseum*. In contrast, zoophilic and anthropophilic dermatophytes have adopted a parasitic existence on the skin of mammalian hosts. Zoophilic species have become obligate parasites on the skin of animals, for example *Microsporum canis* in cats or *Trichophyton ver-rucosum* in cattle. Anthropophilic species have become obligate parasites on human skin. Some of the obligate parasites have become specialized for a single host; for example, *Microsporum nanum* is found almost exclusively in pigs. Other dermatophytes, such as *Trichophyton mentagrophytes*, infect a wide variety of mammals. Zoophilic fungi frequently cause disease in humans, whereas anthropophilic fungi rarely infect non-human hosts. The association with a particular host species is reflected in different types and degrees of inflammatory responses by human skin to anthro-pophilic and zoophilic dermatophytes. In young children, the highly anthropophilic *Microsporum audouinii* elicits a mild inflammatory response that may go unnoticed, whereas the zoophilic *Trichophyton verru-cosum* causes a very pronounced inflammatory response, sometimes accompanied by systemic signs. In general, dermatophytes tend to induce less intense inflammatory responses in their adapted hosts than they do in other hosts.

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Chapter 93

Bacterial causes of bovine mastitis

Mastitis is a common infectious disease in intensively farmed dairy cattle. Although more than 100 microbial species have been isolated from the bovine mammary gland, a relatively small number are responsible for most cases of clinical mastitis. It is usual to designate mastitis according to the origin of the organisms. Contagious mastitis is caused by bacteria that reside primarily in the mammary gland of cows whereas environmental mastitis is associated with microorganisms that are present in the environment. Formerly, contagious mastitis accounted for most outbreaks of the disease but, following the implementation of mastitis control programmes during the past three decades, the incidence of contagious mastitis due to *Staphylococcus aureus* and *Streptococcus agalactiae* declined. Data from the UK show that the incidence of clinical mastitis decreased from approximately 150 cases per 100 cows per year in the 1960s (Wilson and Kingwill, 1975) to between 47 and 65 cases per 100 cows per year in 2004–2005 (Bradley *et al*., 2007). Mastitis control programmes are particularly effective against pathogens that reside in the mammary gland. Control measures for mastitis pathogens which are widespread in the environment are difficult to implement. Mastitis remains the most common and economically important infectious disease of dairy cows (Kossaibati and Esslemont, 1997).

With the exception of *Mycoplasma* species which can invade the mammary gland from the bloodstream, most organisms which cause mastitis enter the gland through the teat canal. Fungal and viral pathogens are occasionally implicated in mastitis. Five bacterial pathogens are responsible for most cases of bovine mastitis (Box 93.1).

Mammary gland defence mechanisms

The teat orifice and the teat canal are the first barriers to infection of the mammary gland. Some pathogens such as S. *aureus* colonize the teat skin and

teat canal, increasing the probability of intramammary infection. Desquamation of keratinized cells from the epithelial surface of the teat canal may contribute to the mechanical removal of bacteria from this location. In addition, fatty acids present in the keratinized layer exert a bacteriostatic effect. The flushing action of milk through the gland also acts as a natural defence mechanism, and frequent stripping of the gland is recommended for treatment of mastitis caused by Gram-negative bacteria. Teat length may be important in determining susceptibility to infection. In heifers, short teats in association with oedema of mammary tissue at calving may predispose to mastitis (Waage *et al*., 2001). Superficial teat lesions also increase the likelihood of infection due to impairment of mechanical barriers. Even relatively mild hyperkeratosis of the teat orifice caused by incorrect milking machine function is associated with increased subclinical mastitis (Lewis *et al*., 2000).

Non-specific antibacterial factors found in the mammary gland are listed in Box 93.2. Lactoferrin exerts a bacteriostatic effect by binding free ferric ions which are then unavailable for bacterial utilization.

Box 93.1 Bacterial pathogens frequently isolated from cows with clinical mastitis.

- *Escherichia coli*
- *Streptococcus uberis*
- *Staphylococcus aureus*
- *Streptococcus dysgalactiae*
- *Streptococcus agalactiae*

Box 93.2 Non-specific soluble factors in the mammary gland with antibacterial activity.

- Lactoferrin
- Lactoperoxidase–thiocyanate–hydrogen peroxide system
- Complement
- Lysozyme

Because of the low concentration of lactoferrin in the lactating mammary gland, its principal function appears to relate to protection against coliform infection during the drying-off period. Lysozyme is a bactericidal protein active against Gram-positive bacteria but, because it is present in low concentrations in bovine

milk, its significance compared with other defence mechanisms is uncertain. The lactoperoxidase-thiocyanate–hydrogen peroxide system is bacteriostatic for Gram-positive bacteria and bactericidal for Gram-negative bacteria. This system depends on adequate concentrations of all three components within the mammary gland. Lactoperoxidase is synthesized in mammary epithelium whereas thiocyanate levels are influenced by the dietary intake of certain green feeds. Hydrogen peroxide may be produced by a variety of enzymatic activities in milk and by the metabolic activity of certain bacteria, if present. Complement, activated by the alternate pathway, may contribute to a limited extent to defence against Gramnegative bacteria. The role of other elements of innate immunity, including nitric oxide synthase and acute phase proteins such as serum amyloid A, in defence of the mammary gland is not yet clearly defined. Antimicrobial peptides are likely to have important immunomodulatory functions but their exact role in protection of the mammary gland against mastitis is uncertain (Bowdish *et al*., 2005).

Cell counts in the non-infected lactating mammary gland are usually less than 10^5 somatic cells per ml. The cell types include macrophages, with lesser numbers of lymphocytes and neutrophils and small numbers of epithelial cells. Cell counts tend to be elevated during early and late lactation and the percentage of neutrophils increases late in lactation. The speed of recruitment of cells to the udder is an important factor in susceptibility to mastitis, and cows with low somatic cell counts before infection are at greater risk of developing severe disease than cows with high cell counts. The inflammatory response is initiated by macrophages which recognize the invading pathogens. The macrophages release cytokines such as IL-1β and TNF-α which stimulate the inflammatory response and increase the bactericidal properties of neutrophils. Neutrophils are the principal cells involved in eliminating bacteria from the mammary gland. Recruitment of neutrophils from the blood to the site of infection in response to a number of inflammatory mediators, such as cytokines and prostaglandins, is one of the first steps in the inflammatory response. Epithelial and endothelial cells play an important role in recruitment of neutrophils as it is proposed that adhesion of bacteria to epithelial cells and the action of bacterial toxins on these cells induce the synthesis of cytokines such as TNF-α and IL-6 (Rainard and Riollet, 2003). These cytokines then activate expression of a number of adhesion molecules by endothelial cells. Binding of neutrophils to endothelial cells follows. Migration of neutrophils along the endothelium and into the tissues is facilitated by chemoattractants such as C5a, C3a, LPS, IL-1, IL-2 and IL-8

(Rainard and Riollet, 2006) Cell numbers in milk increase within hours of infection, with counts of several hundred cells per ml common in subclinical infections. In clinical mastitis, millions of cells per ml may be present. Neutrophils act by engulfing invading bacteria and subsequently killing them by oxygen-dependent or oxygen-independent systems. Oxidative damage is generally effective against Gram-negative bacteria but organisms such as *Staphylococcus aureus*, which produce catalase, can resist oxidative damage. Oxygen-independent killing is mediated through hydrolytic enzymes within lysosomes. However, the functioning of this mechanism may be less efficient in milk due to ingestion of casein and fat particles by neutrophils. Tissue damage and impaired mammary function may result from the respiratory burst and enzyme release by the activity of the accumulating neutrophils.

The role of lymphocytes in the protection of the mammary gland is the subject of much research. The proportion of T lymphocytes present, which varies with the stage of lactation, is greatest in late lactation, and the ratio of T lymphocyte subpopulations also changes throughout lactation. The functional significance of these changes, which is unclear, appears to correlate with reduced resistance to infection in the postpartum period (Sordillo *et al*., 1997).

The predominant immunoglobulin isotype in normal bovine milk, IgG_1, is selectively transferred into milk from serum but concentrations in normal milk are low, approximately 0.6 mg/ml (Butler, 1981) and concentrations of the other immunoglobulins are even lower. During mastitis, there is an increase in permeability of the epithelium separating the milk space from the interstitial space and this allows passage of molecules from blood into milk. This increase in permeability is important as it allows the concentrations of immunoglobulins in milk to rise during mastitis. The IgG_1 isotype opsonizes bacteria for phagocytosis by macrophages. As neutrophils are recruited into the affected tissue, the importance of IgG_2 increases because this isotype can opsonize bacteria for phagocytosis by neutrophils. IgM can also act as an opsonin. IgA agglutinates bacteria, prevents bacterial adherence to epithelium and neutralizes bacterial toxins.

Contagious mastitis

The bovine mammary gland is the principal reservoir of infectious agents which cause contagious mastitis, namely *Staphylococcus aureus, Streptococcus*

agalactiae, Mycoplasma bovis and *Corynebacterium bovis*. The source of infection is usually an infected mammary gland. Transmission of infection and appropriate control measures relate to factors such as the ability of a particular pathogen to survive in the host. Because streptococci and mycoplasmas are susceptible to environmental influences, they survive for much shorter periods outside the host than staphylococci. The severity of local systemic responses in mastitis depends directly on the virulence attributes of the pathogen.

Staphylococcus aureus

Infection with *S. aureus* is a common cause of clinical and subclinical mastitis in many modern dairy herds despite the implementation of mastitis control measures. *Staphylococcus aureus* can colonize the teat skin and teat canal and this may predispose to intramamary infection. However, the udder is considered to be the main source of infection as strain typing using techniques such as multilocus sequence typing has shown that strains of *S. aureus* derived from the udder are different from those isolated from other sites on the body. Smith *et al*. (2005) reported that the majority of strains causing intramammary infection in three countries (United Kingdom, United States of America and Chile) belonged to a single clonal complex. This finding suggests that certain strains are adapted to the bovine udder. Although staphylococci are resistant organisms which can survive in the environment for weeks, transmission of infection occurs mainly at milking through contaminated milkers' hands, teat cup liners and udder cloths. Adhesins such as fibrinogen-binding protein and fibronectin-binding protein A facilitate adhesion to the internal mucosal surfaces, and the organism produces a number of virulence factors which allow it to establish in spite of local immune responses. Enzymes such as hyaluronidase, staphylokinase and proteinases assist tissue invasion. Antiphagocytic factors such as a capsule allow staphylococci to resist phagocytosis and, even if engulfed, organisms can persist and multiply within phagocytes. When this occurs, *S. aureus* outlives the phagocytic cells and may be released into the tissues where it can cause further damage. Persistence within the mammary gland is likely to be enhanced for those strains that survive within mammary epithelial cells and by the production of biofilms (Melchior *et al*., 2006). Virulence factors such as exoenzymes, leukocidins and haemolysins, augment tissue damage.

Mastitis caused by *S. aureus* ranges in severity from peracute to subclinical. Chronic subclinical disease interspersed with periodic clinical episodes is the

most common form observed. There are no apparent differences in virulence factor production between isolates from acute and chronic staphylococcal mastitis, and variation in disease manifestations is likely to be influenced by the stage of lactation at which infection occurs. Severe disease usually develops early in lactation. In the most severe form, peracute gangrenous mastitis, the infection causes venous thrombosis with local oedema and congestion of the udder leading to tissue necrosis. In this uncommon form of staphylococcal mastitis, onset is sudden, and clinical signs include high fever, profound depression and anorexia. The affected quarter is swollen and sore on palpation. Udder discoloration becomes evident, and gangrenous black areas are obvious within 24 hours. Toxaemia may result in death unless appropriate treatment is instituted early. The acute form is characterized by severe swelling of the affected gland and a purulent secretion which often contains clots. Extensive fibrosis is a common sequel.

In chronic or subclinical staphylococcal mastitis, episodes of bacterial shedding from affected quarters occur along with elevated somatic cell counts. Clinical detection of this form of mastitis relates to the extent of tissue damage. Bacterial multiplication occurs principally in the collecting ducts and, to a limited extent, in the alveoli. The inflammatory response results in duct blockage and atrophy of the associated alveoli. Influx of phagocytic cells may lead to abscess formation and fibrosis which further limits effective clearance of the organisms and also interferes with antibiotic penetration during treatment. Accordingly, although some intramammary infections caused by *S. aureus* are cleared by immune mechanisms, the majority become chronic, low-grade or subclinical resulting in substantial production losses.

Streptococcus agalactiae

As a cause of mastitis, *S. agalactiae* has been encountered less frequently in recent years than in the past. However, it continues to be a problem in individual herds with high cell counts. This pathogen is an obligate parasite of the bovine mammary gland which can also survive to a limited extent in the environment. In herds with poor hygiene, environmental sources of infection may be important. The course of infection is similar to that of chronic *S. aureus* infection with cycles of bacterial shedding and high somatic cell counts. Few data are available on the virulence factors of *S. agalactiae* which are important in bovine mastitis. Various surface proteins are likely to be important in adherence; a

capsule offers protection against phagocytosis and exoenzymes including haemolysins contribute to the production of tissue damage. Following introduction into the mammary gland, *S. agalactiae* multiplies and invades the lactiferous ducts. Passage through the duct walls into the lymphatic system and the supramammary lymph node occurs. An influx of neutrophils into the gland follows and the inflammatory reaction results in blockage of the teat ducts and atrophy of secretory tissues. These inflammatory cycles occur periodically with progressive loss of secretory tissue. A relatively mild systemic reaction occurs coinciding with the first phase of replication and inflammation. Subsequently, clinical signs are usually mild and confined to the mammary gland. When the inflammation of the acini and ducts begins to resolve, the epithelial lining is shed, contributing to clot formation in the milk. Most udder damage has already occurred before clinically detectable changes in milk are evident.

Mycoplasma species

Although a number of *Mycoplasma* species have been isolated from outbreaks of bovine mastitis, the most important pathogen is *Mycoplasma bovis*. Mycoplasmal mastitis is particularly common in large dairy herds. The reservoir of infection appears to be clinically healthy calves and young cattle which harbour *M. bovis* in the respiratory tract. Infection may be introduced into a herd free of infection by accidental inoculation of the organisms with teat syringes or cannulae. Once infection becomes established, transmission to other animals occurs during milking. Affected cows can shed 10^5 to 10^8 CFU per ml of milk, contaminating milking machines, milkers' hands and cloths which are then important sources of infection for other animals in the herd. Haematogenous spread of infection between quarters occurs. *Mycoplasma bovis* can also cause congenital infection, thus maintaining the infection within a herd. The pathogenesis of mastitis caused by *Mycoplasma* species is unclear. It is thought that surface proteins are likely to be important for adherence and immune evasion as has been shown for mycoplasmal infections of other tissues. A purulent interstitial exudate is present throughout the gland resulting in degeneration of alveolar epithelium. This is followed by epithelial hyperplasia with fibrosis and atrophy in the late stages of the disease.

Clinical signs do not develop in all affected cows, and subclinical carriers are important sources of infection. When present, clinical signs include a dramatic alteration in milk consistency and a rapid decrease in milk yield within days of

infection. The secretion appears normal but, on standing, a deposit of sandlike or flocculent material settles out leaving a whey-like supernatant. Later in the disease, the secretion may be scanty and thick or serum-like, containing curds. As response to treatment is variable, infection often results in agalactia.

Coagulase–negative staphylococci and *Corynebacterium bovis*

These bacteria are minor mastitis pathogens but they may cause subclinical infections or mild clinical disease. They can be classified as contagious pathogens because coagulase-negative staphylococci are considered to be part of the normal flora of animals and *C. bovis* is an inhabitant of the bovine mammary gland and teat ducts. Infections with these bacterial pathogens are more prevalent in herds in which teat dipping is not practised nor dry cow therapy used, than in herds in which effective control measures are in place. Infection with coagulase-negative staphylo-cocci, apparently acquired during the dry period, is most common at calving. Although a number of different species of coagulase-negative staphylococci may be isolated from mastitis cases (see Chapter 14), *S. chromogenes* and *S. hyicus* are the species most often isolated from outbreaks of intramammary infection and clinical mastitis. Many infections with coagulase-negative staphylococci are eliminated shortly after calving but, if infection becomes established, it tends to persist. Some strains of coagulase-negative staphylococci isolated from cases of mastitis are invasive and produce toxins (Anaya-Lopez *et al*., 2006). Infection can result in increased somatic cell counts or in mild clinical disease in herds in which the other major mastitis-producing pathogens are under control. It has been suggested that cows harbouring coagulase-negative staphylococci are less likely to be affected with other major pathogens which cause contagious mastitis. Infection with *C. bovis* may protect cows against infection with *S. aureus* but protection against streptococci does not occur.

Environmental mastitis

Bacteria commonly present in the environment, especially *E. coli* and *Streptococcus uberis*, are the organisms most frequently isolated from cases of clinical mastitis in many countries. Contamination of teat ends is a major predisposing factor in the development of environmental mastitis. Because

environmental pathogens can survive and multiply in organic bedding materials, housing conditions can influence teat contamination rates. Mastitis caused by *S. uberis* is particularly associated with bedding of cows with straw. When sawdust and wood shavings are used for bedding, *E. coli* and *Klebsiella* species are often isolated from infected cows. The rate of infection is usually higher in housed cattle than in those on pasture although *S. uberis* has been isolated from intensively grazed pasture in numbers similar to those found in bedding material (Harmon *et al*., 1992). Infection can be transmitted by environmental contamination of intramam-mary tubes. Outbreaks of mastitis with organisms such as *Pseudomonas aeruginosa* or fungi, which are resistant to antibiotics, are particularly associated with this mode of transmission. Many infections caused by environmental pathogens occur during the drying-off period and in the weeks before calving. The majority of infections with *E. coli* occur during the 7 to 10 days before calving which may be explained in part by the ability of the organism to acquire iron from the dry mammary gland through the enterobactin iron-acquisition system (Smith and Hogan, 1993) Hogan and Smith, 2003). Typically, environmental mastitis is of shorter duration than contagious mastitis. In addition, infections caused by coliform bacteria are likely to be clinically evident and clinical signs may be severe. Bacterial pathogens periodically isolated from cows with mastitis are listed in Box 93.3.

Escherichia coliv and other coliform bacteria

An association between herds with low bulk milk somatic cell counts and an increased incidence of toxic mastitis has been recorded. The majority of these outbreaks are caused by infection with *E. coli* (Green *et al*., 1996; Menzies *et al*., 2000). The relationship between bulk milk cell counts, individual cow cell counts and the occurrence of toxic mastitis has not been fully elucidated. Recent studies suggest that slow recruitment of cells to the udder is a factor predisposing to mastitis and that the level of somatic cell counts in individual cows before infection can influence the course and severity of the subsequent mastitic episode (Hill, 1981; Shuster *et al*., 1996) Green, 2000). Somatic cell counts are not readily correlated with the risk of coliform mastitis.

Box 93.3 Environmental bacterial pathogens periodically isolated from cows with mastitis.

- *Arcanobacterium pyogenes*
- *Bacillus species*
- *Enterobacter aerogenes*
- *Enterococcus faecalis*
- *Klebsiella pneumoniae*
- *Leptospira serovars*
- *Mannheimia haemolytica*
- *Mycoplasma bovis*
- *Peptoniphilus indolicus*
- *Pseudomonas aeruginosa*

The pathogenesis of coliform mastitis is largely attributed to the effects of endotoxin. Endotoxic injury to the microvasculature of the alveolar walls and interstitial tissue of the mammary gland causes hyperaemia, haemorrhage and oedema of the affected quarter. Coliform bacteria do not usually invade the tissue and, if cows survive the effects of endotoxin, affected quarters can return to partial production in the same lactation. Severity of the disease is dependent on the neutrophil response and the pre-existing level of neutrophils in the gland. Clinical signs may be dramatic if the response is delayed and the existing level of neutrophils is low, a feature of disease in newly-calved cows. In peracute coliform mastitis, onset is sudden and the cow becomes toxaemic, anorexic, depressed and pyrexic. Within hours, recumbency and a drop in temperature to normal or subnormal levels is followed by profuse diarrhoea and dehydration. Usually, the affected quarter is not markedly swollen or hot. The serous secretion contains small flakes of necrotic tissue. This is a serious disease and death may occur within one or two days after the onset of signs. Animals that survive can recover within a few days. Some surviving animals remain recumbent for several days and resulting complications may require euthanasia. Acute disease is characterized by less severe systemic signs with a watery or serous secretion from the affected quarter. Recovery is the usual outcome. Chronic and subclinical infections occur and recurring infections with *E. coli* may be more common than previously thought (Bradley and Green, 2001).

Streptococcus uberis

Many sites in the cow including the tonsils, gastrointestinal tract and genital tract and the coat can yield *S. uberis*. Contaminated bedding is considered to be an important source of the organism facilitating transmission from the teats of one animal to another. Bacterial concentrations are high in straw, moderate in

sawdust and wood shavings and low in inorganic bedding such as sand. *Streptococcus uberis* can be isolated from heavily used pasture in numbers comparable to those present in contaminated bedding.

It is considered that adherence to undamaged mammary tissues is not of primary importance in the colonization of the gland by *S. uberis*. However, a specific adhesion molecule involved in attachment to bovine mammary epithelial cells has been identified by Almeida *et al*. (2006). Successful colonization may relate to an ability to resist phagocytosis by neutrophils and the acquisition of nutrients from milk. Streptococci are nutritionally fastidious organisms and *S. uberis* is unable to synthesize a number of amino acids which are essential for growth. This organism secretes a plasminogen activator which converts plasminogen to plasmin, a protease which can hydrolyse casein to peptides. It is thought that *S. uberis* can then use these peptides for growth. This proposed pathogenic mechanism is unconfirmed, but the partial success of the use of plasminogen activator as a vaccine suggests that it may have a role in the pathogenesis of *S. uberis* (Leigh, 1999). Resistance to both phagocytosis and killing by neutrophils is attributed to the presence of a capsule. Abnormalities in the milk are the only obvious clinical features of infection in most cows. Signs of systemic disease characterized by fever and anorexia are observed in less than 10% of affected cows.

Other environmental microorganisms

Streptococcus dysgalactiae appears to occupy an intermediate position between the contagious and environmental groups of mastitis pathogens. This organism can be found in the environment of cattle and has been isolated from the tonsils, mouth and vagina of cows. However, these bacteria can persist in the mammary gland, possibly due to their ability to invade bovine mammary epithelial cells. These pathogens can be transmitted from cow to cow during milking and are also implicated in summer mastitis of dry cows and heifers.

Box 93.4 Environmental microorganisms infrequently associated with bovine mastitis.

- *Campylobacterjejuni*
- *Clostridium perfringens* type A
- *Corynebacterium ulcerans*
- Fungal agents

- *Histophilus somni*
- *Listeria monocytogenes*
- *Mycobacterium bovis*
- *Nocardia asteroides*
- *Pasteurella multocida*
- *Prototheca zopfii*
- *Serratia marcescens*
- *Streptococcus zooepidemicus*
- *Streptococcus pyogenes* group A
- *Yersinia pseudotuberculosis*

Less common causes of environmental mastitis are often associated with the accidental introduction of the causative organism during intramammary infusion. Spores of *Bacillus cereus* or fungi may be introduced into the udder inadvertently through careless intramammary infusion techniques. *Pseudomonas aeruginosa* is an environmental organism which may be present in water and occasionally may contaminate commercial products such as teat wipes. In addition, antibiotics infused into the udder are ineffective against *Bacillus* spores and fungi, and are unlikely to be effective against *Pseudomonas* species. Clinical signs relate to the causative agent and range from the acute haemorrhagic mastitis caused by *B. cereus* to the mild, low-grade infections as observed in mastitis caused by *Listeria monocytogenes*. However, low-grade infections are important as a cause of decreased production. In addition, infection with *L. monocytogenes* has zoonotic implications. Microorganisms infrequently associated with bovine mastitis are listed in Box 93.4.

Summer mastitis

The term ‘Summer mastitis’ is used to describe an acute suppurative mastitis which occurs in dry cows and heifers at pasture during the summer and early autumn. It occurs mainly in northern and western Europe although sporadic outbreaks of a clinically similar disease occur elsewhere. *Arcanobacterium pyogenes* and *Peptoniphilus (Peptostreptococcus) indolicus* are considered to be the two principal pathogens involved in this mixed infection. A primary role for *S. dysgalactiae* has been suggested, and other bacteria, especially anaerobes, are frequently isolated from affected quarters. The high seasonal incidence of the disease suggests that risk factors for the disease increase in summer. Flies, especially *Hydrotoea irritans*, may transmit the bacteria involved in summer mastitis between cows, and colonization and infection are most likely to occur

when teats are damaged due to rough grazing. Dry cows and heifers are frequently grazed on less intensively managed farmland adjacent to woods and scrub, which form a suitable habitat for flies. Invasion of the mammary gland occurs via the teat canal and results in severe clinical mastitis with marked systemic effects. The virulence factors of *A. pyogenes* include a pyolysin which is cytolytic for cells of the immune system and is a putative vaccine candidate. Bacterial adherence to mammary epithelial cells is facilitated by neuraminidases and extracellular matrix-binding proteins. Animals affected by summer mastitis are pyrexic, anorexic, weak and depressed; some may abort. The affected quarter is swollen, hard and painful and the secretion is watery with clots. Later it becomes purulent with a foetid odour. If the cow survives the toxaemia, an abscess may form and eventually discharge to the exterior. There is usually complete loss of function of the quarter and affected cows are culled.

