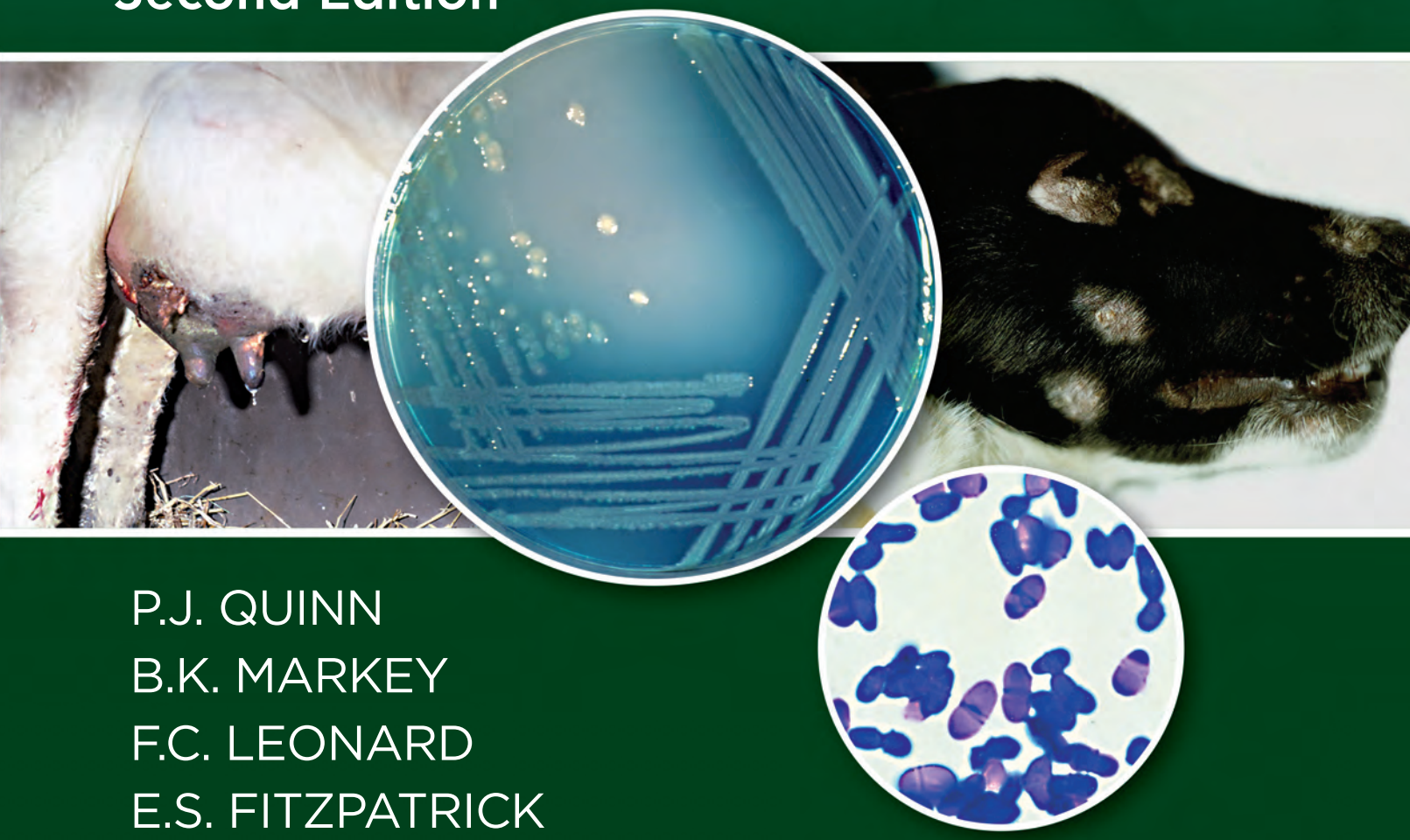
Concise Review of Veterinary Microbiology

Second Edition



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WILEY Blackwell

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Contents

Preface

The first edition of this book provided undergraduate veterinary students with a brief introduction to veterinary microbiology and diseases caused by pathogenic microorganisms. Since its publication in 2003, there have been many changes in veterinary microbiology, some related to the classification of pathogenic microorganisms and others associated with the increased understanding of the pathogenesis of infectious diseases. Developments in molecular aspects of microbiology have broadened the scope of diagnostic methods and have improved our understanding of the epidemiological characteristics of many infectious diseases. New developments relating to the emergence of antibacterial resistance are of particular importance in veterinary therapeutics and in public health.