Diagnosis

The criteria for definition of mastitis have changed from those first set by the International Dairy Federation in 1967 to a current threshold of 100,000 somatic cells per ml (Hamann, 2005). However, regulatory limits for bulk milk vary between countries and thus cell counts taken as significant differ from country to country. A somatic cell count of greater than 200,000 per ml is a threshold frequently used to define sub-clinical mastitis in an individual animal. Establishment of an aetiological diagnosis is dependent on the quality of the milk sample submitted. If more than one organism is isolated from a milk sample, such a sample is regarded as contaminated and the results are unreliable. An exception occurs when mastitis follows severe traumatic injury to the teats in which mixed infections are relatively common. The correct sampling procedure should be followed for milk collection:

- Teats which are obviously dirty should be washed and dried immediately.
- Each teat end should be treated with 70% ethyl alcohol and left for 1 minute before sampling.
- As the first milk expressed may be contaminated, it should be discarded.
- The sterile container used to collect the milk sample should be held almost parallel to the ground at an angle close to 90° to the teat being sampled. This minimizes the risk of contamination from the udder or abdomen.
- The container should be capped tightly, labelled with the cow number and

date and submitted for immediate culture. If this is not possible, it should be stored at 4°C until it can be sent to the laboratory. Many mastitis pathogens survive freezing at -20°C and, accordingly, samples can be frozen and submitted in batches for culture. Some bacteria may not survive freezing and, if difficulty in isolating pathogens arises, samples should be submitted for culture immediately after collection.

Most mastitis pathogens can be isolated easily using routine culture methods. Media used for primary culture are blood agar, Edward's medium, which is selective for streptococci, and MacConkey agar. Colony morphology, patterns of haemolysis and growth characteristics on these media often allow a presumptive identification to be made. A definitive identification of a suspect pathogen can be made using tests specific for that organism as described in the chapters on individual pathogens. Diagnostic kits for the identification of the more common mastitis pathogens are available. These include miniaturized biochemical systems which test the ability of an organism to utilize different sugars. Other metabolic reactions can also be detected. These tests are frequently used for the identification of streptococci and members of the *Enterobacteriaceae*.- Commercial agglutination test kits are also available for Lancefield grouping of streptococci and for agglutination assays to differentiate *S. aureus* from coagulase-negative staphylococci.

Occasionally, bacteria cannot be isolated from mastitic milk samples. Reasons for this include:

- Treatment with antibiotics before sampling.
- Destruction of the bacteria in the course of the inflammatory reaction. In some forms of mastitis caused by *E. coli* or other environmental organisms, systemic effects of endotoxin continue in the absence of viable bacteria in the milk.
- Chronic mastitis in which the organisms have been eliminated but with persistence of the pathological changes.
- Failure to isolate pathogens may relate to the media and cultural methods used. Some microorganisms such as *Mycoplasma* species, *Leptospira* serovar Hardjo and fungi require suitable media, specialized isolation procedures and appropriate incubation conditions.
- Traumatic mastitis.

The history accompanying the samples may provide background information relating to samples which are bacteriologically negative. Further investigation may be required if the history does not match the results obtained. Molecular methods increase the sensitivity of detection and can detect injured or dead organisms. A recent study by Taponen *et al*. (2009) using real-time PCR techniques found that 43% of mastitis samples showing no bacterial growth on culture contained between 10^3 to 10^7 genome copies of mastitis pathogens per

ml of milk.

Molecular diagnostic methods

Molecular methods for the detection of mastitis pathogens have been developed and are particularly useful for pathogens which are difficult to culture. Cai *et al*. (2005) reported the successful use of a real-time PCR for the detection of *Mycoplasma bovis* in milk, an organism that requires specialized culture methods. In a review of molecular methods described in the literature, Cai *et al*. (2003) suggested that real-time PCR methods may be most useful as these methods are quantitative and thus may be able to differentiate between true pathogens and small numbers of contaminating organisms. Multiplex PCR procedures have been described for the detection and differentiation of the principal Gram-positive mastitis pathogens (Gillespie and Oliver, 2005).

Treatment

Antimicrobial agents are used extensively for the treatment and control of bovine mastitis. Intramammary antibiotic preparations are available to farmers in many countries and this easy access is likely to result in excessive antimicrobial chemotherapy. In countries such as Norway, where antibiotic preparations are available only on prescription, indiscriminate use of these therapeutic agents is less likely to occur. It is important that the rationale for use of antibiotics in the treatment of mastitis is thoroughly understood by veterinarians prescribing treatment and that this information is communicated clearly to farmers. Antibiotics used in the treatment of mastitis can be administered by parenteral or intramammary routes. In most cases, administration by the intramammary route is advised in order to achieve therapeutic concentrations in milk (Constable *et al*., 2008). Intramuscular or intravenous injection is likely to be beneficial for the treatment of acute clinical mastitis caused by *S. aureus* or for dealing with bacteraemia associated with severe *E. coli* mastitis. In acute mastitis, antimicrobial compounds given by the intramammary route may fail to reach the affected site due to occlusion of milk ducts by inflammatory exudates. Chemotherapeutic agents given by the parenteral route for the treatment of mastitis should, ideally, have appropriate characteristics (Box 93.5) which render them suitable for treatment of mastitis (Sandholm, 1995; Ziv, 1980).

Box 93.5 Desirable characteristics of drugs for parenteral administration in the treatment of mastitis.

- Low minimal inhibitory concentration for pathogens causing mastitis
- High bioavailability and distribution in mammary tissue after intramuscular or intravenous administration
- Chemical structure favouring accumulation in milk
- Low serum protein-binding activity
- Long half-life

Interactions between host, pathogen and antimicrobial agent

In bovine mastitis, the choice of antimicrobial agent is influenced by the nature of the pathogen and its location within the mammary tissues, the host reaction to the pathogen and the pharmacokinetics and mechanism of action of the drug. Because individual cases of mastitis are usually caused by a single bacterial species, the therapeutic agent selected should be as specific as possible. Accordingly, antimicrobial combinations and broad-spectrum antibiotics should be avoided. Collection of samples for isolation of the causative organism and determination of the antimicrobial susceptibility pattern should be completed before treatment commences. However, the clinical signs and the herd history may indicate which agent is most likely to be involved and treatment of clinical mastitis usually begins before identification of the causal agent. Treatment can be changed, if necessary, in the light of *in vitro* antibiotic sensitivity testing.

The effectiveness of antimicrobial drugs for treating mastitis caused by *Escherichia coli* is considered to be questionable because the main clinical manifestations of the condition relate to endotoxin activity and the subsequent release of inflammatory mediators. In experimental studies of acute *E. coli* mastitis, significant improvement in recovery rates was not demonstrable following antimicrobial therapy. Moreover, the rate of spontaneous recovery in subacute and mild infections with *E. coli* may approach 90%. Treatment with oxytocin along with frequent stripping of the mammary gland may be as beneficial as the administration of antibiotics.

Table 93.1 Chemotherapy used for the treatment of bacterial pathogens which

cause bovine mastitis.

Pathogen	Antimicrobial agents used for treatment	Comments
Staphylococcus aureus	Cephalosporins, cloxacillin, erythromycin, penicillin (if organism is susceptible), penicillin combined with novobiocin, pirlimycin, tetracyclines, tylosin, tilmicosin	Because of inadequate drug penetration at site of infection, clinical recovery is not assured and elimination of bacteria is unpredictable
Streptococcus agalactiae	Cephalosporins, cloxacillin, macrolides, penicillin	Successful treatment can be carried out during lactation. Eradication of the organism from a herd, using intensive antibiotic therapy, is possible
Mycoplasma bovis	Tetracyclines, tylosin	Because antibiotic treatment is usually unsuccessful, control is based on culling of infected animals
Escherichia coli	Ampicillin–cloxacillin, cephalosporins, gentamicin, marbofloxacin, tetracyclines	Antibiotic usage is of questionable benefit but may improve recovery rates in animals with impaired immune defences. Supportive therapy is essential in acute disease
Environmental streptococci	Ampicillin, cephalosporins, cloxacillin, novobiocin, penicillin, pirlimycin, tetracyclines, tilmicosin	Clinical cases respond well to treatment with antibiotics given by the intramammary route
Arcanobacterium pyogenes	Penicillin, tetracyclines	The suppurative reaction induced by *A. pyogenes* infection results in poor antibiotic penetration and treatment is usually ineffective

Antibiotics which accumulate in extracellular spaces are effective chemotherapeutic agents for treating streptococcal mastitis because streptococci are not intracellular pathogens. In contrast, staphylococcal infections are difficult to eliminate because *Staphylococcus aureus* can survive in phagocytes. Moreover, the abscess formation and fibrosis which are features of these infections limit penetration of antimicrobial agents. Furthermore, β-lactam antibiotics may be inactivated by the oxygen burst in phagocytes stimulated by the presence of staphylococci.

Antibiotic susceptibility testing can determine the therapeutic agent which is most effective *in vitro* against a particular pathogen. However, *in vitro* effectiveness may not match the results obtained)*in vivo*. Moreover, many antimicrobial agents lose much of their activity when milk is incorporated into the medium used for antibiotic susceptibility testing. Macrolides are up to 90% less effective and tetracyclines up to 75% less effective against staphylococci when tested in a medium containing milk (Sandholm, 1995).

Apart from resistance to penicillin encountered in staphylococci, many pathogens of the mammary gland remain susceptible to the majority of antimicrobial agents. Although an ideal chemotherapeutic agent is not available for the treatment of mastitis, antimicrobial compounds currently used are presented in Table 93.1.

Antibiotic resistance

The resistance of staphylococci to antibiotics is a major obstacle to the successful treatment of mastitis in dairy cattle. Although there are many reasons

for the failure of treatment in staphylococcal mastitis, production of β-lactamase by certain staphylococcal strains is the principal reason for failure. The prevalence of penicillin-resistant strains of *S. aureus* differs from country to country. Approximately 20% of strains isolated in Norway were resistant to penicillin (Brun, 1998); 36% of strains tested were resistant in a study conducted in a number of European countries, the US and Zimbabwe (de Oliveira *et al*., 2000). In a study conducted in Sudan, 73% of *S. aureus* isolates exhibited multiple resistance (Kuwajock *et al*., 1999). Resistance to macrolide antimicrobial agents which are commonly used for the treatment of *S. aureus* mastitis is much less prevalent than resistance to penicillin, with rates of 14 to 17% being reported. The use of cloxacillin may select for methicillin-resistant *S. aureus*.

Antibiotic therapy in lactating cows

During lactation, antimicrobial therapy is generally used for treatment of clinical mastitis, whereas dry cow therapy is employed for controlling subclinical disease. Mastitis caused by *S. agalactiae* is exceptional as both clinical and subclinical disease can be successfully treated during lactation. *Streptococcus agalactiae* is usually treated by the intramammary route, with success rates approaching 100%. Saturation therapy can be used to eradicate the disease from some herds. This entails identification and treatment of all infected cows combined with strict hygienic measures to prevent the spread of infection. Any cows still infected after this regime will require further treatment. A second option entails treating all lactating cows in the herd.

Treatment of *S. aureus* infections during lactation results in clinical recovery rates of 30 to 60%. However, complete elimination of the organism is rarely achieved during lactation and invariably requires dry cow therapy. Treatment of subclinical mastitis during lactation is usually considered uneconomic although recent work by Swinkels *et al*. (2005a, b) suggests that treatment may be profitable in some herds. Antibiotic treatment of mycoplasmal mastitis is generally not effective although the successful treatment of an outbreak of *M. californicum* and *M. canadense* mastitis with a combination of intramammary chlortetracycline and intramuscular tylosin has been reported (Mackie *et al*., 2000). There is uncertainty about the value of antibiotic therapy for the treatment of *E. coli* mastitis. However, when treatment is given early enough to limit endotoxin production, antibiotics may improve recovery rates during the early

postpartum period when a degree of immunosuppression may be present. In addition, in cases of acute coliform mastitis in which bacteraemia develops, systemic antimicrobial therapy may be required (Wenz *et al*., 2001).

Dry cow therapy

Administration of intramammary antibiotics at the beginning of the dry period is used for the treatment of mastitis caused by contagious pathogens, principally *S. aureus*. Treatment of subclinical cases of mastitis due to environmental organisms such as *S. uberis*, which are detected late in lactation, may be deferred until the dry period. Rates of clearance of *S. aureus* achieved with dry cow therapy range from 25 to 75%. Treatment is unlikely to be successful in older cows, and in cows with high somatic cell counts when infection has persisted and when more than one quarter is infected.

Other therapeutic measures

To combat the effects of endotoxin, supportive therapy in the form of intravenous fluids and anti-inflammatory drugs is important in the treatment of peracute and acute *E. coli* mastitis. Oxytocin, in combination with hand stripping, assists in the removal of organisms, their toxins and inflammatory debris. In addition, oxytocin has been found to be as effective as antibiotics for treating experimentally induced *S. aureus* mastitis (Knight *et al*., 20005. Homeopathic, herbal and other remedies are used for the treatment of mastitis but their efficacy is difficult to assess due to lack of objective published data.

Prevention and control

Measures appropriate for the prevention and control of bovine mastitis differ depending on whether the causative organisms are contagious or environmental in origin. Although some measures such as a correctly functioning milking machine are useful in the prevention of predisposing conditions, it is essential that the major pathogens causing mastitis on a farm are identified in order to formulate effective control strategies. A general plan for the control of bovine mastitis is presented in Box 93.6.

Contagious mastitis

The reservoir of infection for contagious mastitis is infected cows and measures aimed at elimination of infection from the mammary glands are of major importance in control.

- The efficacy of dry cow antibiotic therapy depends on the susceptibility of the infecting pathogens. Dry cow therapy is effective against up to 80% of streptococcal infections but is effective against an average of only 50% of *S. aureus* infections.
- Elimination of teat lesions helps to reduce colonization of the teat skin, especially by *S. aureus*.
- Culling persistently-infected cows is important for the control of staphylococcal mastitis and for the control of mycoplasmal mastitis.

Box 93.6 Control plan for bovine mastitis.

- Properly maintained milking equipment
- Hygienic milking practices
- Post-milking teat disinfection
- Antibiotic therapy for clinical cases and for dry cows
- Culling of persistently-infected animals

Prevention of new infections requires measures to exclude introduction of pathogens into the teat and to reduce exposure of the teats to pathogens.

- Correctly maintained milking equipment minimizes liner slip and incorrect vacuum levels. A properly functioning milking machine reduces the risk of introducing pathogens into the teat canal because of vacuum fluctuations and abnormal pressure gradients within the teats. In addition, milking machine performance can directly affect teat tissues. Prevention of minor lesions such as hyperkeratosis of the teat orifice helps to reduce the incidence of mastitis. The milking machine cluster can transmit contagious pathogens and efficient designs can reduce transmission. Research suggests that low-volume claws, absence of air transmission and low milk-flow rates increase transfer of pathogens between teats (Woolford, 1995).
- Hygienic milking practices, which include washing visibly dirty teats with a disinfectant solution, followed by drying with paper towels, and effective washing and disinfection of the milking machine, reduce the likelihood of infection.
- Effective post-milking teat dipping or spraying is a major control measure for contagious mastitis. A limited number of chemical disinfectants can be used as teat dips. These include chlorine-releasing compounds, iodophors, quaternary ammonium compounds and chlorhexidine gluconate. The range of suitable disinfectants available is limited because teat dips should fulfil a

number of criteria in order to be useful and safe. They should be non-irritating and non-toxic. In addition, they should remain active in the presence of organic matter such as milk and should not be absorbed into the tissues or leave undesirable residues in milk.

- Milking clinically affected cows last reduces the likelihood of transmission of infection. If it is not possible to segregate clinically affected cows, disinfection of milk clusters immediately after removal from an affected cow or use of separate clusters for infected cows can help to reduce the spread of infection.

Control measures appropriate for the prevention of contagious bacterial pathogen transmission are summarized in Fig. 93.1. Increasing the resistance of the cow through vaccination would be an obvious way of preventing and controlling pathogens causing contagious mastitis. Although research is continuing into vaccines against *S. aureus* and a commercial inactivated vaccine is available in the USA, efficacy of such vaccines is limited and their use is unlikely to be economically worthwhile where the prevalence of *S.aureus* mastitis has been reduced using other established control measures.

Environmental mastitis

Reduction of the number of pathogens in the environment depends on maintenance of satisfactory conditions while cows are housed or at pasture. Housing facilities should be correctly designed and well maintained for both lactating and dry cows. Facilities for dry cows and those that are calving are especially important for the control of *E. coli* mastitis as, in many instances, acquisition of infection occurs just before calving. Some important measures for reducing the reservoir of pathogens in the environment of the cow include the following:

- Provision of housing facilities designed to ensure correct lying behaviour with good cubicle usage to avoid teat injuries.
- Clean, dry bedding minimizes the multiplication of pathogens. Bacterial numbers are lower in dry inorganic bedding such as sand or mats than in organic bedding such as straw or sawdust.
- Well ventilated buildings prevent wet conditions which encourage the build-up of potential pathogens.

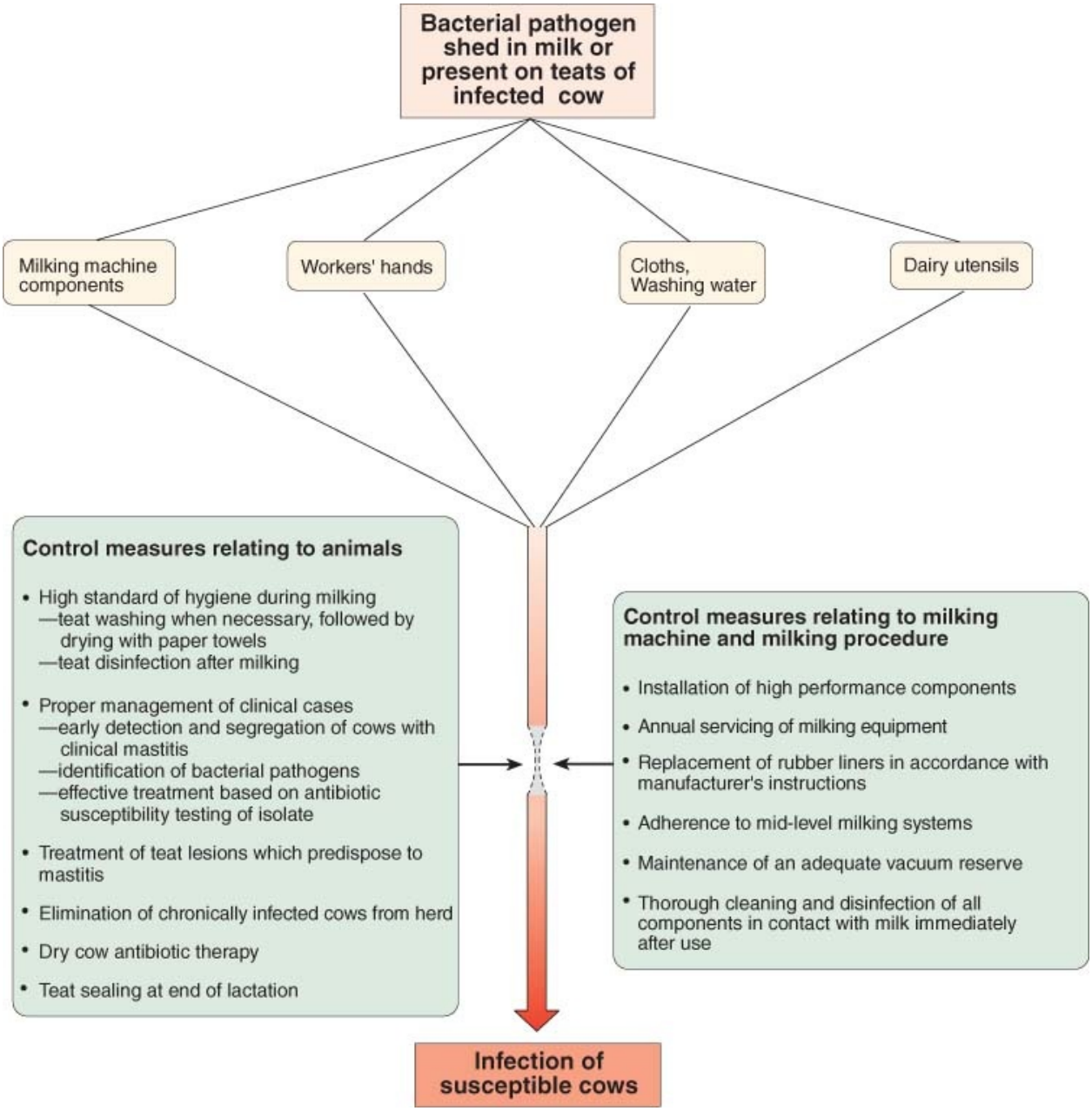
Reduction of new infections can be achieved by using:

- Correctly functioning milking equipment to prevent the introduction of

environmental pathogens into the teat canal and udder.

- Internal teat seals at drying off reduce the rate of new infections both during the dry period and at calving (Woolford *et al*., 1998;Berry and Hillerton, 2007). Dry cow antibiotic therapy can also be helpful in the prevention of new infections but it has no effect on the reservoir of mastitis-producing bacteria of environmental origin.
- Keeping animals standing after milking until the teat sphincter has fully closed may decrease the risk of infection. This can be achieved by feeding animals after milking.
- Teat dipping before milking is reported to reduce new infection rates with environmental pathogens by as much as 50% in some studies; in other studies improvement was not observed.
- A commercial vaccine is available for preventing mastitis caused by *E. coli*. This vaccine is a rough mutant *E. coli* strain which lacks the O antigen and consists only of core antigen. This core antigen, which is highly conserved among Gram-negative bacteria, is composed of lipid A and some common core polysaccharides. The core antigen accounts forthe cross-protection afforded by this vaccine against a wide range of Gram-negative bacteria (Dosogne *et al*., 2002). However, although vaccination of cows during the dry period and early lactation reduces the severity of clinical coliform mastitis, it does not protect against infection and does not always reduce the incidence of clinical mastitis (Wilson *et al*., 2007). Another benefit of vaccination, in addition to reduced severity of clinical disease, is reduced loss of milk production following clinical infection in vaccinated cows (Wilson *et al*., 2008). Commercial vaccines against streptococci causing bovine mastitis are not available but research is ongoing into the development of vaccines for *S. uberis*, including investigation of T cell responses (Denis *et al*., 2009). The stimulation of cell-mediated immune defences in combination with a humoral response may also be important for the development of vaccines against *S. aureus* (Denis *et al*., 2009).

Figure 93.1 Transmission of contagious bacterial pathogens which cause mastitis in dairy cows and relevant control measures.



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Chapter 94

Disinfection, biosecurity and other aspects of disease control

Advances in the development of chemotherapeutic drugs and effective vaccines have provided the veterinary profession with improved methods for controlling and preventing many infectious diseases in animals, especially in intensively-reared animals. Despite these advances, infectious diseases remain a major obstacle to increased productivity. As a consequence of the limitations of chemotherapy and vaccination for controlling transmission of infectious agents, improved biosecurity measures combined with effective disinfection programmes are recognized as essential components of animal management systems on farms and production units in many regions of the world.

Many infectious diseases of animals are spread not only directly by the infected animal but also indirectly, through environmental contamination. Intensive management systems may contribute to the occurrence of enteric and respiratory diseases, particularly in young animals. Effective control measures are required to minimize the spread of infectious agents in susceptible animal populations, especially when intensive production systems are used. Vaccination is one of the preferred methods for preventing infectious diseases caused by specific pathogens. However, many major diseases cannot as yet be controlled by vaccination. In addition, some ‘complex’ diseases of mixed or uncertain aetiology cannot be controlled by this method.

Measures for the control of infectious diseases in domestic animals include accurate identification of animals and restrictions on their movement either into or within a country. Following an outbreak of an infectious disease, isolation of infected and in-contact animals is used to limit spread. If the disease is exotic or subject to a national eradication programme, laboratory testing of clinically affected animals is followed by slaughter of infected and in-contact animals. For endemic infectious diseases, vaccination, disinfection, chemotherapy and chemoprophylaxis are employed selectively, depending on the aetiological agents and the methods applicable for their control. In most countries, the

control measures applied to a particular disease relate to its status within the country, its economic importance both nationally and internationally, and its public health significance. Preventive treatment and control measures appropriate for particular infectious agents are presented in Table 94.1.

Despite the availability of a wide range of chemo-therapeutic drugs and a large number of effective veterinary vaccines, infectious diseases still cause substantial losses in animal populations worldwide. In addition to losses as a result of mortality, there are costs arising from decreased productivity of meat, milk and eggs, reproductive failure and treatment programmes. Infected animals frequently shed pathogenic microorganisms, often in large numbers, and the resulting environmental contamination is an important method of transmitting infection to healthy animals. Apparently healthy carrier animals may shed microbial pathogens intermittently if stressed by transportation over long distances, by adverse housing conditions or by severe climate changes (Fig. 94.1). Salmonellosis, paratuberculosis, leptospirosis, parvovirus and rotavirus infections are examples of diseases in which extensive environmental contamination occurs.

Table 94.1 Methods for the prevention, treatment and control of particular infectious agents.

Infectious agent	Disease / Hosts	Methods					Comments
		Movement[a] restriction	Vector control	Chemotherapy	Disinfection	Vaccination	
Bacillus anthracis	Anthrax / Many species	+	–	+	++	++	Endospores survive for many years in soil; vaccination is permitted where disease is endemic
Streptococcus equi	Strangles / Horses	+	–	+	++	±	Efficacy of vaccines uncertain
Clostridium tetani	Tetanus / Many species	–	–	+	±	++	Endospores of *C. tetani* are widely distributed in soil and in faeces of animals
Microsporum canis	Ringworm / Many species	+	–	+	+	+	*M. canis* is transmitted by direct and indirect contact
Histoplasma capsulatum	Histoplasmosis / Many species	–	–	+	+	–	Soil-borne fungus which causes opportunistic infections
Foot-and-mouth disease virus	Foot-and-mouth disease / Many species	++	–	–	++	+	Vaccination is permitted where disease is endemic. Vaccinal strain must match field strain, and duration of protection is limited
African swine fever virus	African swine fever / Pigs	++	++	–	++	–	Soft ticks of the genus *Ornithodoros* are vectors of the virus

a, exclusion from a country, quarantine or restriction of movement on affected farm.

++, effective method; +, effective under defined conditions; ±, of questionable value;–, not applicable.

Movement of animals for sale, breeding, restocking or competitive events often contributes to the spread of infectious agents. In addition to sick animals exhibiting clinical signs, subclinically affected animals may shed infectious agents. Carrier animals, which appear clinically normal, can also shed pathogens intermittently. The role of animal feeds in disease transmission has become a topic of international importance following the unexpected appearance of bovine spongiform encephalopathy (BSE) in British cattle. The extreme resistance of the agent of BSE to thermal and chemical inactivation renders recycling food of animal origin an undesirable practice, especially if derived from ruminants.

Survival of infectious agents in the environment

Infectious agents shed in excretions or secretions of animals, or present in

products of animal origin, may remain viable for long periods in the environment. Buildings, transport vehicles, soil, pasture, water and fomites may become contaminated by the faeces or urine of infected animals containing bacterial or viral pathogens.

Considerable variation in the survival times of animal pathogens in the environment is recorded (Fig. 94.2). Survival times, however, are influenced by many factors including the number of infectious agents excreted by an infected animal, the availability of nutrients, competition from other microorganisms in the same environment and other microenvironmental factors such as the type and amount of organic matter present, temperature, pH, humidity and exposure to ultraviolet light.

Figure 94.1 Modes of transmission of infectious agents from infected to susceptible animals and relevant control measures.

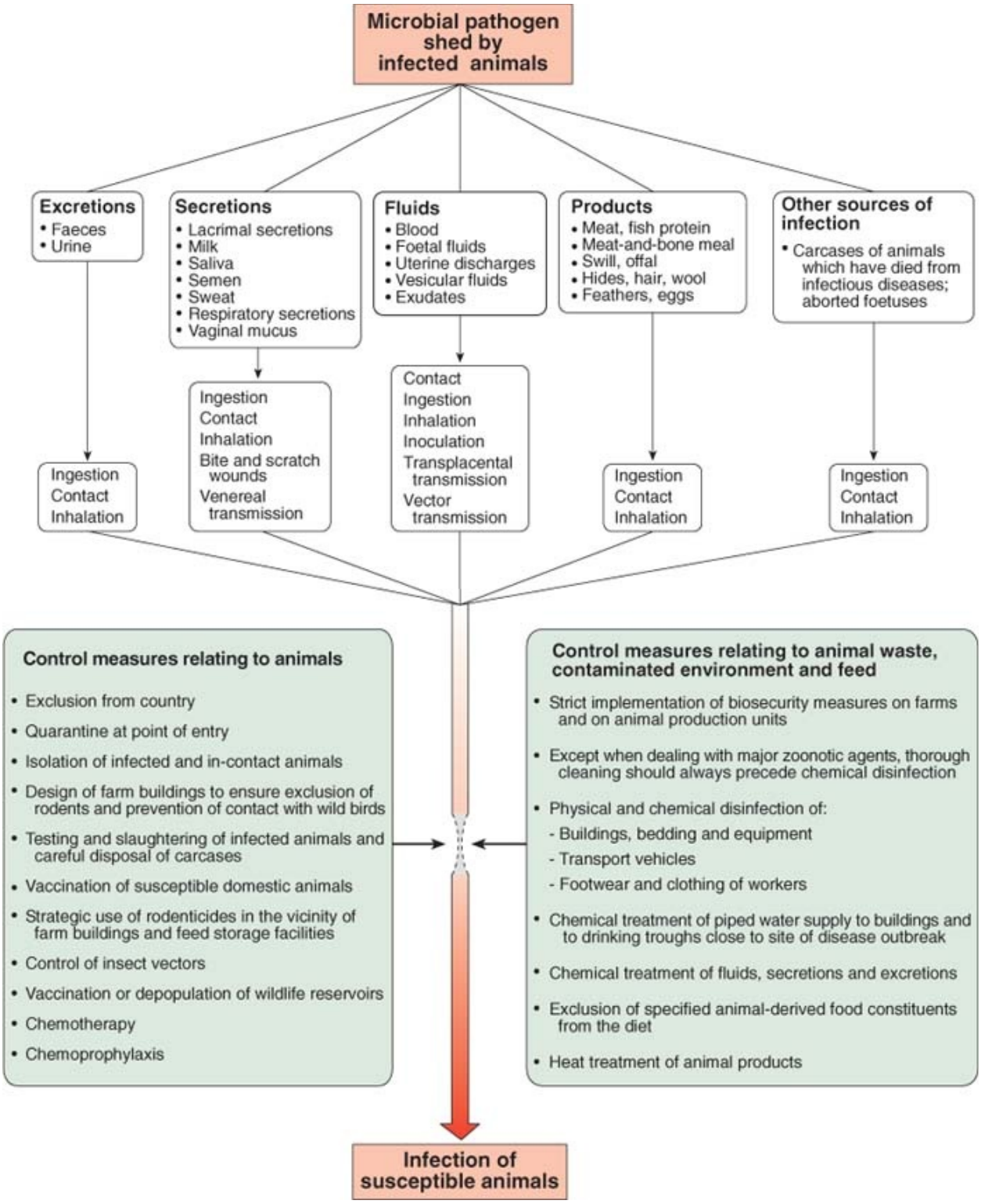
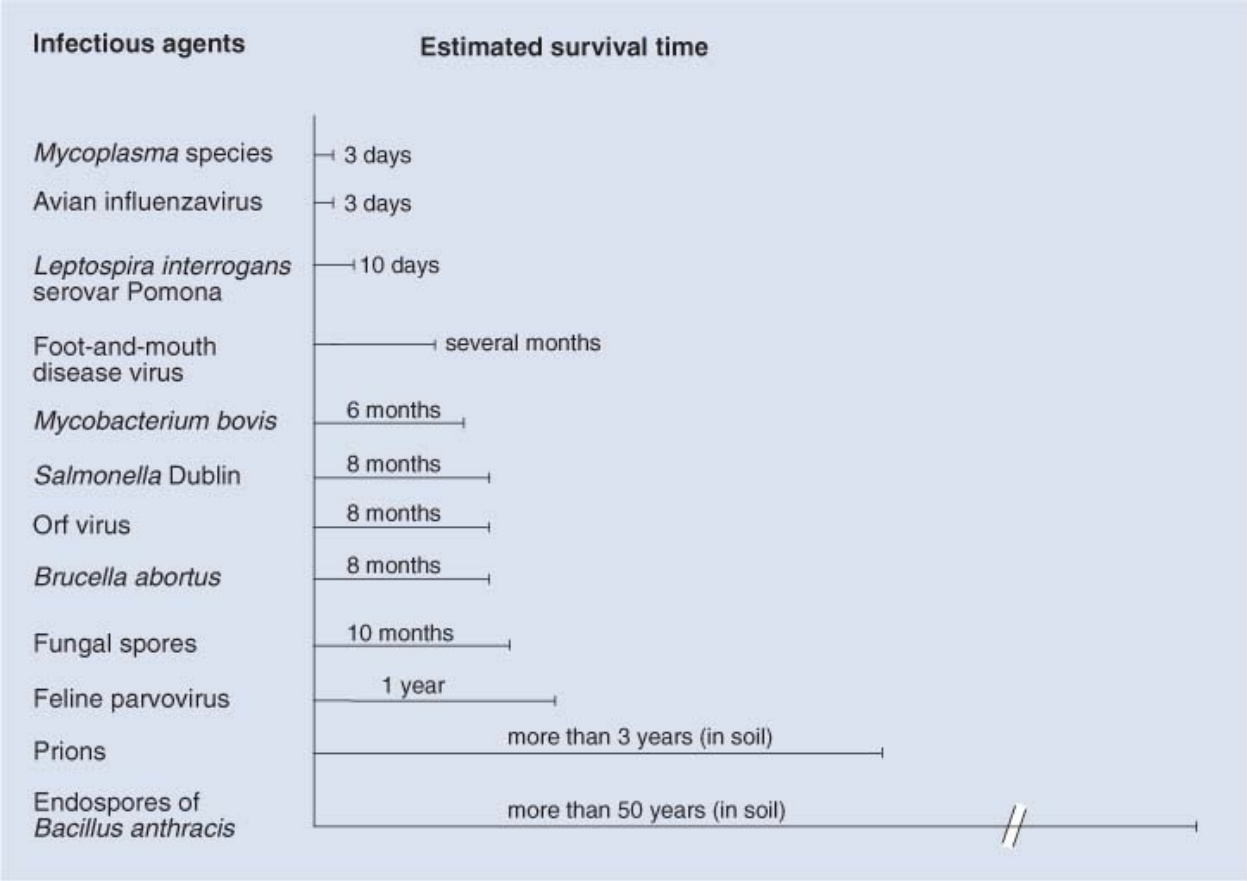


Figure 94.2 Estimated survival times of microbial pathogens in the environment under suitable conditions.



Lability in the environment is a feature of mycoplasmas, many enveloped viruses and spirochaetes. Because of their stability in the environment, pathogenic mycobacteria, salmonellae, fungal spores and parvoviruses remain viable in faeces, soil or contaminated buildings for many months, and in conditions which suit the pathogen's requirements they may survive for more than one year (Quinn and Markey, 2001). Prions and bacterial endospores exhibit exceptional resistance to environmental factors. Scrapie-infected hamster brain homogenates mixed with soil and packed in perforated Petri dishes retained infectivity for more than 3 years when buried in soil (Brown and Gajdusek, 1991). The endospores of *Bacillus anthracis* are considered to be among the most resistant microbial forms encountered in soil. Annual soil sampling on an island off the coast of Scotland, where endospores of *B. anthracis* were released in 1942 during biological weapons trials in World War II, showed that endospore numbers were declining slowly (Manchee *et al*., 1994). More than 40 years after their release, viable endospores were demonstrable in the top layer of soil. A solution of formaldehyde in sea water was used to decontaminate the island.

Transmission of infectious agents

Infectious agents can be transmitted from an infected animal to a susceptible animal by contact, ingestion, inhalation, through bite wounds and sometimes by other routes (Fig. 94.1). The modes of spread, the number of infectious agents shed by an infected animal and the stability of these agents in the environment can influence the rate at which the infectious disease spreads and the efficiency of control measures implemented by the owner of a farm or production unit, or by veterinary intervention at local, national or international level. Insect vectors, which contribute to the dissemination of infectious agents, together with wildlife reservoirs of infectious agents (either mammals or birds), often limit the efficacy of disease control measures and, in some instances, render them ineffective.

The impact of infectious diseases on intensively-reared animals such as pigs and poultry is recognized as one of the major limiting factors relating to the economic production of these species.The ease with which infectious agents can spread between farms may be reduced and in some instances eliminated by the implementation of effective biosecurity measures. These measures are designed to carefully regulate movement of livestock, animal feed, staff and visitors between farms, thereby limiting the risks of disease transmission. Within farms, the spread of endemic disease can be minimized by isolation of sick animals, chemotherapy, chemoprophylaxis, vaccination and practical measures such as cleaning and disinfection of contaminated buildings, equipment and transport vehicles. Where feasible, appropriate measures for the control of wild birds, rodents, insects and companion animals should be implemented (Fig. 94.1).

Biosecurity

The term ‘biosecurity’ includes a wide range of measures aimed at preventing or limiting exposure of domesticated animals on a farm or in a production unit to microbial pathogens from outside sources. It also includes procedures for preventing or limiting the spread of infectious diseases among animals on a farm, in kennels, in poultry production units or wherever animals are housed or reared in close contact with each other. Infectious agents can be transmitted to healthy animals by purchase of infected animals, through contaminated feed, by vectors and from environmental sources. An effective biosecurity programme has many components, all aimed at ensuring that the risks of healthy animals acquiring infection are minimized.

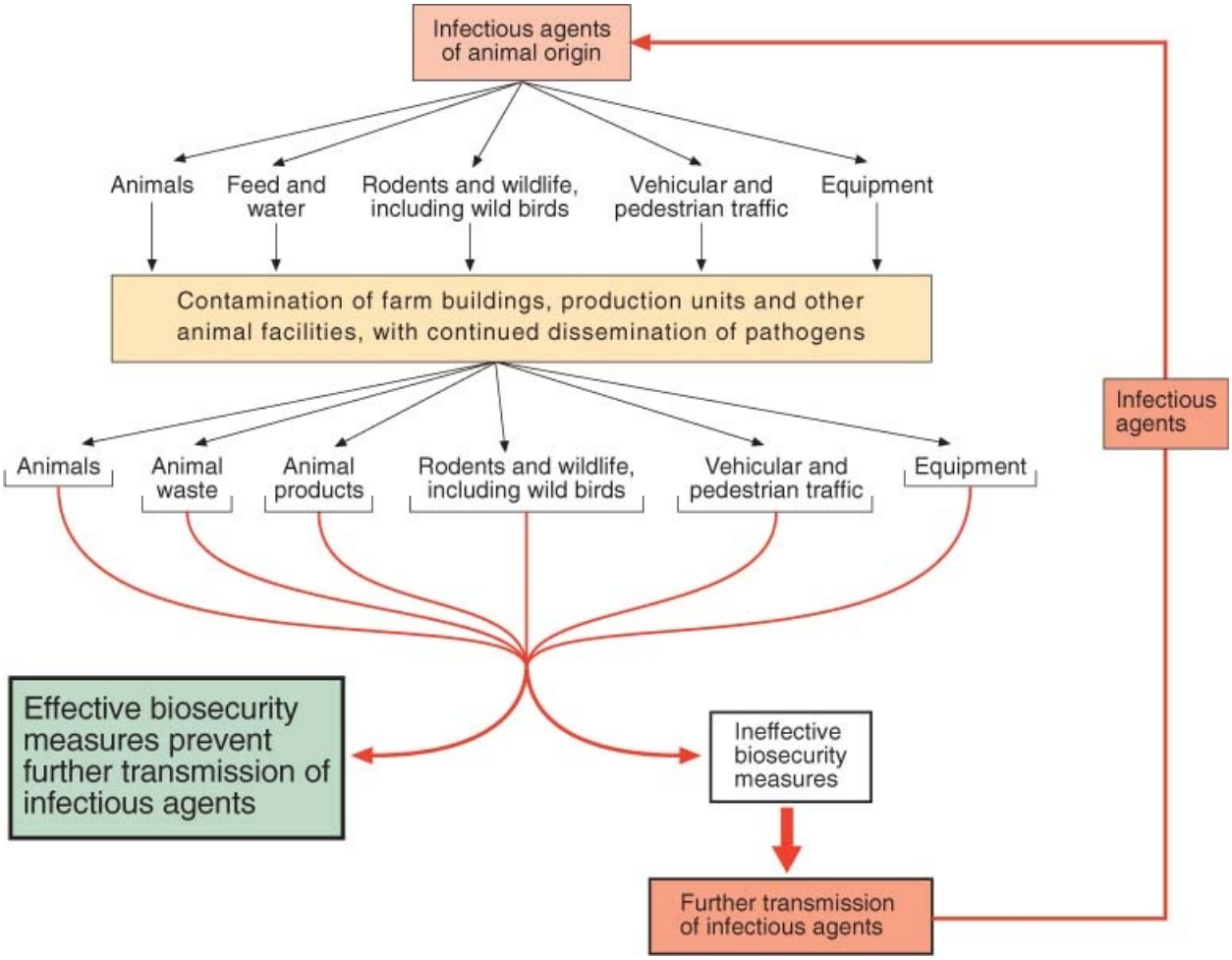
Components of a biosecurity programme include diverse issues ranging from design, location and environment of farm buildings to the purchase of animals, feed supplies and regulation of employees, transport vehicles, service personnel and people visiting the farm either casually or with prior appointments (Table 94.2). The larger the operation, the greater the need for effective biosecurity measures. Joint purchasing of expensive items of farm equipment may be desirable economically but raises many problems when such equipment is moved from farms with questionable standards of animal production to farms with high management standards. The well known adage ‘A chain is only as strong as its weakest link’ applies to the design and implementation of biosecurity procedures. Any lapse in the implementation of an effective biosecurity programme can have serious consequences for the health of the animals involved. A properly formulated and implemented biosecurity programme incorporates those aspects of disease control measures designed to limit the transmission of infectious agents (Fig. 94.3).

Table 94.2 Components of a biosecurity programme for farm animals.

Component	Considerations	Comments
Animals	Replacement animals should be purchased from reputable sources	Newly purchased animals should be isolated for at least 2 weeks and closely monitored
Feed	Source and quality of feed requires close attention	Feed can become contaminated by wild birds and rodents during storage
Water supply	Water quality is influenced by source, climatic factors and local environmental influences	Drinkers within buildings or water troughs for grazing animals can become contaminated with faeces or urine containing microbial pathogens
Environment of animals	Building design should incorporate features which promote animal health and facilitate cleaning and disinfection	Improper building design, inadequate ventilation and insufficient floor space can predispose to stressful conditions
Vehicular and pedestrian traffic	Delivery vehicles should be visibly clean and drivers should be advised at point of entry on the control measures which apply. Staff, service personnel, and others visiting the farm should use protective clothing and use footbaths provided	Particular care is required with vehicles used for transportation of animals, slurry tankers and vehicles used for disposal of used bedding or poultry litter
Equipment used on farms	Sharing of farm equipment such as trailers used for transportation of animals should be avoided	Any equipment used for cleaning farm buildings or spreading animal waste should not be borrowed or loaned
Animal waste	Liquid animal waste is usually stored in slurry tanks; solid waste may be composted on the farm or removed at frequent intervals for dispersal on arable land	An interval of up to 2 months should elapse between the application of slurry to pasture and commencement of grazing
Rodents, wild birds, wildlife	Rodents can act as reservoirs of a number of microbial pathogens; wild birds can transmit avian influenza and other pathogens to commercial poultry flocks; a number of wildlife species can transmit infectious agents to grazing animals	Where feasible, buildings should be rodent proof; wild birds should not have access to poultry houses or feed mills
Cleaning and disinfection	Effective cleaning followed by thorough disinfection is essential for the elimination of microbial pathogens from farm buildings	Cleaning can reduce the number of microbial pathogens in a building but chemical disinfection is required to inactivate residual microbial pathogens

Figure 94.3 Transmission routes of infectious agents from infected to

susceptible animals and the consequences of effective and ineffective biosecurity measures.



Animals

Ensuring that the domestic animal population within a country remains free of major infections is a constant challenge for national and local veterinary personnel and staff in diagnostic laboratories, including support staff. Exclusion of suspect animals from a country, quarantine at point of entry, isolation of infected and in-contact animals on the farm, followed by diagnostic testing and, if necessary, slaughter are widely applied measures for the control of exotic infectious diseases in animal populations. Free movement of animals, including companion animals, between countries or within a country invariably leads to free movement of microbial pathogens also.

Important endemic infectious diseases are dealt with by national governments and by individual animal owners. Schemes for the diagnosis, control and

prevention of important endemic diseases of animals are formulated by national governments and implemented by veterinary personnel at the district level and at farm level. In the event of an outbreak of infectious disease subject to government control, a rigorous testing policy, followed by slaughter of infected animals, segregation, monitoring and retesting of in-contact animals usually applies. Keeping animals free of major infectious diseases requires vigilance on the part of the owner. A closed herd or a closed flock is the most effective method for excluding infectious agents from an animal population.

Replacement animals should be purchased from reputable sources where the history of the herd or flock from which they are derived is known. In addition to the risks associated with replacement animals, there are inherent risks linked to animals returning to their farm of origin following competitions, sales, hospitalization or similar events, where close contact with other animals is unavoidable.

Imported animals should be quarantined at point of arrival in a country and subjected to thorough clinical examination combined with appropriate laboratory test procedures. Even if assurance as to their health status is provided at time of purchase in a market, at sales or when purchased privately, it may be advisable to isolate newly purchased animals after arrival on the farm for at least 2 weeks to monitor their health status. A limitation of both quarantine and isolation of animals after purchase relates to diseases with long incubation periods, such as paratuberculosis in cattle, scrapie in sheep and rabies in dogs and cats. Latent viral infections or latent bacterial infections may not be detectable by clinical examination of animals following a period of isolation on a farm. Serological tests or other diagnostic procedures may be required to detect a carrier state in an apparently healthy animal or to identify an animal with a latent viral infection.

Stud farms have a particular need to ascertain the health status of all mares and stallions used for breeding purposes. There are inherent risks associated with the movement of breeding mares on to a farm. Such animals should have their vaccination status confirmed in advance of acceptance by the stud farm and be certified free of infection with *Taylorella equigeni-talis. Streptococcus equi* is a constant source of concern when horses are brought together for breeding purposes, for competitive events or for sales. A thorough clinical examination together with a detailed clinical history of each animal may aid in the detection and exclusion of suspect horses at point of arrival.

Feed

The source and quality of feed for farm animals requires careful consideration to ensure freedom from microbial pathogens or toxic factors. Feed such as grain can become contaminated during storage with viral or bacterial pathogens before it reaches feed mills. Wild birds and rodents have been implicated in feed contamination, and cats shedding oocysts of *Toxoplasma gondii* in their faeces can contaminate grain in feed mills or on farms. When fed to cattle, silage, hay and harvested crops contaminated with canine faeces containing oocysts of *Neospora caninum* can induce multiple abortions in a herd. Grain and nut crops, harvested during wet seasons, followed by inappropriate storage conditions, can lead to fungal proliferation and mycotoxin production in stored feed. Animal-derived protein should be excluded from the diets of ruminants due to the association of BSE in cattle with the feeding of meat-and-bone meal. Because of the risks associated with swill feeding, this cheap source of food for pigs should be avoided.

Crops grown by the owner on the farm are sometimes a source of infectious agents or biological toxins. *Listeria monocytogenes* can replicate in the surface layers of poor quality silage and produce listeriosis in ruminants. Botulism in farm animals has been associated with feeding baled silage. Ergopeptide alkaloids, produced by *Claviceps purpurea* growing on standing ryegrasses, rye and other cereal crops before harvesting, may be present in silage made from such crops.

Water

The source and quality of water for farm animals may be influenced by farm location, climatic factors and other environmental influences. As water supplies for intensively-reared animals such as pigs and poultry are usually of mains origin, health problems from such a source are usually of little concern. Contamination of a clean water supply on a farm can occur due to pollution of drinkers in buildings or outdoor water troughs by faeces or urine. Drinkers in grazing areas positioned at low levels are more likely to become contaminated than those at higher levels. Header tanks without covers positioned close to roof openings or on platforms may be contaminated by wild birds.

For grazing animals, ponds and larger bodies of water may become contaminated by run-off from slurry spreading or overflowing slurry tanks.

Wildlife, either resident or migratory, can contaminate ponds with either enteric pathogens or leptospires. If poultry houses are located close to lakes or large ponds, migratory water fowl or seabirds can transmit avian influenza or Newcastle disease to domestic poultry (Lister, 2008).

Water troughs for grazing animals may attract wildlife, such as badgers, shedding *Mycobacterium bovis* in their urine. Rats shedding leptospires can contaminate water troughs, and other wildlife reservoirs can transmit a range of waterborne microbial pathogens to domestic animals. Raising water troughs for grazing animals to a level not accessible to wild mammals may reduce the risk of contamination. Frequent cleaning of drinkers for intensively-reared animals should form part of the management system.

Environment of domestic animals

The immediate environment of farm animals can have a positive or negative influence on their state of health. Buildings, farm yards, paddocks and other grazing areas can be planned so as to promote animal health. Conversely, improper building design, inadequate ventilation and insufficient floor space for the animal population can predispose to stressful environmental conditions for intensively-reared animals. Temperature control is a requirement for neonatal pigs and newly-hatched chicks. Poultry and pig units should operate close to their optimal temperatures. Building design should incorporate features which facilitate cleaning and disinfection at the end of a production cycle or following an outbreak of disease. Space for isolation facilities should be an integral part of buildings designed for domestic mammals. Floors and walls with a moderately smooth finish facilitate cleaning and minimize trauma to animals without predisposing to slipping. Food storage areas designed to exclude rodents and wild birds not only decrease the risk of food contamination but also limit waste. Windows, doors and ventilation systems should be rodent-proof. The installation of waterproof power points in buildings reduces the risk of electric shocks for employees. On large farms, consideration should be given to the inclusion of facilities for washing and disinfecting vehicles for transportation of animals.

Dusty paddocks and heavily grazed pastures can lead to a build-up of *Rhodococcus equi* which can result in suppurative bronchopneumonia in foals up to 4 months of age. Rough pastures offer cover for many tick species such as *Ixodes ricinus*. The acquisition of tick-borne diseases such as louping ill and tick-borne fever is usually associated with animals grazing rough pasture.

A secure perimeter fence is an essential component of any biosecurity system. For pig and poultry units, the fence should incorporate features suitable for the control of vehicular and pedestrian traffic. Perimeter fences around farms should ensure that grazing animals are contained within a defined area and not allowed access to adjacent properties. Cost is a major limiting factor in the erection and maintenance of effective boundary fences, especially on large farms.

Vehicular and pedestrian traffic

The control of vehicles calling at farms, especially pig and poultry enterprises, is a fundamental requirement of effective farm management. Delivery vehicles transporting feed, bedding or other supplies should be visibly clean and drivers should be advised on the biosecurity systems operating on the farm. Drivers of milk tankers usually adhere to a defined routine when calling at dairy farms. Particular attention should be paid to drivers of vehicles used for collection of animals, slurry tankers and vehicles used for disposal of animal waste. These drivers should be given clear instructions on procedures to be followed and, if necessary, their work should be supervised. Wheel baths at farm entrances are sometimes used as part of a biosecurity strategy. Unless they are built to defined specifications and maintained on a regular basis, their value as part of a disease control programme is questionable.

Staff working on the farm, service personnel, veterinarians and all others on official business should adhere strictly to the wearing of protective clothing and footwear at all times. In some instances, waterproof footwear may have to be provided for those visiting the farm. Footbaths, strategically positioned for all pedestrians, should be used by persons entering the farm, irrespective of their reason for being present. If farm buildings are arranged as discrete units, the entrance to each building should have a footbath at the point of entry. On farms with high levels of biosecurity, visitors are required to indicate their names and addresses and sign a register before entering the premises.

Equipment

The sharing of farm equipment between groups of farmers with different management standards and different disease problems in their livestock is an arrangement which should be avoided. Equipment used for cleaning buildings,

slurry spreading and the transportation of animals are among the items which should be excluded from joint purchasing agreements.

Animal waste

The form and amount of animal waste generated on a farm are determined by the number and species of animals present, building design and the type of material used as bedding for large animals or as poultry litter. In buildings with slatted floors, animal waste is stored in slurry tanks. These tanks should be constructed to high specifications and have ample capacity to ensure that overflowing of contents does not occur and they should be emptied at regular intervals. Slurry spreading is usually restricted to defined times of the year when ground conditions are suitable for slurry tankers and when the risk of run-off is low. In many European countries, slurry spreading is restricted by government regulations to specified times of the year. An interval of up to 2 months should elapse between the application of slurry to pasture and the commencement of grazing. A longer interval between the application of slurry and the commencement of grazing may be required if enteric pathogens such as *Salmonella* species or pathogenic acid-fast bacteria are likely to be present in the slurry.

Straw used for bedding animals and litter from poultry houses should be stored at a site remote from farm buildings and composted for up to 2 months before spreading takes place on land used for tillage. Composting should take place on a site where run-off is minimized by location and by the design of the holding facility. Alternatively, the material can be removed at regular intervals and dispersed on arable land. If an infectious disease has occurred on the farm from which the bedding material derived, composting should be carried out for at least 2 months before dispersal of the material on arable land.

Wild mammals and birds

Rodents

Rats and mice are often attracted to farm buildings because they provide shelter in cold weather and because of the abundance of food available in such buildings. Rodents act as reservoirs of *Salmonella* species, and mice often

harbour *Salmonella* Typhimurium which they excrete in their faeces. Rats sometimes shed leptospires in their urine and can transmit these virulent pathogens to domestic animals and also to humans.

Farm buildings, especially feed storage facilities, should be designed to exclude rodents. Feed bins should be rodent-proof and feed spillages should be cleared up promptly to lessen the attraction of rodents and wild birds to farm buildings. Strategic use of rodenticides in the vicinity of farm buildings is an effective method for controlling rodent populations.

Wild birds

The availability of grain and shelter often attracts wild birds to farm yards and farm buildings. Migratory waterfowl can transmit avian influenza and Newcastle disease to commercial poultry flocks. Native species of wild birds can transmit microbial pathogens such as *Salmonella* species, *Yersinia* species and *Mycoplasma* species to poultry flocks. Poultry houses should have all openings, including ventilation shafts, covered with wire mesh to exclude wild birds.

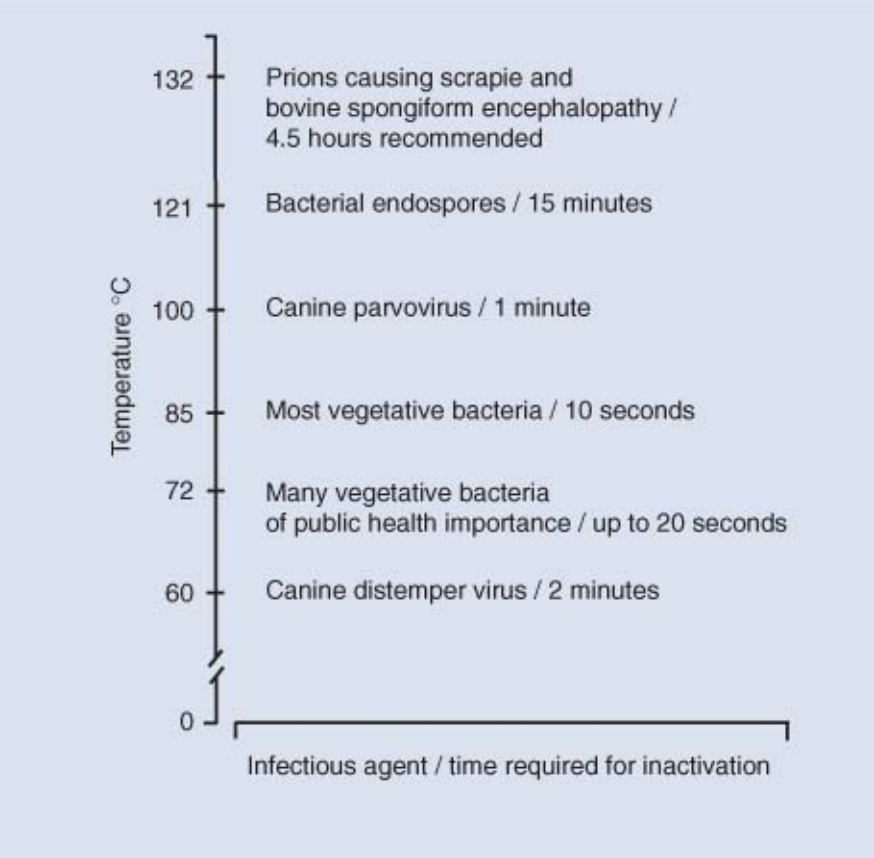
Wildlife

The close interaction between grazing animals and wildlife can result in the transfer of infectious agents between wild animals and domestic animals. Farms bordering game reserves, national parks or other land designated for the preservation of wildlife species provide an opportunity for common grazing by domestic and wild ruminants. Transmission of the foot-and-mouth disease virus from persistentlyinfected African Cape buffalo to cattle has been documented. Badgers and possums, which live either on farms or in close proximity to grazing land, may share the same general environment as grazing cattle and also share common diseases such as tuberculosis caused by *Mycobacterium bovis*. Transfer of infection to cattle from badgers or possums terminally ill with tuberculosis can occur through licking, sniffing or biting moribund animals on pasture. Nocturnal observations of badger behaviour on cattle farms have demonstrated that badgers were attracted to feed sheds, barns, haystacks and cattle troughs in their search for food (Garnett *et al*., 2002). It was also noted that contamination of cattle feed with badger faeces occurred and that badgers came into close contact with cattle.

Cleaning and disinfection of farm buildings

Effective cleaning and disinfection at the end of a production cycle or following an outbreak of disease is an essential component of a biosecurity programme. If carried out in a competent manner, cleaning alone can substantially reduce the number of pathogens in a building, thereby decreasing the risk of a heavy challenge to the animals in that environment. One of the principal reasons for the failure of a disinfection procedure is the presence of residual organic matter due to inadequate cleaning. Accordingly, cleaning is a prerequisite for the implementation of a successful disinfection programme. Building design and the materials used for construction influence cleaning methods. Fittings such as food receptacles and drinkers require special attention. If an important infectious disease has occurred on the premises, the system supplying water should be emptied, cleaned and disinfected.

Figure 94.4 Thermal inactivation of infectious agents by moist heat. The number of infectious agents initially present influences the time required for inactivation. The system used to determine survival or inactivation may alter the reliability of the results. Requirements for the thermal inactivation of prions are not yet conclusively demonstrated.



Selection of an effective and economical disinfectant for the terminal disinfection of a farm building requires consideration of the infectious agents likely to be present, the amount of organic matter remaining on surfaces, the antimicrobial spectrum of the compound selected, the volume of disinfectant required, the method of application, safety aspects of the procedure for workers and the cost involved.

Thermal inactivation of microbial pathogens

Infectious agents vary widely in their susceptibility to thermal inactivation (Fig. 94.4). Although both moist and dry heat can be used for inactivating microorganisms, moist heat is more effective and requires less time to achieve inactivation than dry heat. Many vegetative bacteria are killed in less than 20 seconds by heating at 72°C. At temperatures above 80°C, most vegetative bacteria are killed within seconds. Bacterial endospores are remarkably thermostable, and moist heat at 121°C for at least 15 minutes is required for their destruction. Many viruses are labile at temperatures close to 70°C. Canine parvovirus is a notable exception: a temperature of 100°C for 1 minute is

required to inactivate this resistant virus. Foot-and-mouth disease virus in milk can survive pasteurization at 72°C for 15 seconds and further heating at 72°C for 5 minutes (Blackwell and Hyde, 1976). The virus also survives heating at 93°C for 15 seconds in cream. At temperatures close to 100°C, more than 20 minutes may be required to inactivate this resistant virus in milk (Walker *et al*., 1984). Heat treatment of milk at 148°or 3 seconds reliably inactivates the virus.

The prions that cause transmissible spongiform encephalopathies are extremely resistant to thermal inactivation. Dry heat at 160°ot inactivate these agents but autoclaving at 132°5 hours may be effective.

Disinfection, antisepsis and sterilization

There are a number of well defined measures that can be applied for the prevention and control of infectious diseases within a country or in regions of a country. These include exclusion of suspect animals, quarantine at point of entry, and isolation and slaughter of infected animals if exotic disease is confirmed by clinical or laboratory tests. When infectious diseases are endemic in a country, control measures include vaccination, chemotherapy and chemoprophylaxis (Fig. 94.1, Box 94.1). During the implementation of disease eradication programmes, vaccination may in some circumstances be permitted alongside a slaughter policy. Effective control measures relating to the environment, animal waste and animal products are central to the success of disease eradication programmes (Fig. 94.1, Box 94.2). Chemical decontamination can be used for buildings, equipment, transport vehicles, footwear and clothing. Heat treatment of milk, milk products and waste food of animal origin such as swill, and chemical treatment of fluids, secretions and excretions, are also essential for effective disease control.

Box 94.1 trategies for the prevention, treatment or control of infectious diseases in animal populations.

- Exclusion of animals from a country or continent
- Quarantine of imported animals at point of entry
- Accurate identification of farm animals, especially ruminants, using ear tags or microchip implantation; colour markings can be used for identification of horses while dogs and cats may require detailed written descriptions with accompanying

photographs

- Isolation of infected or in-contact animals on the farm of origin or on the premises being inspected
- Exclusion of animal-derived food components from the diet of ruminants
- Clinical or laboratory confirmation of exotic infectious disease followed by slaughter and careful disposal of infected carcasses
- Vaccination of susceptible domestic animals before exposure to possible sources of endemic or exotic diseases
- Either vaccination or depopulation of wildlife reservoirs depending on the importance of the disease and the feasibility of implementing control measures
- Chemotherapy for animals with endemic disease
- Chemoprophylaxis for prevention of predictable infectious disease in animal populations when vaccination is either impractical or ineffective

Box 94.2 Control measures relating to the environment, animal waste and animal products.

- Chemical disinfection of:
  –buildings, bedding and equipment
  –transport vehicles
  –footwear and clothing of workers
- Chemical treatment of water supply following disinfection of building
- Chemical treatment of fluids, excretions, secretions
- Heat treatment of milk and milk products; mandatory boiling of waste food if swill feeding to pigs is permitted

Disinfection implies the use of physical or chemical methods for the destruction of microorganisms, especially potential pathogens, on the surfaces of inanimate objects or in the environment. Antisepsis can be defined as the destruction or inhibition of microorganisms on living tissues by chemicals which are non-toxic and non-irritating for the tissues. Disinfectants and antiseptics differ fundamentally from systemically active chemotherapeutic agents in that they do not exhibit selective toxicity. Most chemicals used as disinfectants are toxic not only for microbial pathogens but also for host cells. Disinfectants are therefore used only to reduce the microbial population on inanimate surfaces or in organic materials, whereas antiseptics can be applied topically to living tissues.

Because of the great diversity of microbial pathogens, complete destruction of

bacteria, fungi and viruses by sterilization requires carefully controlled conditions. Steam under pressure generating a temperature of 121°C for 20 minutes, dry heat at 160°C for 2 hours or ionizing radiation (gamma radiation) effectively inactivate conventional microbial pathogens. Glutaraldehyde and peracetic acid at specified concentrations are used as sterilizing agents. Sterilization methods, which require strict adherence to well-defined procedures, are used for surgical instruments, fluids for systemic administration, media for culture of microorganisms, and inactivation of microbial pathogens in specimens submitted for laboratory identification. In contrast, disinfection methods used for decontaminating buildings, equipment, transport vehicles, footwear and clothing are not implemented to the same strict guidelines.

Characteristics, modes of action and selection of chemical disinfectants

Although a number of potentially useful physical methods including dry and moist heat, ionizing radiation and mechanical methods may be used for disinfection in the laboratory, at farm level, in clinical facilities and in locations where animals are assembled for sporting events or for sale, chemical disinfection procedures find wider application than physical methods.

Many chemicals with antimicrobial activity can be used for the inactivation of microbial pathogens in buildings, stockyards and transport vehicles and on equipment. Chemicals used include acids, alkalis, alcohols, aldehydes, halogens, phenols and quaternary ammonium compounds. Characteristics of an ideal disinfectant are presented in Box 94.3. None of the currently available compounds possesses all of these characteristics. Selection of a disinfectant agent should be based on its spectrum of activity, its efficacy and its susceptibility to inactivation by organic matter. Additional considerations include compatibility with soaps and detergents, toxicity for personnel and animals, contact time required, optimal temperature, residual activity, corrosiveness, effects on the environment and cost.

Box 94.3 Characteristics of an ideal chemical disinfectant.

- Broad antimicrobial spectrum with activity at low concentrations against vegetative

bacteria (including mycobacteria), bacterial endospores, fungal spores, enveloped and non-enveloped viruses and prions
- Effective against bacteria in biofilms or dried on surfaces
- Absence of irritancy, toxicity, teratogenicity, mutagenicity and carcinogenicity
- Stability, with a long shelf life at ambient temperatures
- Solubility in water to the concentration required for effective antimicrobial activity
- Compatibility with a wide range of chemicals including acids, alkalis, anionic and cationic compounds
- Retention of activity in the presence of organic matter
- Absence of corrosiveness or chemical interactions with metals or other structural materials
- Retention of antimicrobial activity over a wide range of temperatures
- Absence of tainting or toxicity following application to surfaces or equipment in dairies, meat plants or food preparation areas
- Non-polluting for ground water and biodegradable
- Moderately priced and readily available

Selection and use of a disinfectant requires consideration of the infectious agents likely to be present and the conditions prevailing in the location where microbial contamination has occurred. If the pathogen that caused the disease outbreak has been identified, a disinfectant with known activity against that agent should be selected (Table 94.3). The activity of complex disinfectants may vary in accordance with their formulation, and the efficacy of individual compounds listed in Table 94.3 relates to their use under ideal conditions. Before the application of a disinfectant, surfaces should be thoroughly cleaned. This physical procedure, if properly carried out, removes a high percentage of accessible infectious agents. Staff training and proper supervision are essential for the successful implementation of a cleaning and disinfection programme. Effective cleaning should always precede disinfection of buildings, with the exception of those that have housed animals with major zoonotic diseases such as anthrax.

Infectious agents vary in their susceptibility to the chemical disinfectants (Fig. 94.5). Most vegetative bacteria and enveloped viruses are readily inactivated by disinfectants; fungal spores and non-enveloped viruses are less susceptible. Mycobacteria and bacterial endospores are resistant to many commonly used disinfectants. Prions are extremely resistant to chemical inactivation. High concentrations of sodium hypochlorite or heated strong solutions of sodium hydroxide are reported to inactivate these unconventional infectious agents.

Chemical inactivation of microbial pathogens

Among microbial pathogens, there is considerable diversity not only in their morphological appearances but also in their nutritional requirements and metabolic activities. It is not surprising, therefore, that these diverse microorganisms also exhibit wide variation in their resistance to chemical disinfectants. Among vegetative bacteria, fungi and viruses there are marked differences in susceptibility to many chemical compounds. Some Gram-negative bacteria are resistant to a number of commonly used disinfectants such as quaternary ammonium compounds and biguanides. *Pseudomonas aeruginosa* and *Burkholderia cepacia* can withstand treatment with povidone – iodine solutions. Of greater concern, however, is the increasing number of Gram-negative bacteria which exhibit acquired resistance to chemical disinfectants. The ability of some strains of *E. coli* to inactivate formaldehyde has been reported. Acquired resistance to biguanides, phenolic compounds and quaternary ammonium compounds has been observed in both Gram-positive and Gram-negative bacteria.

Because of their complex layered structure and their unique chemical components such as dipicolinic acid, bacterial endospores are inherently resistant to a wide range of chemical compounds. They are also capable of surviving in the environment for prolonged periods and they are resistant to high temperatures which readily destroy vegetative bacteria.

Enveloped viruses are moderately susceptible to many commonly used chemical disinfectants. A number of non-enveloped viruses exhibit marked resistance to a range of chemical compounds. Parvoviruses are exceptionally resistant to chemical inactivation and a limited number of disinfectants are effective against these non-enveloped viruses. These viruses are also stable in the environment and can withstand temperatures up to 100°C for short periods.

Table 94.3 The antimicrobial spectrum of chemical disinfectants[a]

Disinfectant	Microbial pathogens							
	Bacteria				Fungal spores	Viruses		Prions
	Gram-positive	Gram-negative	Myco-bacteria	Endo-spores		Enveloped	Non-enveloped	
Acids (mineral)	++	+	–	±	+	+	±[b]	–
Alcohols	++	++	++	–	+	+	–	–
Aldehydes	++	++	+	++	++	++	++	–
Alkalis	++	++	+	+	+	+	±[b]	±[c]
Biguanides	++	+	–	–	+	+	–	–
Halogens								
Chlorine compounds	++	++	+	+	+	++	++	±[d]
Iodine compounds	++	++	+	+	+	++	+	–
Peroxygen compounds								
Hydrogen peroxide	++	++	±	++	+	++	±	–
Peracetic acid	++	+ +	+	++	++	++	+	–
Phenolic compounds	++	++	+	–	+	+	–	–
Quaternary ammonium compounds	++	+	–	–	+	+	–	–

a, the antimicrobial activity of complex disinfectants may vary in accordance with their formulation. The data presented relate to the use of chemical compounds at appropriate concentrations under ideal conditions

b, acids and alkalis inactivate the virus of foot-and-mouth disease

c, hot 2 mol/litre NaOH is reported to be effective

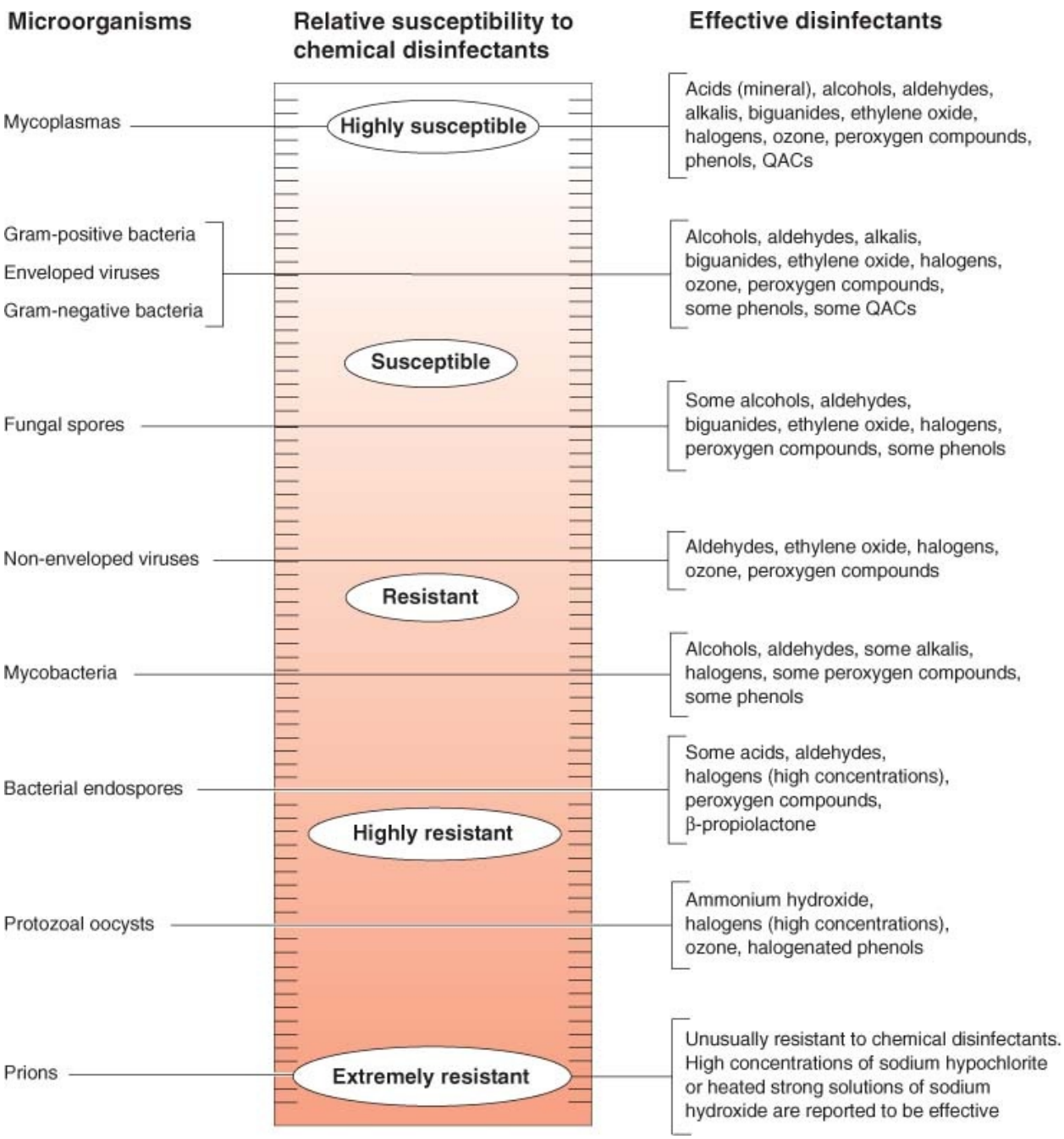
d, high chlorine concentrations are required for inactivation

++, highly effective; +, effective; ±, limited activity;–,no activity

Fungal pathogens are generally less susceptible to chemical disinfectants than vegetative bacteria. Intrinsic resistance of fungal cells to chemical compounds may relate to the composition of fungal cell walls which renders them less permeable than bacterial cells to chemicals such as cationic compounds and alcohols. Wall thickness and the presence of glucan and mannoproteins are considered to be factors in the impermeability of fungal cell walls to some chemical disinfectants. Conventional infectious agents such as bacteria, fungi and viruses share some common characteristics including their susceptibility to inactivation by chemical disinfectants. A group of infectious agents, termed unconventional infectious agents, have characteristics which distinguish them from typical microbial pathogens. These unconventional infectious agents, which have been assigned the name prions, appear to be devoid of nucleic acid,

can infect animals and humans without inducing a detectable immune response and exhibit resistance to physical and chemical methods of inactivation far beyond the most resistant forms of conventional infectious agents. Prions are implicated in a group of unusual neurological diseases referred to as transmissible spongiform encephalopathies. Currently, it is widely accepted that prions are composed of altered protein, and the nature of the denaturation may account in part for the exceptional resistance of these infectious agents to physical and chemical methods of inactivation. As prions have not been isolated like conventional infectious agents, it is unclear if their resistance derives in part from innate characteristics of these agents or from their close association with host tissues which confer protection against physical and chemical methods of inactivation.

Figure 94.5 Microorganisms ranked according to their relative susceptibility or resistance to chemical disinfectants. The disinfectants which are effective against particular groups of microorganisms are listed. The composition and concentration of the disinfectant, the ambient temperature and the contact time influence the effectiveness of the disinfection procedure. The presence of organic matter on the surfaces of buildings, on vehicles used for transportation of animals, on feeding utensils and equipment and on the footwear and clothing of workers interferes with the microbiocidal activity of many chemical disinfectants. QACs, quaternary ammonium compounds.



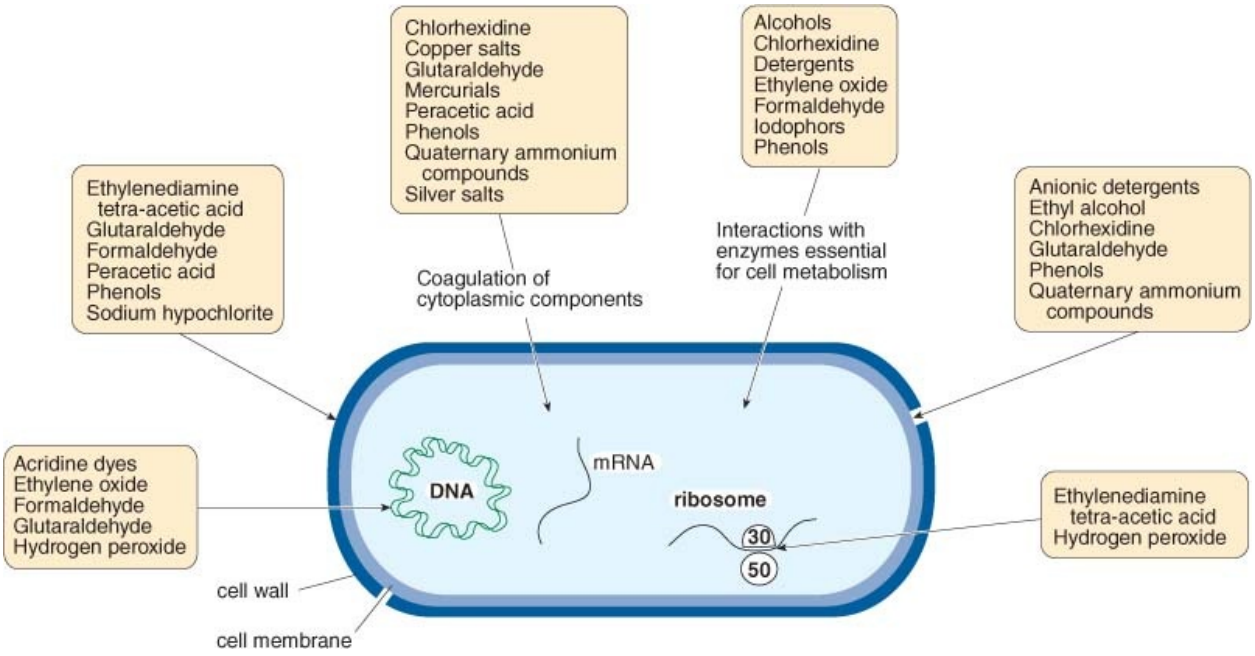
Bacteria

Unlike antibiotics or sulphonamides which usually exert their effects on specific target sites within the bacterial cell, chemical disinfectants often act at a number of sites. Chemicals with antibacterial activity may react with the cell wall, cell membrane, DNA, ribosomes or cytoplasmic components. Activity of chemical disinfectants depends on the microorganisms present, physical and chemical

properties of the disinfectant, the concentration of the compound and environmental factors. Sites of action or changes induced in cytoplasmic components of a bacterial cell by chemical disinfectants are illustrated in Fig. 94.6.

Gram-positive bacteria are usually more susceptible to the action of chemical disinfectants than Gram-negative bacteria. Structural differences in their cell walls, especially in the higher lipid content of the Gram-negative bacterial cell wall, may account for some of the differences in their susceptibility to chemical inactivation. Although staphylococcal cell walls normally have a low lipid content, an increase in their cell wall lipids leads to increased resistance to phenolic compounds and to other disinfectants. Among the Gram-negative bacteria, *Proteus* species and *Pseudomonas aeruginosa* are recognized for their resistance to quaternary ammonium compounds and also to chlorhexidine. The prolonged survival of *P. aerugi-nosa* and *Burkholderia cepacia* in povidone—iodine solution has been attributed to the presence of organic matter or biofilm formation on items immersed in the solution. Acquired bacterial resistance to disinfectants has also been observed in both Gram-positive and Gram-negative bacteria in recent years. The constant repetitive use of some chemical compounds in particular buildings, such as hospitals or clinics, may contribute to the selection of bacteria resistant to such chemicals. To minimize the risk of resistance developing in such circumstances, rotational use of different classes of disinfectants has been recommended.

Figure 94.6 Sites of interaction or changes induced in a bacterial cell by chemicals with antibacterial activity.



Mycobacteria

In terms of their relative resistance to chemical compounds, mycobacteria occupy a position between Gram-negative bacteria and bacterial endospores (Fig. 94.5). Mycobacterial resistance to disinfectants is linked to the cell wall composition of these bacteria; their high lipid content renders them hydrophobic, thereby preventing hydrophilic agents from entering the cells. Other components of mycobacterial cell walls which distinguish them from conventional bacteria include mycolic acid derivatives and mycobacterial peptidoglycans. Differences in the resistance of mycobacteria have been reported, with *Mycobacterium chelonae* resistant to treatment with glutaraldehyde which is usually mycobactericidal. The exceptional ability of *M. chelonae* to adhere to smooth surfaces may contribute to this organism' s ability to withstand treatment with glutaraldehyde.

Quaternary ammonium compounds, some dyes and biguanides inhibit *M. tuberculosis* and other mycobacterial pathogens but do not inactivate these bacteria. Although some phenolic disinfectants including bisphenols are ineffective against pathogenic acid-fast bacteria, *ortho*-phenylphenol is particularly effective against these pathogens. The chemical compounds reported to be effective against pathogenic mycobacteria include glutaraldehyde, formaldehyde, alcohols, ethylene oxide and a number of halogen disinfectants. The mycobactericidal activity of hydrogen peroxide and other peroxygen

compounds is not well established (Hawkey, 2004). Investigation of the activity of a commercial peroxygen compound indicated that it was ineffective as a mycobactericidal agent (Griffiths *et al*., 1999). Peracetic acid, however, has been reported to be effective against mycobacteria.

Table 94.4 Chemical disinfectants with sporicidal activity.

Disinfectant	Structural or functional components affected	Comments
Ethylene oxide	DNA and protein components	Alkylating agent with marked sporicidal activity
Formaldehyde	DNA and protein components	A 5% solution of formalin (formaldehyde gas dissolved in water) inactivates endospores of *B. anthracis* in soil
Glutaraldehyde	Although not clearly identified, target sites are probably nucleic acid and proteins	A 2% solution of alkaline glutaraldehyde is an effective sporicide
Hydrogen peroxide	Outer and inner spore coats, cortex and probably small acid-soluble DNA-binding proteins	Sporicidal activity of hydrogen peroxide is slow; damage to sensitive enzymes may interfere with the outgrowth required for return to a vegetative state
Iodophors	Protein components are the probable sites of iodine binding; damage to spore coats and cortex may facilitate entry to the spore core	High iodine concentrations and long contact times are required for sporicidal action
Ortho-phthalaldehyde	Enzymes associated with spore germination are probable target sites	The sporicidal activity of this cyclic dialdehyde is limited
Ozone	Reacts with amino acids, RNA and DNA; outer spore coat is the probable target site	This allotropic form of oxygen is unstable in water but production of free radicals prolongs its antimicrobial activity
Peracetic acid	Reported to react with small acid-soluble DNA-binding proteins; high concentrations cause structural damage to endospores	In some published reports, there are conflicting results on the sporicidal activity of peracetic acid; despite these uncertainties, the sporicidal activity of this strong oxidizing agent against *B. anthracis* in soil has been demonstrated
Sodium hypochlorite and other chlorine-releasing compounds	Spore coats and cortex are the initial target sites; subsequently, components of the spore core are affected	Many chlorine-releasing compounds such as chlorine dioxide are sporicidal; inactivation by organic matter is not as marked with chlorine dioxide as with sodium hypochlorite

Decontamination of farm buildings following an outbreak of *M. bovis* infection in cattle requires consideration of the building design, size, surfaces which require cleaning prior to disinfection and the most appropriate mycobactericidal chemical disinfectant for the prevailing conditions. Because of the toxic vapour liberated by formaldehyde and glutaraldehyde, neither compound is appropriate for routine farm use. Phenolic compounds containing *ortho*-phenylphenol, or other phenolic formulations referred to as clear-soluble solutions, are mycobactericidal. If surfaces are cleaned to a level at which no residual organic matter is evident, sodium hypochlorite or other suitable halogen disinfectants can be used for decontamination.

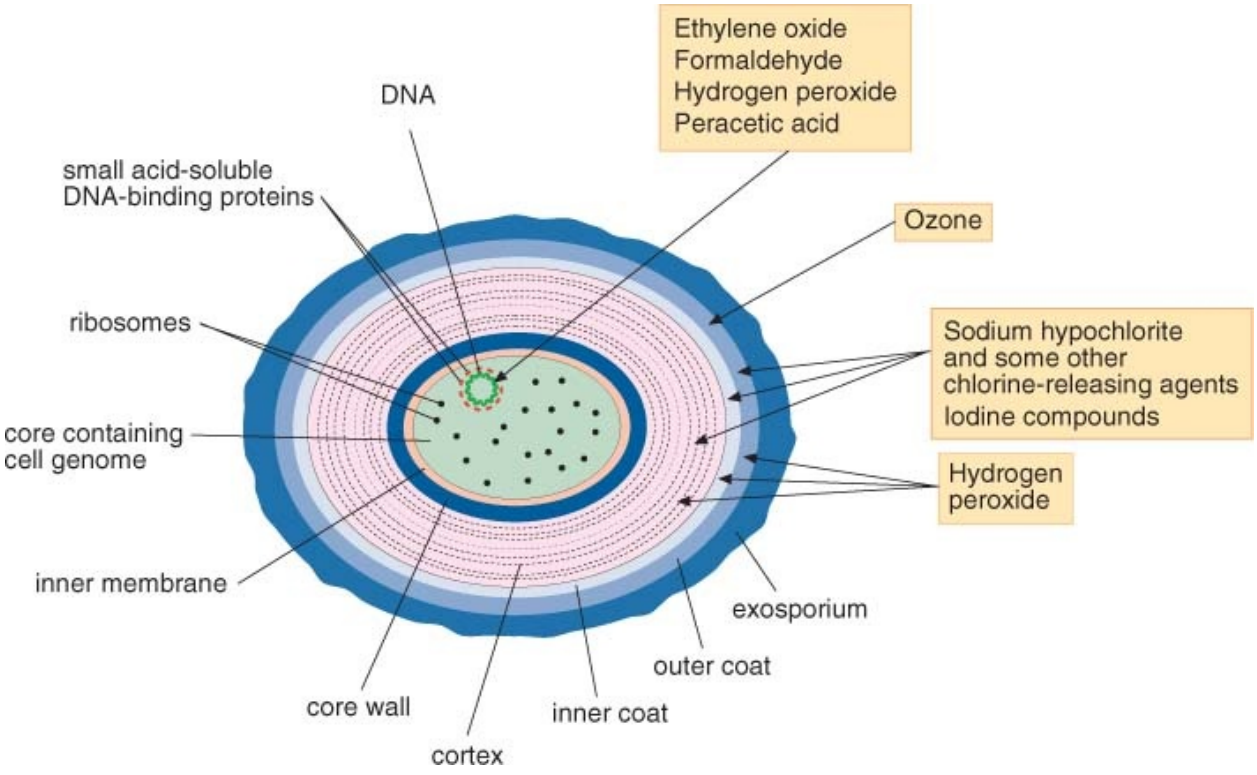
Bacterial endospores

Endospore-producing bacteria include *Clostridium* species and *Bacillus* species. Bacterial endospores are much more resistant than their corresponding vegeta-

tive cells to adverse environmental conditions, heat and chemical disinfectants. The ability of particular bacteria such as clostridia and *Bacillus anthracis* to form spores confers on them unique attributes which include prolonged survival under adverse conditions and resistance to chemical disinfectants. Although disinfectants such as alcohols, biguanides, phenolic compounds and quaternary ammonium compounds may be sporostatic, they are not sporicidal.

Chemical disinfectants with sporicidal activity include alkylating agents, oxidizing agents and halogens (Table 94.4). Although some uncertainty remains about the sporicidal activity of a limited number of these chemicals, many are potent sporicides. The ability of formaldehyde and glutaraldehyde to inactivate endospores is well-established. Likewise, sodium hypochlorite, chlorine dioxide and other chlorine-releasing compounds are effective sporicides in the presence of limited amounts of organic matter. The effectiveness of the oxidizing agents hydrogen peroxide and peracetic acid as sporicidal agents may be influenced by the concentration at which they are used and also by the presence of organic matter.

Figure 94.7 Sites or structures of a bacterial endospore which are the probable targets of chemicals with sporicidal activity. The target sites of other sporicidal chemicals such as glutaraldehyde and *ortho* phthalaldehyde are not clearly defined.



The sites or structures of a bacterial endospore which are targets of chemicals with sporicidal activity are shown in Fig. 94.7. Target sites of some chemicals which inactivate endospores are not well defined. Individual chemicals may first damage external endospore structures before entering the core where they may react with DNA or with enzymes required for outgrowth and return to the vegetative state.

Fungal pathogens

Although many chemical disinfectants have both antibacterial and antifungal activity, bacteria and fungi have many distinct structural features. Fungal cell walls contain chemical compounds such as glucan polymers, mannoproteins and chitin. The fungal cell membrane is rich in ergosterol which is the target site of many antifungal drugs. Because of the complexity of their cell walls, fungal pathogens are more resistant to inactivation by chemical disinfectants than non-sporulating bacteria.

The antifungal activity of quaternary ammonium compounds is reported to depend on the type of compound used, its concentration and the fungal cells being treated. Phenolic compounds with antifungal activity include halogenated phenols but many phenolic compounds are reported to be ineffective against pathogenic fungal spores.

The oxidizing agents peracetic acid and hydrogen peroxide possess strong fungicidal activity. Chlorhexidine has some fungicidal activity against yeast cells but high concentrations of this compound may be required to ensure inactivation. Formaldehyde and glutaraldehyde-based formulations have strong fungicidal activity. Sodium hypochlorite and a number of other halogens are effective against spores of pathogenic fungi. Twelve disinfectants including chlorine-releasing compounds, ethyl alcohol, chlorox-ylenol, quaternary ammonium compounds, chlorhexi-dine and glutaraldehyde were evaluated for their ability to inactivate spores of *Microsporum canis* in naturally-infected feline hair (Rycroft and McLay, 1991). Sodium hypochlorite, benzalkonium chloride and glutaraldehyde-based formulations were the most effective disinfectants. Phenolic compounds, ethyl alcohol and anionic detergents were ineffective against these fungal spores.

Viruses

Many chemical compounds which are bactericidal are also virucidal. However, when the activity of these disinfectants is examined more closely, major differences are evident. With the exception of mycobacteria, most vegetative bacteria are more readily inactivated by chemical compounds than are non-enveloped viruses. Enveloped viruses are usually considered to have a pattern of susceptibility similar to that of vegetative bacteria (Fig. 94.5). Many viruses shed by infected animals are found in association with organic matter such as faeces, exudates or body fluids which may shield them from chemical inactivation. In addition, some resistant animal viruses, notably canine and feline parvoviruses, are shed in enormous numbers, thereby enhancing their opportunity to infect susceptible animals in the immediate environment and also facilitating transfer by vehicle or vectors to other distant locations. Viral diseases of animals which are spread mainly by contaminated surfaces, equipment or fomites are more amenable to control by chemical disinfectants than viral infections spread primarily through aerosols, secretions, body fluids or animal products. The ability of a virucide to function in an effective manner is influenced by many factors including the nature of virus particles present, the presence or absence of organic matter, contact time, the concentration of chemical used and the environmental temperature. Ultimately, the structure of the virus particles, the antiviral spectrum of the chemical employed and the environment in which the interaction between the viruses and the chemical takes place determine the outcome. Selection and use of chemical disinfectants for particular circumstances requires an understanding of the viral pathogens likely to be present, the prevailing conditions in the building or premises requiring decontamination, the spectrum of activity of the virucide selected, its safety for workers and the cost involved.

The sites and modes of action of chemical disinfectants which either inhibit viruses or have virucidal activity are summarized in Table 94.5. Although acids and alkalis have limited activity against many viruses, their ability to inactivate the virus of foot-and-mouth disease is of considerable importance. This non-enveloped picornavirus resists inactivation by many commonly used disinfectants but is inactivated by sodium carbonate, sodium hydroxide, citric acid and acetic acid. Chemicals with limited virucidal activity include alcohols, biguanides, oxidizing agents such as hydrogen peroxide, quaternary ammonium compounds and many phenolic compounds. Aldehydes, ethylene oxide, β-

propiolactone and halogens, especially sodium hypochlorite and other chlorine-releasing compounds, are effective virucides. Structural and functional components of enveloped and non-enveloped viruses which are targets of chemical disinfectants are shown in Fig. 94.8. The virucidal activity of complex disinfectants is often determined by their exact formulation and the concentration of each active constituent incorporated into the final preparation.

Prions

Unlike conventional infectious agents, prions are resistant to the majority of chemical compounds that have the ability to inactivate bacteria, bacterial endospores, fungi and viruses. The basis of this exceptional resistance is not well understood but probably relates to the fact that prions are altered proteins which can resist further chemical modification by organic solvents, oxidizing agents, phenolic compounds, some halogens and alkylating agents.

Experimental procedures indicate the exceptional resistance of the agents which cause transmissible degenerative encephalopathies to physical and chemical methods of inactivation. Based on published data from many sources, Tables 94.6 and 94.7 summarize the physical and chemical methods that have been evaluated for the inactivation of agents that cause transmissible degenerative encephalopathies.

The lack of consistency among the scientific reports relating to procedures used for the inactivation of the causal agents of transmissible spongiform encephalopathies may be due in part to the selection of biological specimens used for evaluation and also to the methods used to determine whether or not inactivation was achieved (Taylor, 2004).

Prions have been shown to be resistant to several physical methods of inactivation including dry heat, moist heat, gamma irradiation, microwave irradiation and ultraviolet irradiation. From the data presented in Table 94.6, it is evident that the aetiological agents involved are generally not inactivated by dry heat at a temperature of 200°C or moist heat at temperatures close to 130°C. The ashing of brain samples at 600°C failed to fully inactivate the scrapie agent (Brown *et al*., 2000). Treatment of macerated hamster brain infected with the 263K strain of hamster-adapted scrapie at 1,000°C for 15 minutes completely inactivated this strain of scrapie agent (Brown *et al*., 2004). In published reports, paradoxical results which indicate enhancement of the thermostability of the

BSE agent when autoclaved at 138°C compared with 134°C are attributed to smearing and drying of macerated brain tissue on glass surfaces before autoclaving took place (Taylor, 2000). Prior fixation of tissue in ethyl alcohol or formalin has been shown to enhance the resistance of scrapie agents to inactivation.

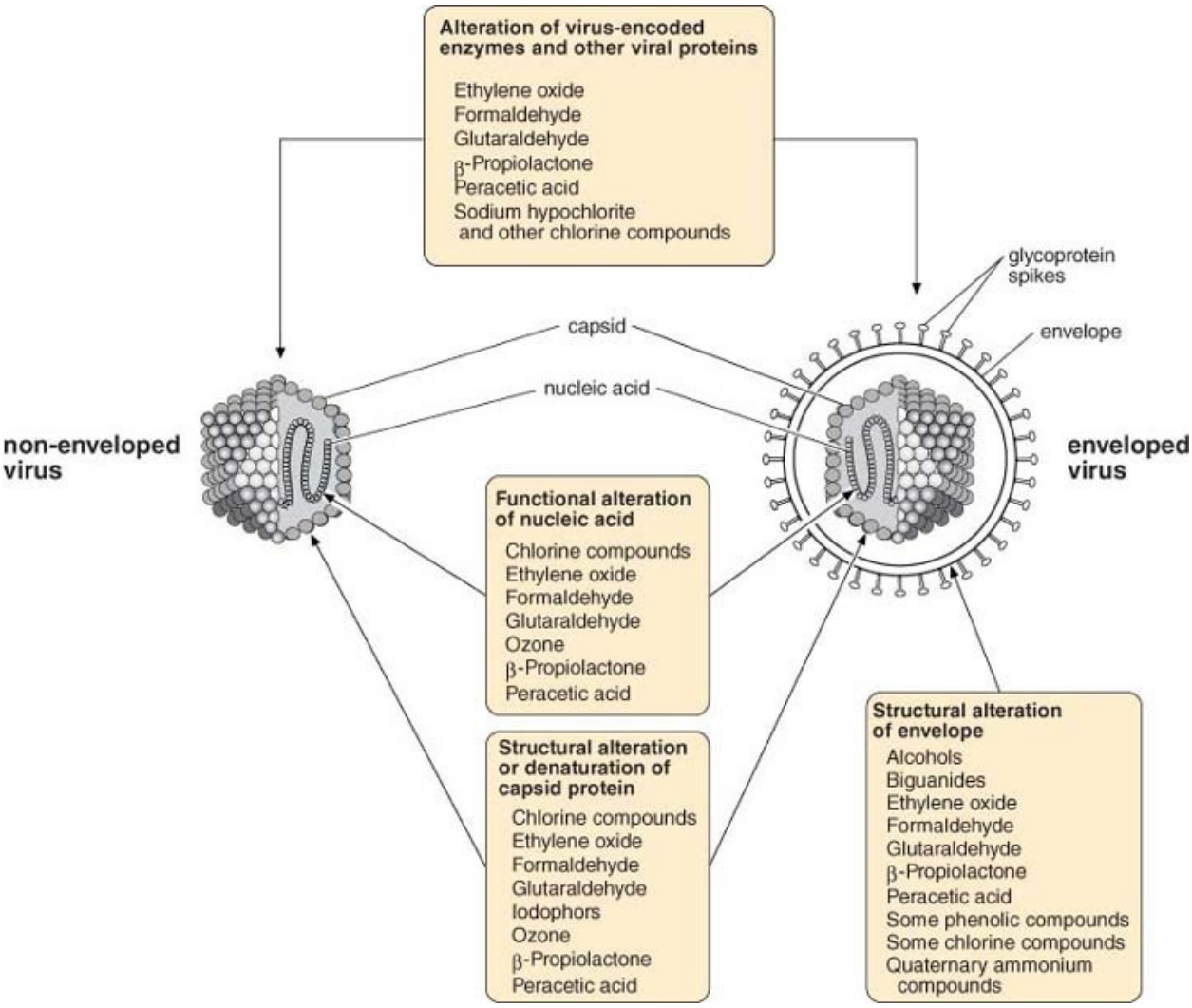
Hydrochloric acid, alkylating agents, detergent, organic solvents and oxidizing agents are ineffective against the causal agents of transmissible spongiform encephalopathies (Table 94.7). Extensive investigations with a wide range of chemical compounds gradually yielded relevant data on the inactivation of these resistant agents. It has been reported that when PrP^{Sc} is bound to metallic surfaces it is more resistant to inactivation than when present in tissue. Sodium hypochlorite solutions and heated sodium hydroxide inactivated the BSE agent, the CJD agent and the scrapie agent. Inactivation of the CJD agent and the scrapie agent occurred following treatment with 2.5% (w/v) sodium hypochlorite for 1 hour (Brown *et al*., 1986). Further evaluation of the activity of sodium hypochlorite demonstrated that solutions yielding concentrations of available chlorine from 8,250 to 16,500 ppm inactivated the BSE agent. Autoclaving the scrapie agent at 121°C for 30 minutes in the presence of 2 mol/litre sodium hydroxide resulted in inactivation of the agent (Taylor *et al*., 1997).

Table 94.5 The sites and modes of action of chemical disinfectants which have inhibitory effects on viruses or have virucidal activity.

Disinfectant	Site of action	Mode of action	Comments
Acids (organic or mineral acids)	Viral capsid	Induce conformational change in susceptible viruses by acting on viral capsid proteins	Citric and phosphoric acids inactivate foot-and-mouth disease virus; hydrochloric acid inactivates rotavirus and vesicular stomatitis virus
Alkalis	Probably the viral capsid	High pH values denature protein such as capsid protein	Solutions of alkalis with high pH values are effective against foot-and-mouth disease virus, adenovirus and swine vesicular disease virus
Alcohols	Act on lipid envelope and on the capsid protein of some viruses	Alteration of viral envelope and protein denaturation	May be effective against some enveloped viruses; cannot be relied on to inactivate non-enveloped viruses
Aldehydes			
Formaldehyde	Viral envelope, viral capsid and viral nucleic acid	Alkylating agent which reacts with amino groups, sulphydryl groups and carboxyl groups; cross-links proteins and reacts with nucleic acids	Wide antimicrobial spectrum; used for fumigation of buildings and in vaccine preparation
Glutaraldehyde	Viral envelope, viral capsid and viral nucleic acid	Alkylating agent which reacts with proteins and nucleic acids	Acts more rapidly than formaldehyde; wide antimicrobial spectrum
Biguanides			
Chlorhexidine	Reported to react with viral envelope	Membrane-active agent	Antiviral activity confined to enveloped viruses
Ethylene oxide	Viral capsid and viral nucleic acid	Alkylating agent which reacts with proteins and nucleic acids	Effective virucide; used for gas sterilization procedures
β-Propiolactone	Viral capsid and viral nucleic acid	Alkylating agent which reacts with proteins and nucleic acids	Effective virucide; widely used in the preparation of veterinary vaccines
Halogens			
Chlorine compounds	Viral capsid and viral nucleic acid	React with protein components and nucleic acid; cause structural alteration of some viruses	Among the most effective virucides available; sodium hypochlorite, chlorine dioxide and organochlorines are widely used as virucides
Iodophors	Viral capsid	Bind to capsid proteins; the amino acids tyrosine and histidine are reported to be targets for iodine binding	Widely used in the dairy industry and for skin and wound disinfection
Oxidizing agents			
Peracetic acid	Reported to react with proteins embedded in the envelope, with viral capsid proteins and also with nucleic acid	Reacts with sulphydryl and amino groups in proteins and also with chemical groupings in nucleic acid	Wide antimicrobial spectrum; powerful oxidizing agent, effective against many virus families
Hydrogen peroxide	Reacts with sulphydryl groups in viral proteins, such as capsid proteins	Potent oxidizing agent; reacts with sulphydryl groups and cysteine residues	Antiviral activity not well documented
Ozone	Viral capsid and viral nucleic acid	Strong oxidizing agent which damages capsid protein and also viral nucleic acid	Marked virucidal activity; used for water disinfection and also in the food industry
Phenolic compounds			
Diverse group of compounds; antiviral activity determined by the formulation of individual disinfectants	Viral envelope damaged by some phenolic compounds; the antiviral activity of most phenolic compounds is limited to enveloped viruses	Denature proteins and react with viral envelope	Although enveloped viruses may be inactivated, activity against non-enveloped viruses is unpredictable
Quaternary ammonium compounds	Viral envelope; capsid proteins of some viruses may be altered	React with viral envelope or with glycoproteins embedded in the envelope; alteration of capsid proteins may occur in some viruses	May be active against enveloped viruses; non-enveloped viruses usually resistant

Figure 94.8 The structural and functional components of viruses with which

chemical disinfectants are reported to interact. These chemicals may have inhibitory effects on viruses or have virucidal activity. The virucidal activity of complex disinfectants depends on their formulation and also on a range of environmental factors.



Studies on the efficacy of hyperbaric rendering procedures for the inactivation of the BSE agent and the scrapie agent have yielded equivocal results (Schreuder *et al*., 1998). Thermal inactivation procedures for these agents using moist heat are not yet conclusively established. The BSE agent is considered to be more resistant to heat inactivation procedures than the scrapie agent (Schreuder *et al*., 1998). Although the aetiological agents of transmissible spongiform encephalopathies exhibit exceptional resistance to chemical inactivation, high concentrations of available chlorine released by sodium hypochlorite and sodium hydroxide at a concentration of 2 mol/litre, heated to 121°C, are effective methods for inactivating these resistant agents.

Table 94.6 Evaluation of physical methods for the inactivation of agents which cause transmissible degenerative encephalopathies.

Treatment method	Agent	Details of procedure employed	Comments
Dry heat	Scrapie agent		
	ME7 strain	160°C for 24 hours	Not inactivated
	ME7 strain	200°C for 20 minutes	Not inactivated
	ME7 strain	200°C for 1 hour	Infectivity not detected
	263K strain	200°C for 1 hour	Not inactivated
	301V strain	200°C for 1 hour	Not inactivated
	Scrapie agent	360°C for 1 hour	Not inactivated
	Scrapie agent 263K strain	600°C for 15 minutes in a muffle furnace	Traces of infectivity detected
	Scrapie agent 263K strain	Incinerated at 1,000°C for 15 minutes	Completely inactivated
Moist heat			
Gravity displacement autoclaving	Scrapie agent		
	139A strain	126°C for 2 hours	Reported to be inactivated
	22A strain	126°C for 4 hours	Inactivated
	Creutzfeld-Jacob disease (CJD) agent	132°C for 1 hour	Reported to be inactivated
	Scrapie agent 263K strain	132°C for 1 hour	Reported to be inactivated
	Bovine spongiform encephalopathy (BSE) agent 301V strain	126°C for 30 minutes	Not inactivated
	Scrapie agent ME7 strain	126°C for 30 minutes	Reported to be inactivated
Porous load autoclaving	Scrapie agent BSE agent	134°C to 138°C for up to 1 hour	Reported to be inactivated but effectiveness questioned
Rendering treatment	Scrapie agent	138°C for 125 minutes	Not inactivated
	BSE agent Scrapie agent	133°C at 3 bar for 20 minutes	Cannot be relied on to completely inactivate these agents
Irradiation			
Gamma irradiation	CJD agent Kuru agent Scrapie agent Transmissible mink encephalopathy (TME) agent	150 kGy	Not inactivated
Microwave irradiation	Scrapie agent 22A strain	650 W of energy at a frequency of 2,450 MHz for 35 seconds	Not inactivated
Ultraviolet irradiation	CJD agent Kuru agent Scrapie agent TME agent	100 kJ/m^2 at 254 nm	Not inactivated

Table 94.7 Evaluation of chemical treatment, either alone or with physical methods, for the inactivation of agents which cause transmissible degenerative encephalopathies.

Treatment method	Agent	Details of procedure employed	Comments
Acid	Scrapie agent 263K strain	1 mol/litre hydrochloric acid at room temperature for up to 153 hours	Not inactivated
	Scrapie agent	8 mol/litre hydrochloric acid for 1 hour	Not inactivated
	Scrapie agent	1 mol/litre hydrochloric acid at 65°C for 1 hour	Substantial inactivation reported but not completely inactivated
Alkali	Scrapie agent 263K strain	2 mol/litre sodium hydroxide for up to 2 hours	Not inactivated
	CJD agent	1 mol/litre sodium hydroxide for 60 minutes, followed by gravity displacement autoclaving at 121°C for 30 minutes	Inactivated
	Scrapie agent 22A strain	Gravity displacement autoclaving at 121°C in the presence of 2 mol/litre sodium hydroxide for 30 minutes	Inactivated
Alkylating agents	Scrapie agent	Immersion of infected brain tissue in 20% formol saline for 974 days	Not inactivated
	CJD agent	10% formol saline for 1 year	Not inactivated
	TME agent	Formol fixed tissue for 6 years	Not inactivated
	BSE agent	10% formol saline for 2 years	Not inactivated
	Scrapie agent	12.5% unbuffered glutaraldehyde for 16 hours	Not inactivated
	CJD agent	5% buffered glutaraldehyde for 14 days	Not inactivated
	CJD agent	88% (v/v) ethylene oxide at 14 psi and at a temperature of 43°C for 4 hours	Not inactivated
Detergent	Scrapie agent 22A strain	5% sodium dodecyl sulphate (SDS) at room temperature for 2 hours	Not inactivated
		Boiling in 5% SDS for 15 minutes	Not inactivated
		Autoclaving at 121°C in 5% SDS for 15 minutes	Not inactivated
Halogens	CJD agent	2.5% sodium hypochlorite (with 25,000 ppm available chlorine) at room temperature for 1 hour	Inactivated
	Scrapie agent 263K strain	2.5% sodium hypochlorite (with 25,000 ppm available chlorine) at room temperature for 1 hour	Inactivated
	Scrapie agent	1.4% sodium hypochlorite (with 14,000 ppm available chlorine) for 30 minutes	Inactivated
	BSE agent	Sodium hypochlorite supplying available chlorine at concentrations ranging from 8,250 ppm to 16,500 ppm for 30 to 120 minutes	Inactivated
	Scrapie agent 263K strain	2% (w/v) iodine composed of a mixture of 2% elemental iodine and 2.4 % sodium iodide for 4 hours	Not inactivated
Organic solvents	Scrapie agent	5% chloroform for 2 weeks	Not inactivated
	Scrapie agent	4% phenol for 2 weeks	Not inactivated
Oxidizing agents	Scrapie agent	50 ppm chlorine dioxide for 24 hours	Not inactivated
		3% (v/v) hydrogen peroxide for 24 hours	Not inactivated
	CJD agent	Potassium permanganate at concentrations ranging from 0.1% (w/v) to 0.4% (w/v) for 60 minutes	Not inactivated
	Scrapie agent ME7 strain	Scrapie-infected murine brain homogenate treated with up to 19% peracetic acid for 24 hours	Not inactivated
		Fragments of intact scrapie-infected murine brain tissue treated with 2% peracetic acid for 24 hours	Inactivated

Chemicals used as disinfectants in veterinary

medicine

Acids

Acidic conditions tend to inhibit bacterial growth, and many organic acids are employed as preservatives in food and pharmaceuticals. The antimicrobial activity of acids is usually pH dependent. Organic acids are highly effective against Gram-positive bacteria but not as active against Gram-negative bacteria.

Viruses show wide variation in their susceptibility to low pH levels, often related to structural features. The capsid proteins of some viruses, which are markedly inhibited by acidic conditions, may undergo changes in their conformational state when treated with organic acids. Many acids including citric acid and phosphoric acid inactivate the virus of foot-and-mouth disease. Mineral acids may also be used for this purpose but they are corrosive and hazardous for workers. Hydrochloric acid inactivates human rotavirus and vesicular stomatitis virus. At a 2.5% concentration, hydrochloric acid has been used for inactivating the endospores of *Bacillus anthracis* on hides.

In food processing industries, mineral acids are used extensively as cleaning agents for removing lime scale, milk stone and other alkaline deposits in pipes and milking machines and on surfaces. The choice of acid may be determined by the types of component being treated and by their ability to withstand high temperatures and acidity.

Acetic acid, an effective antibacterial agent, can be used at concentrations up to 0.5% for treating wounds infected with *Pseudomonas* species. This organic acid, which is non-irritating at low concentrations, has been used topically for the treatment of chronic bacterial conditions including otitis externa.

Organic acids and mineral acids have a defined but limited role in disinfection procedures. Their antimicrobial activity usually correlates with low pH values and they tend to act slowly. Because strong mineral acids are hazardous for workers, eye protection and rubber gloves should be worn when handling these acids.

Alcohols

The antimicrobial spectrum of alcohols includes Gram-positive and Gram-negative bacteria, mycobacteria, some fungal pathogens and some enveloped viruses. They are not sporicidal and non-enveloped viruses are resistant. The

bactericidal property of alcohols increases with carbon chain length but water solubility decreases. Ethyl alcohol and isopropyl alcohol are widely used as disinfectants. The presence of water increases the bactericidal activity of alcohol and the most effective concentration of ethyl alcohol is approximately 70%. Alcohols denature protein, are lipid solvents and exhibit rapid antimicrobial activity against vegetative bacteria. The antifungal activity of alcohols is attributed to reduction in water activity to below the levels at which fungal enzymes, membranes and cells retain structural and functional stability (Maillard, 2004). Because they evaporate rapidly, alcohols exert no residual effects following topical application.

Alcohols are often used alone for topical disinfection. Solutions of iodine or chlorhexidine in 70% alcohol are employed for the preoperative disinfection of skin sites. Published data indicate that preoperative application of 2% chlorhexidine in 70% isopropyl alcohol is superior to an aqueous solution of 10% povidone – iodine for preventing surgical - site infection in human patients (Darouiche *et al*., 2010). In comparison with other disinfectants, alcohols are inexpensive, relatively non-toxic, non-tainting and colourless. The antimicrobial activity of alcohols is limited in the presence of organic matter, especially when dried on surfaces. Because of their solvent activity, rubber and some plastic material may be damaged by alcohol. Due to their inflammability, alcohols should not be applied to surfaces close to naked flames.

Aldehydes

As a group, aldehydes are highly reactive chemicals with a wide antimicrobial spectrum. Three aldehydes, formaldehyde, glutaraldehyde and *ortho* phthalaldehyde are used as disinfectants. A number of chemicals including aldehydes, ethylene oxide and β-propiolactone are alkylating agents: they inactivate enzymes and other proteins with labile hydrogen atoms such as sulphydryl groups. Aldehydes react readily with amino, carboxyl, sulphydryl and hydroxyl groups on proteins, causing irreversible changes in protein structure. Some aldehydes react with amino groups on purine and pyrimidine bases in nucleic acids and with peptidoglycan. Formaldehyde occurs as a gas which is freely soluble in water and also as a solid. At room temperature, formaldehyde polymerizes, forming a colourless solid material called paraformaldehyde. Formaldehyde solution (formalin) is an aqueous solution containing approximately 38% formaldehyde (w/w) with methyl alcohol added to delay

polymerization. Vapour-phase formaldehyde can be used for fumigation of sealed buildings. The vapour can be produced by evaporation of formalin, by the addition of formalin to potassium permanganate crystals or by heating paraformaldehyde. The antimicrobial spectrum of formaldehyde is wide. It is effective against vegetative bacteria including mycobacteria, endospores, fungi and viruses but it acts more slowly than glutaraldehyde. Apart from its use as a disinfectant, formaldehyde is used in the preparation of veterinary vaccines and also in footbaths to prevent or treat foot lameness in cattle and sheep. Even at low levels, the irritating vapour and pungent odour of formaldehyde is evident. It is toxic and the inhalation of vapour may sensitize workers. The use of formaldehyde as a broad-spectrum antimicrobial agent is declining due to its known toxicity and its potential carcinogenicity. Chlorine-releasing agents should not be used in association with formaldehyde as bis-chloromethylether, a potent carcinogen, is produced by the reaction of formaldehyde with hydrochloric acid, sodium hypochlorite or other chlorine-containing compounds.

Glutaraldehyde, a dialdehyde, is usually supplied commercially as a 2%, 25% or 50% acidic solution. Although stable at acid pH, it is more active at values close to pH 8. It has high microbiocidal activity against vegetative bacteria, bacterial endospores, fungal spores and viruses. Unlike formaldehyde, glutaraldehyde reacts rapidly with microbial pathogens. For high - level disinfection, glutaraldehyde is used as a 2% solution. It reacts with proteins, with components of bacterial cell walls and with bacterial endospores. Although recognized as one of the most effective sporicidal agents, its mode of interaction with these resistant structures is not clearly defined. In addition to its disinfectant properties, glutaraldehyde is used for specimen fixation in electron microscopy and also commercially for treatment of leather. It is widely used for the sterilization of thermosensitive medical equipment such as endoscopes and anaesthetic equipment which cannot be subjected to heat sterilization. Even at low levels, glutaraldehyde vapour is irritating for the eyes and mucous membranes. Some workers exposed to glutaraldehyde develop allergic contact dermatitis, asthma and rhinitis. Concerns about the risks of exposure to glutaraldehyde have resulted in a marked decline in its use as a broad-spectrum disinfectant, and alternative chemicals with comparable efficiency and fewer toxic side effects are being considered.

Ortho-phthalaldehyde, an aromatic aldehyde, has been evaluated as a potential replacement for glutaraldehyde. This recently introduced chemical has marked bactericidal and virucidal activity but its sporicidal activity is less than that of

glutaraldehyde. An advantage of *ortho*-phthalaldehyde over other aldehydes is its lipophilic activity which facilitates its uptake through the cell walls of Gram-negative bacteria and mycobacteria (Moore and Payne, 2004). Further work is required to determine safety and efficacy aspects of this aromatic aldehyde.

Alkalis

The antimicrobial activity of alkalis is related to hydroxyl ion concentration. Alkalis are frequently used to increase the pH of industrial sanitizers and cleaners. Sodium hydroxide, potassium hydroxide and sodium carbonate (washing soda) are the alkalis most often employed for cleaning surfaces of buildings and vehicles; calcium hydroxide (slaked lime) is sometimes used for whitewashing surfaces of buildings following an outbreak of an infectious disease. At high concentrations these chemicals have marked microbiocidal properties. Caustic alkaline solutions are effective against many viruses including foot-and-mouth disease virus, adenoviruses and swine vesicular disease virus. Although sodium carbonate at a 4% concentration is used primarily as a cleaning agent, it is particularly effective against foot-and-mouth disease virus. At concentrations over 5%, sodium hydroxide has a wide antimicrobial spectrum including bacterial endospores. Prions, which are resistant to most chemical decontamination procedures, are inactivated by treatment with 2 mol/litre sodium hydroxide at 121°C for 30 minutes. Both sodium hydroxide and potassium hydroxide are corrosive for metals and hazardous for workers. All workers using strong alkaline solutions should be informed of their caustic nature and should wear eye protection, rubber gloves and protective clothing.

Ammonium hydroxide, described as a weak base, inactivates coccidial oocysts which are resistant to the majority of standard chemical disinfectants. Aqueous solutions of this weak base, even at low concentrations, have potent antibacterial activity. Strong solutions of ammonium hydroxide emit intense pungent fumes.

Biguanides

This group of cationic compounds includes chlorhexidine, alexidine and some polymeric forms. Biguanides are widely used as aqueous solutions for hand washing and preoperative skin preparation. Because biguanides are cationic, their activity is greatly reduced by soaps and other anionic compounds. The most important member of this group, chlorhexidine, is available as dihydrochloride,

diacetate and gluconate. Chlorhexidine gluconate, which is water soluble, is the form most commonly used. Absence of toxicity is an important feature of biguanides.

Chlorhexidine is a membrane-active agent which at low concentrations inhibits membrane enzymes and promotes leakage of cellular constituents. When the concentration is increased, cytoplasmic constituents are coagulated and a bactericidal effect is observed. This biguanide has a wide antibacterial spectrum which includes Gram-positive bacteria and many Gram-negative bacteria. It has limited fungicidal activity. Some Gram-negative bacteria such as *Proteus* species and *Pseudomonas* species may be highly resistant to this disinfectant, and in addition it is neither mycobactericidal nor sporicidal. Although it may be active against some enveloped viruses, the antiviral activity of chlorhexidine is variable and it cannot be recommended as an effective antiviral disinfectant. Chlorhexidine – alcohol solutions are particularly effective as topical disinfectants; they combine the antibacterial rapidity of alcohol with the persistence of chlorhexidine at the site of application. Because it has longer residual activity on teat skin than many other disinfectants, chlorhexidine is used extensively in teat dips for mastitis control programmes in dairy cattle. The activity of chlorhexidine is pH dependent and it is more active at alkaline than acid pH values. Its activity is reduced by the presence of organic matter. As some biguanides may be toxic for aquatic species, care should be taken when disposing of chlorhexidine solutions.

Ethylene oxide

At room temperature, ethylene oxide is a colourless gas with a faint odour and an irritating effect on eyes and mucous membranes. It is soluble in water and a range of organic solvents. Ethylene oxide is inflammable and when present in the air at a concentration exceeding 3% it forms an explosive mixture. This safety problem can be overcome by mixing ethylene oxide with carbon dioxide or other suitable gases which are not inflammable. Ethylene oxide is not corrosive for metals and it decomposes spontaneously into methane, ethane and carbon dioxide.

As an alkylating agent, ethylene oxide reacts with amino, carboxyl, sulphydryl and hydroxyl groups, leading to denaturation of microbial proteins including enzymes and also nucleic acids. It is a highly effective antimicrobial agent with bactericidal, fungicidal, virucidal and sporicidal activity. Some vegetative

bacteria such as enterococci are reported to be comparatively resistant to this gas and it does not inactivate prions. Activity against the protozoan parasite *Cryptosporidium parvum* has been reported. Ethylene oxide is used for the low-temperature sterilization of heat-sensitive equipment. A desirable feature of the gas is its ability to penetrate a variety of materials including large packages, bundles of cloth and certain plastics. However, inactivation of microorganisms takes place slowly. The antimicrobial activity of ethylene oxide is influenced by relative humidity, temperature, concentration of the gas, contact time and the presence of water vapour. Substances that interfere with the activity of ethylene oxide include organic residues and salt crystals.

There has been a decline in the use of ethylene oxide in recent years arising from risks to human health following exposure to the gas through work-related activities and also to patients arising from residual gas present in sterilized material. Ethylene oxide has been listed as a mutagen and a human carcinogen since the 1990s. Modern ethylene oxide sterilization equipment and improved work practices have decreased the risks associated with the use of this low-temperature chemical sterilizing agent. Despite these changes, unease with this highly efficient broad-spectrum antimicrobial agent has persisted not only because of its toxicity but also on account of its inflammability. An additional concern relates to the potential risks arising from release of this alkylating agent into the environment in the immediate vicinity of institutions employing ethylene oxide routinely for low-temperature sterilization of equipment.

Halogens

Chlorine and iodine compounds are used extensively in veterinary medicine for their antimicrobial activity. It is claimed that bromine compounds, which are sometimes used for their bactericidal activity, have greater antimicrobial activity than chlorine compounds. Despite these claims, bromine compounds are employed infrequently as disinfectants. In addition to their long-established place in water treatment, chlorine gas and chlorine-releasing compounds are used extensively in food processing industries. Iodine compounds are also used as disinfectants in food processing and as teat dips in dairy cattle. Because they are relatively non-toxic, iodine compounds are used for hand disinfection preoperatively in veterinary and human healthcare facilities, in laboratories and also at farm level.

Chlorine compounds

Many different types of chlorine-releasing compounds are available for general-purpose disinfection for particular applications in food processing industries and for veterinary use. The antimicrobial activity of chlorine preparations is determined by the amount of available chlorine in the solution. The stability of free available chlorine in solution is strongly influenced by chlorine concentration, pH, the presence of organic matter and exposure to light. Chlorine-releasing compounds include sodium hypochlorite and *N*-chloro compounds, also referred to as organic chlorine compounds. Chloramine-T, dichloramine-T, halozone, sodium dichloro-isocyanurate and potassium dichloro-isocyanurate are examples of organic chlorine compounds. The antimicrobial activity of these compounds is reported to be slower than that of hypochlorites but some of these organic chlorine compounds are less susceptible to inactivation by organic matter than sodium hypochlorite. Chlorine dioxide, which is a gas at room temperature, has been proposed as an alternative to sodium hypochlorite. This gas, which is soluble in water, forming a stable solution in the dark, decomposes slowly when exposed to light. At concentrations above 10% in air, chlorine dioxide is unstable and explosive. However, it is an effective antimicrobial compound and non-inflammable and non-explosive at concentrations used for sterilization. Chlorine dioxide has a broad antimicrobial spectrum. It is bactericidal, fungicidal, virucidal (Table 94.5) and spori-cidal (Table 94.4) and reported to be capable of inactivating prions (Table 94.7). A gaseous chlorine dioxide sterilization apparatus, which was developed more than 20 years ago, is available commercially. The sterilizing ability of chlorine dioxide is reported to be equivalent to that of ethylene oxide.

The antimicrobial activity of chlorine and its compounds is due to the formation of hypochlorous acid which occurs when free chlorine is added to water. Hypochlorites and chloramines undergo hydrolysis when added to water, leading to the formation of hypochlorous acid. This acid releases nascent oxygen, a powerful oxidizing agent. Chlorine combines directly with bacterial cytoplasmic proteins and with viral capsid proteins. Although chlorine-releasing compounds damage the genomes of a number of viruses, the nature of these reactions is not clearly defined. Chlorine compounds such as sodium hypochlorite are most effective at pH values below 7 and their antimicrobial activity is inversely proportional to the pH of the environment in which they are used. The optimum pH for hypochlorites is close to pH 5. Because of their

instability, hypochlorites can lose up to 50% of their concentration within a month if stored in open containers. On account of this instability, fresh solutions should be prepared before use.

Chlorine-releasing compounds are potent virucides. Chlorination, a standard water treatment for preventing the spread of infectious disease, is generally considered to be a safe procedure. Chlorine gas has been used for decades for the treatment of public water supplies and sodium hypochlorite is employed for the treatment of water in swimming pools. Household bleaches, which usually contain high concentration of sodium hypochlorite, are used at suitable dilutions in dairies, in food processing plants and for general disinfection of equipment and farm buildings. Sodium hypochlorite is fast-acting, non-staining and inexpensive. However, its corrosive effects and its relative instability limit its application in some circumstances. The two most important factors limiting the antimicrobial activity of hypochlorites are the presence of residual organic matter and the neutralization of hypochlorous acid by alkaline substances. In environments with high residual organic matter due to inadequate cleaning, low levels of chlorine compounds are ineffective.

Health risks arising from the use of chlorine compounds appear to be limited. Detection of trihalom-ethanes in chlorinated water has raised concern about the safety of this form of treatment of public water supplies as trihalomethanes are reported to be carcinogenic in laboratory animals. Few alternative treatment methods for rendering public water supplies safe for human consumption are available at present. Advantages of chlorine compounds over other disinfectants include low toxicity at effective concentrations, a wide antimicrobial spectrum, ease of use and relatively low cost.

Iodine compounds

The antimicrobial activity of iodine compounds has been recognized for more than 180 years. Although iodine and chlorine compounds share some common characteristics as effective antimicrobial agents, they have a number of distinguishing features. Iodine is less reactive chemically than chlorine, and iodine compounds are more active in the presence of organic matter than chlorine compounds. The antimicrobial activity of iodine is greater at acid pH than at alkaline pH.

Elemental iodine, a bluish-black crystalline substance with a metallic lustre, is only slightly soluble in water. Despite its low solubility, iodine was formerly

used for its antimicrobial activity as an aqueous solution. Iodine is readily soluble in ethyl alcohol and in aqueous solutions of potassium iodide and sodium iodide. When dissolved in ethyl alcohol (tincture of iodine), high levels of free iodine are obtained. Disadvantages of using iodine solutions include instability, staining of skin and fabrics, toxicity and skin irritation. Inorganic iodine has been largely replaced by iodophors in which iodine is complexed with surface-active compounds or polymers which allow both increased solubility and sustained release of free iodine. In most iodophor preparations, the carrier is usually a non-ionic surfactant in which iodine is present as micellar aggregates. When complexed, free iodine levels are limited and the disadvantages of using aqueous or alcoholic solutions are avoided. An iodophor in which iodine is complexed with polyvinylpyrrolidone, referred to as povidone – iodine, is a commonly used disinfectant. When an iodophor is diluted with water, dispersion of the micellar aggregates of iodine occurs leading to a slow release of iodine. Because the amount of free iodine in an iodophor solution depends on the concentration used, more concentrated solutions may have less antimicrobial activity than diluted solutions. The increased antimicrobial activity of dilute solutions reflects the level of free iodine present. For maximum antimicrobial effect, iodophor solutions should be diluted in accordance with the manufacturers' instructions.

When used at appropriate dilutions and at pH values below 5, iodophors have a broad range of antimicrobial activity. They are bactericidal, fungicidal and virucidal. It has been suggested that the amino acids tyrosine and histidine in the viral capsid are the specific targets for iodine. These disinfectants are reported to react with sulphydryl groups. Some non-enveloped viruses are less sensitive than enveloped viruses to iodophors. Buffered solutions of iodine are effective against spores of *Bacillus subtilis* but high concentrations and long contact times are required for effective sporicidal action. The spore coat and cortex are the sites affected. Reports of prolonged survival of *Pseudomonas aeruginosa* and *Burkholderia cepacia* in povidone – iodine solution have been attributed to the presence of organic matter, inorganic material or biofilm formation on the items being treated. Addition of alcohol improves the antimicrobial activity of iodophors. Alcoholic solutions of iodophors are widely used in some countries for disinfection of hands and sites prior to surgical procedures. Acidic iodophor solutions are used as sanitizers in the food industry. When employed as disinfectants in dairy plants, the pH of iodophors is kept acidic by the addition of phosphoric acid to ensure the removal of dried milk residues. Effective post-

milking teat dipping is an important control measure for contagious mastitis in dairy cattle, and iodophors are among the common teat dips used for this purpose.

Heavy metals and their derivatives

The term ‘heavy metals’ includes metals such as mercury, lead, zinc, silver and copper. Salts of heavy metals are sometimes used for their antimicrobial activity in farming, horticulture, and human and veterinary medicine. The ability of extremely low concentrations of certain metals such as silver and copper to exert a marked inhibitory effect on bacteria, algae and fungi has been recognized for centuries. The antibacterial and antifungal activity of heavy metal derivatives such as copper salts is attributed to their ability to inhibit enzymatic activity in microbial membranes and within the cytoplasm, by binding to sulphydryl groups.

Copper, which is slowly oxidized in air, forms a series of compounds, some with marked antimicrobial activity. For decades, copper salts have been incorporated into lotions for their antiseptic and astringent activities. In addition to its inhibitory effect on microbial metabolism by combining with sulphydryl groups, copper has a strong affinity for DNA which it can denature reversibly at low concentrations. Copper salts, through their ability to combine with protein molecules, are reported to have an adverse effect on viral capsid proteins. Copper sulphate mixed with lime, referred to as Bordeaux mixture, was introduced in the 1880s as a spray for the control and prevention of fungal infections in susceptible plants such as potatoes. When sprayed on foliage, it adhered to leaves forming a slow-release copper complex with sustained antifungal activity. The algicidal activity of copper sulphate has been used to prevent algal growth on open bodies of water and pools. A number of copper compounds are used as preservatives in wood, paper and paint industries.

A number of mercurial compounds including mercuric chloride, mercurochrome, thiomersal and phenylmercuric nitrate have been used for many years for the treatment of minor wounds and skin infections. Thiomersal, which was formerly used as a skin disinfectant and fungicide, is used as a preservative for biological products such as bacterial and viral vaccines. Concerns arising from the risks of environmental pollution, direct toxicity and the persistence of mercury in the environment have resulted in a progressive decline in the use of mercurial compounds in recent years. The majority of mercurial compounds

have been replaced by disinfectants and therapeutic agents which are less toxic, more effective and less likely to result in environmental pollution. Inclusion of mercury in products for agricultural and industrial use is strongly discouraged by regulatory authorities.

Interference with sulphydryl groups has been proposed as the likely method of interference by silver compounds with bacterial and fungal enzyme systems. Aqueous solutions of silver nitrate, which are bactericidal, have been used to prevent infection of burn wounds in human patients. The antibacterial effect of silver-releasing surgical dressing has been documented. Sand coated with silver has been used in filters for water purification, and silver-coated charcoal has been employed for similar purposes. The virucidal activity of silver compounds against a range of enveloped and non-enveloped viruses has been described. Although the mode of action has not been explained, alteration of capsid proteins was proposed as the likely method of inactivation.

β-Propiolactone

At room temperature β-propiolactone is a colourless non-flammable liquid which is miscible with water. The antimicrobial activity of β-propiolactone depends on its concentration and on relative humidity and temperature. It is bactericidal, fungicidal, sporicidal and virucidal. Microbial inactivation is attributed to alkylation of DNA. It is a highly effective gaseous sterilizing agent. Although β-propiolactone is considerably more active than ethylene oxide, it lacks the penetrating power of ethylene oxide. When organic matter is present, an increased concentration of β-propiolactone is required.

β-Propiolactone has been widely used for the production of inactivated veterinary viral vaccines. It has potential use in decontamination of animal premises and when dealing with contaminated fluids of animal origin. Health hazards associated with the use of β-propiolactone include skin lesions and eye irritation following direct exposure. Of even greater concern are the longer term risks following limited exposure because of the suspected carcinogenic activity of this potent antimicrobial agent.

Peroxygen compounds

Hydrogen peroxide, peracetic acid, ozone and peroxygen-based commercial disinfectants containing sodium or potassium peroxymonosulphates are strong oxidizing agents with broad antimicrobial spectra. A number of published

reports, however, indicate that some peroxygen compounds, including peroxymono-sulphates, are ineffective as mycobactericidal agents. The characteristics of individual compounds determine their usefulness as disinfectants in veterinary medicine.

Hydrogen peroxide, which is available as a solution, is a non-polluting compound that decomposes to oxygen and water. The strength of a solution of hydrogen peroxide is usually specified as 10 volumes or 20 volumes, which indicates the volume of oxygen that evolves from 1 volume of the peroxide solution. For industrial use, higher concentrations of solution are available. Because hydrogen peroxide solutions are unstable, benzoic acid or other suitable substances are added as stabilizers. This oxidizing agent is bactericidal and sporicidal. There are limited data on the virucidal activity of hydrogen peroxide, and some non-enveloped viruses may survive exposure to its oxidizing activity. Hydrogen peroxide is an effective sporicidal agent and its activity is enhanced by increased concentrations and elevated temperatures. In common with some other peroxygen compounds, the activity of hydrogen peroxide against mycobacteria is questionable. The presence of catalase or other peroxidases in some bacteria can increase tolerance to low levels of hydrogen peroxide. Due to the activity of tissue catalase, strong effervescence follows application of hydrogen peroxide to contaminated wounds and this reaction assists in the removal of cellular debris and pus. When applied to tissues, the antimicrobial activity is of short duration. Formation of hydroxyl radicals, which react with cellular components including nucleic acid, account for the antimicrobial activity of this compound. A new process, using radio waves to generate plasma-activated hydrogen peroxide vapour, has been developed in recent years. The microbiocidal activity of the plasma is attributed to the generation of hydroxyl ions and other free radicals. In addition to its use as a disinfectant and antiseptic, hydrogen peroxide is used in the food industry for aseptic packaging.

Peracetic acid, a colourless liquid with a pungent odour, is a strong oxidizing agent. It is miscible with water and has greater lipid solubility and more potent antimicrobial activity than hydrogen peroxide. This disinfectant, which is available commercially as a 15% aqueous solution, is rapidly lethal for a wide range of microorganisms including bacteria and their spores, fungi, algae and viruses. Even at low temperatures, and in the presence of organic matter, it is sporicidal. Unlike a number of other peroxygen compounds, per-acetic acid is mycobactericidal. It oxidizes sulphydryl and amino groups and denatures protein and nucleic acid. Because of its wide antimicrobial spectrum, it is used as a cold

sterilizing agent for some thermolabile medical devices. Peracetic acid can corrode steel, copper and other metals and it can damage natural and synthetic rubber. On account of its strong antimicrobial action at low temperatures and an absence of residues, peracetic acid is widely used in the food-processing and beverage industries. It is also used for disinfecting sewage sludge. At concentrations close to 60%, peracetic acid can be explosive at room temperature. Exposure to vapour phase peracetic acid has an irritating effect on the eyes and respiratory tract. Apart from the irritation caused to mucous membranes, concern has been expressed about safety aspects of peracetic acid. Following prolonged exposure to moderate levels of peracetic acid, there may be an increased risk of tumour development.

Ozone, an allotropic form of oxygen, has strong oxidizing properties. It is bactericidal, sporicidal and virucidal. In water, ozone is chemically unstable but its antimicrobial activity persists due to the production of free radicals, including hydroxyl radicals. Alteration of proteins following treatment with ozone results from reactions with cysteine, tryptophane and methionine. The primary viral changes caused by ozone appear to result from structural damage to viral capsids, followed by inactivation of viral nucleic acid. The sporicidal activity of ozone is attributed to changes induced in the outer spore coat layers. Ozone is sometimes used for disinfection of water and also in the food industry. A number of commercial disinfectants utilize peroxygen compounds in their formulations. One such commercial preparation, Virkon®, has been evaluated for its activity against mycobacteria. Based on the results obtained, it was concluded that Virkon ® was ineffective as a mycobactericidal agent (Griffiths *et al*., 1999).

With the exception of peracetic acid, there is uncertainty about the mycobactericidal activity of a number of peroxygen compounds. Several new low-temperature procedures for sterilization, utilizing hydrogen peroxide gas plasma and ion plasmas formed from peracetic acid, have been described in recent years. These new procedures offer many potential advantages over some of the methods currently available for sterilization.

Phenolic compounds

For more than 150 years, phenol has been employed for its antimicrobial activity. It has the distinction of being one of the first chemicals used as an antiseptic in surgical procedures. Phenol was also the standard disinfectant with which other chemicals with potential antimicrobial use were compared in a

procedure known as the phenol-coefficient technique. Formerly, most of the phenolic compounds used for the manufacture of disinfectants were obtained from tar, hence the use of the term coal-tar disinfectants. Tar itself, a by-product of the destructive distillation of coal, contains a number of products. Fractionation of tar yields phenols, organic bases and neutral products. The temperature employed for fractionation determines the range of products produced and their biological activity. A progressive increase in desirable biological properties of coal-tar phenols is observed with increasing boiling point but this is accompanied by decreased solubility in water. Formerly, some well known patented coal-tar products such as Jeyes Fluid and Lysol, solubilized by the addition of soap, were popular general-purpose disinfectants. Today, many phenolic compounds are synthesized. In addition to a wide range of coal-tar phenols, non-coal-tar phenols such as 2-phenylphenol (*ortho*-phenylphenol) are also available. Simple and substituted phenols constitute a vast group of chemicals but their antimicrobial activity cannot be readily related to their chemical structures.Halogenation and nitration increases the antibacterial activity of phenol but nitration also increases systemic toxicity. In the bisphenol series, halogenation confers additional antimicrobial activity. Phenolic compounds range from phenol and cresylic acid to a collection of high-boiling tar acids. The term black fluids is applied to coal-tar fractions solubilized with soaps; white fluids are emulsions prepared from tar fractions. Because of differences in formulation, generalizations concerning the antimicrobial activity of phenolic compounds are inappropriate. The antimicrobial activity and also the toxicity of individual phenolic disinfectants depend on the exact formulation and concentration of each active constituent in the preparation. By binding to amino acid residues and displacing water molecules, phenols denature protein. Conformational changes in membrane proteins result in cytoplasmic membrane damage leading to leakage of intracellular components. Some phenolic compounds also react with thiol groups, causing metabolic inhibition.

When employed at recommended concentrations, phenolic compounds are bactericidal. *Ortho*-phenylphenol and black fluids are particularly effective against mycobacteria but bisphenols are not mycobactericidal. When halogenated, phenols and cresols have antifungal activity. As a group, phenolic compounds are not sporicidal and their activity against viruses is formulation-dependent; enveloped viruses may be inactivated but non - enveloped viruses are often resistant. *Ortho*-phenylphenol is active against enveloped and many non-enveloped viruses. Due to the great diversity of phenolic compounds available

and the wide variation in their formulations, this group of disinfectants cannot be recommended as reliable virucidal agents. Phenolic compounds are usually moderately priced and not seriously affected by the presence of organic matter. Contact with the skin should be avoided because of the irritation and depigmentation produced by some compounds. As pigs and cats are particularly susceptible to the toxic effects of phenolic disinfectants, all treated surfaces should be thoroughly rinsed before the reintroduction of animals. Phenolic compounds impart a tarry odour and leave a residual film on surfaces which can cause tainting of food and agricultural products. Accordingly, these disinfectants should not be used in meat plants, dairies or food storage areas. They are unsuitable for disinfecting surfaces or containers which come into direct contact with food for human consumption. Disinfectants containing phenolic compounds are potentially toxic for workers, domestic animals and wildlife. In circumstances where substantial amounts of these disinfectants are used in farm buildings, run-off should not be discharged into ponds, lakes, rivers or streams. Fluid containing phenolic disinfectants should be collected in slurry tanks or other suitable holding facilities and applied to arable land remote from water courses.

Quaternary ammonium compounds

The structure of quaternary ammonium compounds is related to that of ammonium chloride and these chemicals can be considered as organically substituted ammonium compounds. These cationic compounds have surface-active properties but they are incompatible with a wide range of chemical agents including non-ionic and anionic surfactants such as soaps, phos-pholipids and other substances. Quaternary ammonium compounds (QACs) are most effective at neutral or slightly alkaline pH values. They have low toxicity and high solubility in water. These disinfectants exert their antimicrobial action by reacting with anionic lipids in the cytoplasmic membrane and with the outer membrane of Gram-negative bacteria. Low concentrations of QACs cause membrane damage with resultant leakage of cytoplasmic components, a consequence of the disruption of the interactions between lipids and proteins in membrane structures. High concentrations of QACs induce coagulation of cytoplasmic components. Interactions of these compounds with enzymes essential for cell metabolism interfere with metabolic processes. QACs are attracted to the negatively charged surfaces of microorganisms where they bind

to phospholipids in the cell membrane and denature proteins, thereby impairing permeability. They should be applied to clean surfaces as their antimicrobial activity is reduced by organic matter such as blood, milk or faeces.

In comparison with many other disinfectants, QACs are moderately expensive. They have a limited antimicrobial spectrum and they exhibit greater activity against Gram-positive bacteria than against Gram-negative bacteria. Some Gram-negative bacteria such as *Pseudomonas* species and *Serratia marcescens* can survive and grow in solutions of QACs. These cationic compounds are neither mycobactericidal nor sporicidal. Although some of these compounds have activity against enveloped viruses, non-enveloped viruses are resistant and as a group they are considered unreliable virucides.

One of the most commonly used QACs, benzalkonium chloride, which is bactericidal and fungicidal, is incompatible with components of some commercial rubber preparations but not with silicone rubber. This compound is employed for preoperative preparation of skin and mucous membranes. Benzethonium chloride is employed for treatment of superficial wounds and also for controlling algal growth in swimming pools. Ethyl alcohol potentiates the action of QACs.

High concentrations of QACs can cause skin irritation. Because they are non-staining, odourless, non-toxic and usually non-corrosive, QACs are used extensively as disinfectants in food processing industries. They are also used for disinfection of automatic calf feeders and included in sheep dips for controlling microorganisms associated with fleece problems.

Characteristics of chemicals employed as disinfectants, not only in veterinary medicine and human medicine but also for procedures related to public health and for use in the food industry, are summarized in Table 94.8.

Microbial resistance to disinfection

Disinfectants play an important role in limiting the spread of bacterial pathogens (Russell, 2003). The continuous use of these agents in veterinary medicine and elsewhere could potentially lead to the emergence of bacteria resistant to these important antimicrobial compounds. Bacteria with increased tolerance to disinfectants have been reported and can be selected for under defined laboratory conditions (McMurry *et al*., 1998; Braoudaki and Hilton, 2004 ; Randall *et al*., 2004). When a disinfectant is used under suboptimal conditions, treated bacteria

may survive. This may occur following dilution, through inactivation of the disinfectant by organic material present in the environment being treated, or through incorrect application of the disinfectant.

Resistance to disinfectants is less common among bacteria than antibiotic resistance (Poole, 2002). Nonetheless, resistance to different classes of disinfectants has been reported for pathogens of veterinary importance including *E. coli*, *Campylobacter* species, *Listeria* species, staphylococci and *Pseudomonas* species (Randall *et al*., 2001, 2003 ; Brenwald and Fraise, 2003). When used in accordance with the recommended guidelines, commercial disinfectants are usually effective.

Several mechanisms of resistance to disinfectants have been described and many of these overlap with the mechanisms that facilitate antibiotic resistance. There is a concern that bacteria resistant to various classes of disinfectants may develop cross-resistance to important antibiotics used to treat animals.

Resistance to disinfectants following target site modifications

In the laboratory, it is possible to select bacteria which have acquired resistance to disinfectants. Mutants of *E. coli*, *Salmonella* Typhimurium and *S. aureus* demonstrating reduced susceptibility to triclosan can be selected *in vitro*. Triclosan is a widely used biocide and, at low concentrations, it is the only biocide known to inhibit a specific bacterial enzyme, FabI, that encodes an enol acyl-carrier protein (ACP) reductase involved in fatty acid biosynthesis. Levels of resistance conferred by *fabI* mutants with MIC ranges of between 1 and 64 mg/ml are significantly below concentrations of the disinfectant applied in practice (from 2,000 to 20,000 mg/ml). Interestingly, Braoudaki and Hilton (2004) described an *E. coli* O157 strain that exhibited an MIC to triclosan of more than 1,024 mg/ml. This strain did not contain any alteration in the *fabI*-encoding gene, suggesting that an undescribed mechanism may be involved. In *Pseudomonas aeruginosa*, a second enol-ACP reductase, denoted as FabV, was recently described (Zhu *et al*., 2010).

Table 94.8 Characteristics of disinfectants based on their chemical structures, antimicrobial activities or other descriptive features.

Disinfectant group	Characteristics	Uses	Comments
Acids	At low concentrations, organic acids such as citric acid and acetic acid are non-irritating; mineral acids such as hydrochloric acid and sulphuric acid are corrosive and hazardous for workers	Organic acids used as preservatives in food and pharmaceuticals; mineral acids used as cleaning agents and for decontaminating farm buildings	Organic acids inhibit Gram-positive bacteria; both organic and mineral acids inactivate the virus of foot-and-mouth disease
Alcohols	Two alcohols, namely ethyl alcohol and isopropyl alcohol, are widely used as disinfectants; they are relatively non-toxic, non-tainting and colourless; their solvent activity can damage rubber and some plastic material; they are inflammable and should not be used close to naked flames	Alcohols denature protein and are lipid solvents; at 70% concentration, ethyl alcohol is rapidly bactericidal; alcohols are not sporicidal	The antimicrobial spectrum of alcohols includes Gram-positive and Gram-negative bacteria, mycobacteria, some fungi and many enveloped viruses
Aldehydes	This group of highly reactive chemicals are alkylating agents which react with proteins, peptidoglycans and nucleic acids; three aldehydes, formaldehyde, glutaraldehyde and *ortho*-phthalaldehyde are used as disinfectants; the irritating vapour and pungent odour of aldehydes is an undesirable feature of these broad-spectrum disinfectants	Highly effective bactericidal and virucidal agents; glutaraldehyde is an effective sporicide; formaldehyde is used in the preparation of veterinary vaccines and in footbaths for cattle and sheep	These chemicals are highly effective disinfectants; their use is declining due to concerns about their toxicity and potential carcinogenicity
Alkalis	The antimicrobial activity of these chemicals is related to hydroxyl ion concentration; many are highly corrosive for metals and hazardous for workers; caustic alkaline solutions can cause tissue injury	Frequently used to raise the pH of industrial cleaners and sanitizers; some, such as sodium carbonate, are used for cleaning surfaces of buildings and transport vehicles	At high concentrations have marked microbiocidal properties; heated solution of sodium hydroxide inactivates prions; workers should wear protective clothing, eye protection and gloves
Biguanides	These cationic compounds include chlorhexidine and alexidine; they are relatively non-toxic and are widely used for hand washing and preoperative site preparation; soaps and anionic compounds inhibit their antimicrobial activity	As a group, biguanides have wide antibacterial activity; chlorhexidine has limited antifungal activity and is neither mycobactericidal nor sporicidal; biguanides cannot be considered as effective antiviral disinfectants	Some Gram-negative bacteria are resistant to chlorhexidine; chlorhexidine-alcohol solutions are highly effective as topical disinfectants; chlorhexidine is widely used in teat dips for dairy cattle
Ethylene oxide	This colourless gas has a faint odour and an irritating effect on eyes and mucous membranes; it is inflammable and at concentrations over 3% in air it forms an explosive mixture	This alkylating agent is bactericidal, fungicidal, virucidal and sporicidal; it is used for the low-temperature sterilization of heat-sensitive equipment	Ethylene oxide is listed as a mutagen and as a human carcinogen; toxicity following exposure to ethylene oxide vapour may be acute or chronic; prolonged exposure to the gas may result in increased occurrence of neoplastic conditions and other tissue changes

Disinfectant group	Characteristics	Uses	Comments
Halogens			
Chlorine compounds	Chlorine-releasing compounds include sodium hypochlorite, *N*-chloro compounds and chlorine dioxide; these compounds, which are widely used in veterinary medicine, have a wide antimicrobial spectrum which includes bacteria and their spores, fungi, viruses and, at specified concentrations, prions. The presence of organic matter or alkaline substances limits the antimicrobial activity of these compounds	As chlorine-releasing compounds are potent virucides, they have been used for water treatment for decades; they are used in dairies, food processing plants and for general disinfection of buildings and equipment; chlorine dioxide is used for sterilization of equipment, including packaged material	Due to their instability, fresh solutions of many chlorine-releasing compounds have to be prepared before use; inadequate cleaning of surfaces or equipment can result in failure of these compounds to inactivate microorganisms
Iodine compounds	Although less reactive chemically than chlorine compounds, iodine compounds are effective antimicrobial agents; iodophor preparations offer many advantages over inorganic iodine and they are widely used for disinfection of hands and sites prior to surgical procedures. Iodophors are bactericidal, fungicidal, sporicidal and virucidal; addition of alcohol improves the antimicrobial activity of iodophors	Iodophors are used for disinfection of hands and surfaces preoperatively; they are widely used in the food industry, in dairy plants and on farms; iodophor teat dips are commonly used as a control measure for contagious mastitis in dairy cattle	Prolonged survival of some Gram-negative bacteria in povidone–iodine solutions has been reported; organic matter, biofilm formation or other factors may have contributed to bacterial survival in the iodophor solution
Heavy metals	Salts of heavy metals were formerly used in farming and human and veterinary medicine for their antimicrobial activity; the antibacterial and antifungal activity of copper salts is attributed to inhibition of enzymatic activity; interference with sulphydryl groups is proposed as the basis for the antibacterial and antifungal activity of silver compounds	Copper salts have been used in lotions for their antiseptic activities; copper sulphate mixed with lime was introduced as a spray for the prevention of fungal infections in susceptible plants in the 1880s; silver compounds have been used for treating burn wounds and also in filtration systems for water purification	Mercurial compounds were formerly used for treatment of minor wounds and skin infections; they were also used as preservatives in biological products such as vaccines; concerns arising from the toxicity of these compounds have resulted in a progressive decline in their use
β-Propiolactone	This alkylating compound has a wide antimicrobial spectrum; it is bactericidal, fungicidal, sporicidal and virucidal and has been widely used for production of inactivated veterinary vaccines; there are a number of health hazards associated with the use of this compound	This compound has potential use as a disinfectant when dealing with contaminated fluids of animal origin; it has a particular role in the preparation of inactivated veterinary viral vaccines	The suspected carcinogenic activity of β-propiolactone has limited its use as a disinfectant in veterinary medicine

Disinfectant group	Characteristics	Uses	Comments
Peroxygen compounds	Hydrogen peroxide, peracetic acid, ozone and some peroxygen-based disinfectants are strong oxidizing agents with broad antimicrobial spectra; some of these agents have questionable activity against mycobacteria; peracetic acid is rapidly lethal for bacteria, their spores, fungi, algae and viruses and is used as a cold sterilizing agent	Hydrogen peroxide is used for wound irrigation, topical disinfection and aseptic packaging; peracetic acid is used for sterilizing thermolabile medical devices and for disinfecting sewage sludge; ozone is used for water disinfection and also in the food industry	At concentrations close to 60%, peracetic acid can be explosive; it has an irritating effect on the eyes and mucous membranes; long-term exposure to peracetic acid may increase the risk of tumour development
Phenolic compounds	This diverse group of disinfectants ranges from phenol and cresylic acid to a collection of high-boiling-point tar acids. The antimicrobial activity and the toxicity of individual phenolic disinfectants depend on formulation. These disinfectants are bactericidal but not sporicidal; some inactivate enveloped viruses; they are not seriously affected by the presence of organic matter	Phenolic disinfectants are suitable for surfaces, buildings and transport vehicles with residual organic matter; because of their tarry odour and the residual film left on surfaces, they are unsuitable for surfaces or containers which come into direct contact with food	Toxicity for humans and animals, especially cats and pigs, is a feature of phenolic compounds; contact with skin should be avoided and run-off from buildings treated with these disinfectants should not enter ponds, lakes, rivers or streams
Quaternary ammonium compounds (QACs)	These cationic compounds have surface-active properties; they are most effective at neutral or alkaline pH values. They interfere with cell metabolism by causing membrane damage and they have a limited antimicrobial spectrum which includes Gram-positive and some Gram-negative bacteria; they are neither mycobactericidal nor sporicidal and are considered unreliable virucides; they are non-toxic and usually non-corrosive	Some QACs are used for pre-operative skin preparation and for treatment of wounds; used extensively in food processing industries; they are sometimes included in sheep dips for controlling microorganisms which cause fleece problems	QACs are incompatible with a wide range of chemicals including non-ionic and anionic surfactants such as soaps and phospholipids

Because the *inhA* gene present in *Mycobacterium tuberculosis* and *M. smegmatis* is a common target for both triclosan and isoniazid, an antimicrobial therapeutic-agent cross-resistance between these two unrelated compounds may arise (McMurry *et al*., 1999).

Resistance to disinfectants arising from alterations in cell permeability

Intrinsic bacterial resistance is common among Gram-negative bacteria, such as the *Enterobacteriaceae* and among *Pseudomonas* species. This is often attributed to mechanisms that limit the intracellular concentration of a disinfectant. Structural components of the outer cell membranes of these bacteria present an

effective barrier to the entry of some disinfectants (Table 94.9).

In Gram-positive bacteria, the mechanically strong, thick, outer cell wall with an open network structure of peptidoglycan appears to offer little resistance to the diffusion of small molecules, such as antibiotics and other antimicrobial compounds, into these cells. A triclosan-resistant *S. aureus* strain demonstrating cross-resistance to ciprofloxacin was recently described (Tkachenko *et al*., 2007).

In contrast to Gram-positive bacteria, the envelope of Gram-negative bacteria is composed of a multi-layered structure that exhibits an increased resistance to disinfectants (Stickler, 2004). The cell wall of Gram-negative bacteria has an outer membrane (OM), in which structural components function to limit the entry of disinfectants into the cytoplasm.

Water-filled outer-membrane protein channels known as porins facilitate the selective entry of small molecules into the cell and simultaneously restrict the influx of hydrophobic and large hydrophilic disinfectant molecules (Poole, 2002). In *E. coli*, the outer membrane protein F (OmpF) is an example of one such porin channel. *Pseudomonas* isolates demonstrating reduced susceptibility to QACs and chlorhexidine have been reported and this phenotype was linked to altered cell membrane permeability (Tabata *et al*., 2003).

Table 94.9 Nature of the changes in bacterial cells which contribute to the emergence of resistance to disinfectants.

Disinfectant type / Example	Nature of resistance	Bacterial species	Comments
Aldehydes			
Formaldehyde	Restriction of entry into bacterial cell due to changes in outer membrane protein	*E. coli*	
	Inactivation of disinfectant by formaldehyde dehydrogenase	*E. coli* *Klebsiella pneumoniae*	Plasmid-encoded resistance Resistance demonstrable in some strains
Glutaraldehyde	Changes in cell wall polysaccharides	*Mycobacterium chelonae*	
Biguanides			
Chlorhexidine	Bacterial degradation of disinfectant	*Achromobacter xylosoxidans*	Efflux genes are typically plasmid-encoded
	Resistance to entry into bacterial cell attributed to change of surface hydrophobicity	*Pseudomonas stutzeri*	
	Active efflux of disinfectant from bacterial cell	*Klebsiella pneumoniae*	
Phenolic compounds			
Triclosan	Resistance due to mutation in genes encoding target site molecules	*E. coli, S. aureus, Mycobacterium tuberculosis, Pseudomonas aeruginosa*	
	Active efflux of disinfectant from bacterial cell	*E. coli, P. aeruginosa*	Multiple efflux determinants identified for *P. aeruginosa*
Quaternary ammonium compounds	Resistance to entry into bacterial cell associated with changes in outer membrane protein, surface change and hydrophobicity	*P. aeruginosa*	
	Surface changes associated with decrease in outer membrane protein	*E. coli*	
	Active efflux of disinfectant from bacterial cell	*S. aureus, Enterococcus faecalis, Klebsiella pneumoniae, E. coli, P. aeruginosa*	

The role of lipopolysaccharide (LPS) as a permeability barrier in Gram-negative bacteria has been reported extensively. LPS is a unique component of the OM in Gram-negative bacteria and forms a rigid barrier that limits penetration of lipophilic compounds into the cell (Stickler, 2004). Other membrane proteins and membrane characteristics, such as surface hydrophobicity, have also been shown to influence the entry of disinfectants into the cell.

The electrostatic charge associated with bacterial cell surfaces also plays a role in resistance to disinfectants, especially in the case of compounds such as QACs (Bruinsma *et al.*, 2006).

Gram-positive endospore-forming bacteria display an intrinsic resistance to disinfectants (Gilbert *et al.*, 2004). Spores are resistant to most disinfectants,

such as alcohols, biguanides, organic acids, phenolic compounds, organomercurials and QACs, that would inactivate vegetative cells. The endospore structure provides a robust physical barrier, rendering many disinfectants ineffective as sporicidal agents.

Resistance to disinfectants mediated by efflux pumps

Efflux pumps are membrane-associated proteins that extrude a range of structurally dissimilar toxic compounds from the cytoplasm of the cell. Five classes of efflux pumps are recognized and are widely distributed among bacteria. These include the small multidrug resistance (SMR) family (now part of the drug/ metabolite transporter (DMT) superfamily), the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the resistance – nodulation — division (RND) family and the multidrug and toxic drug extrusion (MATE) superfamily. Additional information on efflux pump structures is provided in Chapter 12.

In Gram-positive bacteria, genes encoding SMR and MFS families of efflux pumps are frequently located on plasmids. Reduced susceptibility of *S. aureus* to QACs, phenolic compounds and intercalating agents was linked with the efflux pumps QacA to D (Wang *et al*., 2008). In *Acinetobacter* species, *E. coli*, *Salmonella* species, *Pseudomonas* species and other Gram-negative bacteria, transporters such as QacE and QacE ?1 are also located on plasmids or in some instances on the chromosome. The latter are generally associated with integrons that may account for their broad dissemination. *Salmonella* Typhimurium DT104 contains a 43-kbp *Salmonella* Genomic Island 1 (SGI 1) (described in Chapter 12), wherein a *qacEΔ1*-encoding gene linked to a class 1 integron is located on the chromosome. This structure or its derivatives have been identified in more than 15 different *Salmonella* serovars and also in *Proteus mirabilis*.

The following Gram-negative bacteria possess well characterized membrane transport proteins: *Pseudomonas aeruginosa*, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexJK (Poole, 2007); *E. coli* and *Salmonella*, AcrAB-TolC, AcrEF-TolC and EmrE; and *Campylobacter jejuni* and *C. coli*, CmeABC (Piddock, 2006). Despite their ability to export a range of structurally diverse chemical compounds, chromosomally-encoded efflux pumps are not known to promote acquired resistance to biocides (Poole, 2004). Over-expression of the AcrAB-TolC efflux pump in *E. coli* confers resistance to triclosan and pine oils (Moken *et al*., 1997).

Bacteria resistant to various classes of disinfectants can exhibit cross-resistance to some important antibiotics used to treat animals. The relationship between biocide export and antibiotic resistance remains to be determined (Poole, 2004).

Mercury resistance has been attributed to the plasmid-encoded *mer* genes (Poole, 2004). Chlorhexidine resistance due to degradation of the biocide has also been reported (Ogase *et al*., 1992), as has resistance to formaldehyde due to the plasmid-encoded formaldehyde dehydrogenase found in *E. coli* (Kummerle *et al*., 1996) and *Seratia marcescens* (Kaulfers and Marquardt, 1991).

Resistance to disinfectants mediated by bacterial populations within biofilms

Many bacteria are associated with surfaces and grow as a biofilm rather than as planktonic cells. Population-based resistance to disinfectants may play an important role *in vivo*. Populations of bacteria in biofilms have been consistently described as displaying biocide resistance orders of magnitude higher than their planktonic counterparts (Smith and Hunter, 2008). This has major implications for the control of infectious agents of importance in veterinary medicine, where biofilms may form on surgical equipment, implants and elsewhere. Bacteria within a biofilm are organized into a three-dimensional structure consisting of multiple bacterial species embedded within an exopolysaccharide matrix or glycocalyx. Biofilms containing *Salmonella* and *Proteus* exhibit an increased resistance to triclosan. *Listeria* species present in a biofilm show increased resistance to peroxides. Within these bacterial communities, metabolism is altered and the organisms exhibit a biofilm-associated pheno-type, including a reduction in disinfectant penetration due to the extracellular polysaccharide matrix (Pan *et al*., 2006), enzymatic inactivation of the biocide (Huang *et al*., 1995) and induction of efflux pumps (Maira-Litran *et al*., 2000).

Increased resistance to biocides within a biofilm has been attributed to several factors. The extracellular matrix acts as a physical barrier, limiting diffusion of the disinfectant into the bacterial cell. This barrier also facilitates the inactivation of the biocide through chemical reactions that are often mediated by bacterial enzymes (Stickler, 2004). The glycocalyx contains trapped extracellular enzymes which are concentrated within this matrix (Gilbert *et al*., 2004). If a species capable of producing an inactivating enzyme to a particular disinfectant is present within a biofilm, it will confer resistance upon that entire bacterial

community.

Bacteria present in biofilms have a slower growth rate than planktonic cells due to the limited nutrients in this environment. Therefore, bacterial cells in such a microenvironment may exist as metabolically inactive or dormant cells. Slowly growing cells are generally more resistant to biocidal compounds than rapidly growing cells (Stickler, 2004). Similarly, the biofilm-associated phenotype involves an alteration in OM proteins that reduces the permeability of the cell and up-regulates efflux pump activity.

Disinfection procedures

The correct choice of disinfectant is fundamental to the success of a disinfection programme. For optimal activity, disinfectants should be used at the correct concentration and allowed sufficient contact time with the surfaces or equipment. Thorough cleaning of all surfaces before application of disinfectant is essential for the inactivation of infectious agents as the antimicrobial activity of many chemical compounds is seriously impaired by residual organic matter such as faeces, blood, exudates, food and bedding. Moderate amounts of organic matter interfere with the activity of halogen disinfectants, particularly sodium hypochlorite, whereas phenolic disinfectants retain much of their activity under similar conditions.

A pressure washer set at low pressure can be used for applying disinfectant to the surfaces of buildings at a rate of 0.4 litres/m^2. A knapsack sprayer may suffice for small areas. Fumigation may be the preferred method for disinfection of buildings if an outbreak of a notifiable disease has been confirmed. As many disinfectants such as formaldehyde are ineffective at low temperatures, buildings should be heated to approximately 20°C in cold weather. Transport vehicles should be included in disinfection programmes as pathogens can be transferred over long distances in the cargo area, in the cab or, occasionally, on the outside of the vehicle. High-pressure cleaning with warm water containing detergent should be followed by rinsing with hot water. Surfaces should be allowed to dry before the application of disinfectant at the correct concentration to all parts of the vehicle, including the bodywork and wheels. A contact time of at least 30 minutes is required. Waste water from vehicles should be disposed of in a slurry tank and applied only to land used for tillage.

Attention to detail is essential for the success of a disinfection programme.

Failure to inactivate infectious agents present in buildings, on equipment or in transport vehicles may be due to the selection of an ineffective disinfectant, careless use of a potentially effective disinfectant, environmental factors or reintroduction of infectious agents by clinically affected animals, healthy carrier animals, food or rodents (Table 94.10).

Footbaths

Many infectious agents shed in faeces or urine of animals can be transferred on footwear from one location to another. Footbaths located at entry points to a farm or building should be used by all staff and visitors. To ensure compliance with footbath use, all pedestrians entering the premises should wear clean, waterproof footwear. Footbaths should be large enough to accommodate the largest size of footwear worn by workers or visitors. Disinfectants suitable for footbath use include iodophors, phenolic compounds and formalin. If a specific infectious agent is identified as the cause of a disease outbreak, a disinfectant known to be effective against that agent should be used in all footbaths on the premises.

Wheel baths

Wheel baths are sometimes positioned at farm entrances as part of a disease control programme (Quinn and Markey, 2001). The design of wheel baths should be such that there is adequate contact with disinfectant for a sufficient time to ensure destruction of infectious agents on wheel surfaces. The tyre of the largest wheel entering the bath should be completely immersed in disinfectant in one revolution.

Installation of a properly designed wheel bath is expensive and may impart an unrealistic impression of biosecurity. In many instances, the contents of vehicles, including animals, their secretions and excretions, animal feed and bedding, pose a greater threat of transferring infectious agents than vehicle wheels.

Table 94.10 Factors that may contribute to the failure of disinfection programmes or limit their effectiveness.

Disinfectant factors	Environmental factors	Comments
Selected chemical ineffective against the pathogen	Residual organic matter due to inadequate cleaning	Despite effective disinfection of a contaminated place, antimicrobial activity does not persist
Too dilute to be effective	Improper application of disinfectant to surfaces, equipment or transport vehicle	Infectious agents may be reintroduced by infected animals, fomites, personnel, transport vehicles and by other means

Insufficient contact time sallowed Temperature too low for optimal activity	Lack of contact with pathogen due to nature of surface	
Relative humidity too low for gaseous disinfectants	Biofilm formation on surfaces	
	Inactivation of quaternary ammonium compounds and biguanides by residual soaps and detergents	
	Inadequate treatment of water supply in contaminated buildings	

Practical aspects of disinfection procedures

To ensure success, disinfection procedures require careful planning and efficient implementation. Many chemicals used as disinfectants are corrosive, toxic or hazardous. Highly reactive chemicals such as formaldehyde and glutaraldehyde may be carcinogenic. Those supervising disinfection programmes should ensure that prolonged contact with toxic chemicals by workers is avoided. Disinfectants should be kept in cool, dark storage areas and used before they reach the expiry date specified on containers. They should be diluted by trained personnel in accordance with the manufacturers' instructions. All staff working with strong solutions of acids or alkalis should wear face shields and rubber gloves. Chlorine-releasing compounds and formaldehyde should never be used together or immediately after each other because a potent carcinogen is formed if the two chemicals are allowed to interact. Fumigation procedures require careful planning and should not be carried out by inexperienced staff.

Care is required when cleaning buildings before disinfection proceeds. Power hosing can generate aerosols containing viable pathogens which may be inhaled by workers unless properly fitting face masks are worn. After an outbreak of anthrax, the building should be sealed and all drains blocked. The contents of the building including bedding, fittings and equipment should be sprayed with 5% formalin which should be left to act for at least 10 hours before items are removed. Subsequently, the entire building and its fittings should be cleaned and disinfected. As no single chemical disinfectant can be considered suitable for every purpose and circumstance, the selection and use of disinfectants for particular microbial pathogens require a clear understanding of their antimicrobial spectrum and their limitations. Some disinfectants such as aldehydes have a wide antimicrobial spectrum while other compounds such as biguanides are limited in their antimicrobial activity. Cost, availability, stability, inactivation by organic matter and toxicity are features that influence the selection, safety and general use of many chemical compounds on the farm, in

food processing industries or for specific aspects of disease control programmes. Chemical disinfectants are indispensable for the successful implementation of disease eradication programmes. To ensure success, the use, application and limitations of these compounds should be understood by those implementing such programmes. With careful selection, accurate dilution and judicious use of disinfectants, tissue residues, food tainting and environmental pollution can be readily avoided while still ensuring the destruction of microbial pathogens of veterinary importance.

In addition to the intrinsic resistance of bacterial endospores, mycobacteria and some Gram-negative bacteria to particular chemical compounds, the ability of a number of pathogenic bacteria to acquire resistance to chemical disinfectants has been observed in recent years. There is evidence that resistance to disinfectants and antibiotics is genetically linked in a number of instances. Although not an issue of major concern at present, this development confirms the adaptability of bacterial pathogens to measures aimed at controlling environmental microbial populations through chemical intervention. This observation also emphasizes the need for careful selection and prudent use of chemical compounds as part of disease control programmes for animal and human populations. Education of farmers, producers, food processors and others engaged in the development and implementation of biosecurity programmes requires a coordinated effort on the part of veterinary, medical and health science professionals so that each can offer informed advice on the careful selection, proper use and safe disposal of chemical compounds with antimicrobial activity.

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Appendix

Relevant websites

General topics

American Society for Microbiology (ASM): http://www.asm.org
American Tissue Culture Collection (ATCC): http://www.atcc.org
Animal diseases: http://www.oie.int/
Antimicrobial susceptibility testing: http://www.clsi.org
Centers for Disease Control and Prevention: http://www.cdc.gov

Genomic analysis

Bacterial genome listing: http://www.ncbi.nlm.nih.gov/genomes/static/eub_g.xhtml and http://www.genomesonline.org/
BLAST alignment tool: http://www.ncbi.nlm.nih.gov/BLAST
In silico simulation of molecular biology experiments: http://insilico.ehu.es
J. Craig Venter Institute (formerly TIGR): http://www.jcvi.org/
Kyoto Encyclopedia of Genes and Genomes: http://www.genome.jp/kegg
Multi Locus Sequence Typing (MLST): http://www.mlst.net
Ribosomal Database Project: http://rdp.cme.msu.edu

Bacteriology

American Society for Microbiology – Approved Lists of Bacterial Names: http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=bacname&part=A60
Collection of bacterial taxonomic and genomic information at PathoSystems Resource Integration Center: http://patricbrc.vbi.vt.edu/portal/portal/patric/Home
List of Prokaryotic Names with Standing in Nomenclature: http://www.bacterio.cict.fr/

Taxonomic outlines for Bergey's Manual, Volumes 3, 4 and 5: http://www.bergeys.org/outlines.xhtml

Mycology

Index Fungorum, names of fungi: http://www.indexfungorum.org/

Virology

European Advisory Board on Cat Diseases (ABCD) with useful review articles on infectious diseases of cats: http://www.abcd-vets.org/

International Committee on Taxonomy of Viruses (ICTV) with latest information on viral nomenclature: http://ictvonline.org/virusTaxonomy.asp?bhcp=1

Infectious diseases

Department for Environment, Food and Rural Affairs (DEFRA), animal diseases fact sheets: http://www.defra.gov.uk/foodfarm/farmanimal/diseases/atoz/index.htm

European Food Safety Authority, Biohazard Panel. Many useful documents on selected zoonoses: http://www.efsa.europa.eu/en/panels/biohaz.htm

Fact files on infectious diseases of animals at Center for Food Security and Public Health, Iowa State University: http://www.cfsph.iastate.edu/DiseaseInfo/index.php

Food and Agriculture Organization of the United Nations: http://www.fao.org/

Material Safety Data Sheets – Canadian Laboratory Center for Disease Control: www.phac-aspc.gc.ca/id-mi/index-eng.php

ProMED mail, a global electronic reporting system for outbreaks of emerging infectious diseases, maintained by the International Society for Infectious Diseases (ISID): http://www.promedmail.org/pls/apex/f?p=2400:1000

US-FDA Foodborne Pathogenic Microorganisms and Natural Toxins Handbook: http://vm.cfsan.fda.gov/~mow/intro.xhtml

US-FDA Pathogen Annotated Tracking Resource Network: http://www.patrn.net

World Organisation for Animal Health (OIE) with useful information on infectious disease occurrence: http://www.oie.int/wahis/public.php?page=home

and online manual of diagnostic tests and vaccines for animals: http://www.oie.int/eng/normes/mmanual/A_summry.htm
World Health Organization: http://www.who.int/en/

Index

famciclovir
foscarnet
ganciclovir
idoxuridine
penciclovir
trifluridine
valacyclovir
inhibitor of RNA polymerase
ribavirin
reverse transcriptase inhibitors
delaviridine
lamivudine
nevirapine
stavudine
zidovudine
neuraminidase inhibitors
oseltamivir
zanamivir
protease inhibitors
amprenavir
ritonavir
saquinavir
Aphthovirus
Aquabirnavirus
Aquareovirus
Arboviruses
Arcanobacterium pyogenes
Archaea
Archaebacteria
Arcobacter
Argentina virus
Arteriviridae
Arterivirus
Arteriviruses

contagious agalactia
contagious bovine pleuropneumonia
contagious caprine pleuropneumonia
enzootic pneumonia of pigs
haemotropic mycoplasmas
mycoplasmal diseases of poultry
veterinary significance
Mycoplasma agalactiae
Mycoplasma bovis
Mycoplasma capricolum subsp. *capripneumoniae*
Mycoplasma capricolum subsp. *capricolum*
Mycoplasma dispar
Mycoplasma gallisepticum
Mycoplasma haemocanis
Mycoplasma haemofelis
Mycoplasma hyopneumoniae
Mycoplasma hyorhinis
Mycoplasma hyosynoviae
Mycoplasma leachii
Mycoplasma meleagridis
Mycoplasma mycoides subsp. *capri*
Mycoplasma mycoides subsp. *mycoides* (large colony type)
Mycoplasma mycoides subsp. *mycoides* (small colony type)
Mycoplasma ovis
Mycoplasma suis
Mycoplasma synoviae
Mycotic abortion
Mycotoxic leukoencephalomalacia
Mycotoxic lupinosis
Mycotoxic oestrogenism
Mycotoxicoses
aflatoxicosis
citrinin toxicosis
cyclopiazonic acid toxicosis