The second edition of this book includes new chapters on bacterial genetics, antibacterial resistance, immunology, antifungal chemotherapy, biosecurity and vaccination. Topics of particular importance in veterinary medicine are given extended coverage. Important changes which have occurred in veterinary microbiology in recent years are presented in relevant chapters. There are five sections in this book and the Appendix includes a list of relevant websites to facilitate readers requiring additional information on topics referred to in the book. Colour has been used to enhance the quality of the illustrations and to facilitate interpretation of complex diagrams.

Acknowledgements

We wish to acknowledge the assistance provided by colleagues who reviewed chapters, provided scientific, technical and editorial advice or who assisted in other ways: James Buckley, Rory Breathnach, Louise Britton, Jill Bryan, Marguerite Clyne, Hubert Fuller, James Gibbons, Stephen Gordon, Laura Luque-Sastre, Aidan Kelly, Pamela Kelly, Dores Maguire, Marta Martins, Kerri Malone, Jarlath Nally, David Quinn, Michael Quinn, Eoin Ryan, John Ryan, Patrick Raleigh, Shabarinath Srikumar, Graham Tynan, Patrick Wall and Annetta Zintl.

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Dublin, January 2015

Abbreviations and definitions

AGID	agar gel immunodiffusion
ATP	adenosine triphosphate
BCG	bacille Calmette–Guérin
bp	base pairs
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CFT	complement fixation test
CNS	central nervous system
cELISA	competitive enzyme-linked immunosorbent assay
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FA	fluorescent antibody
Fc	crystallizable fragment, portion of an immunoglobulin without an antigen combining site
IFA	indirect fluorescent antibody
IFN	interferon
Ig	immunoglobulin
LPS	lipopolysaccharide
KOH	potassium hydroxide
MBC	minimal bactericidal concentration
MHC	major histocompatibility complex
MIC	minimal inhibitory concentration
MLST	multi-locus sequence typing
mRNA	messenger RNA
MRSA	methicillin-resistant *Staphylococcus aureus*
MZN	modified Ziehl–Neelsen
nm	nanometre, 10^{-9} metre
NK cells	natural killer cells
OIE	Office International des Épizooties (World Organization for Animal Health)
ORF	open reading frame
PAS	periodic acid–Schiff
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
RTX	repeats-in-toxin
SMEDI	stillbirth, mummification, embryonic death, infertility
V factor	nicotinamide adenine dinucleotide
VP	viral protein
UK	United Kingdom
µm	micrometre or micron, 10^{-6} metre
USA	United States of America
UV light	ultraviolet light
X factor	haemin
ZN	Ziehl–Neelsen
°C	degrees Celsius

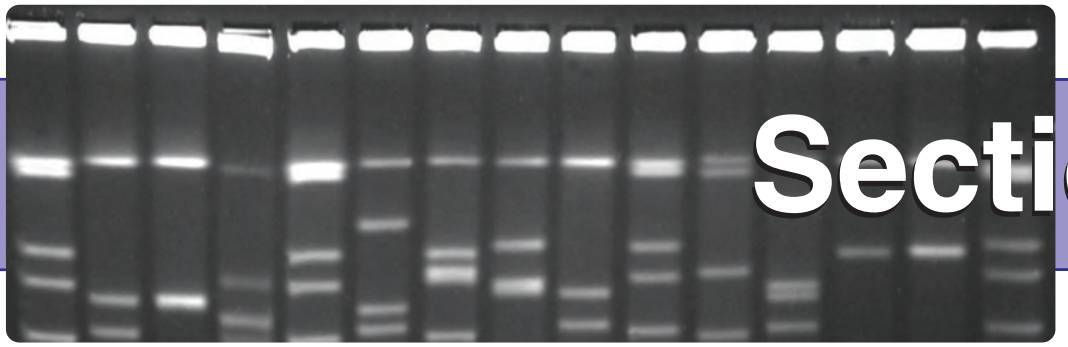
About the companion website

This book is accompanied by a companion website:

www.wiley.com/go/quinn/concise-veterinary-microbiology

The website includes:

- PowerPoint figures from the book for downloading

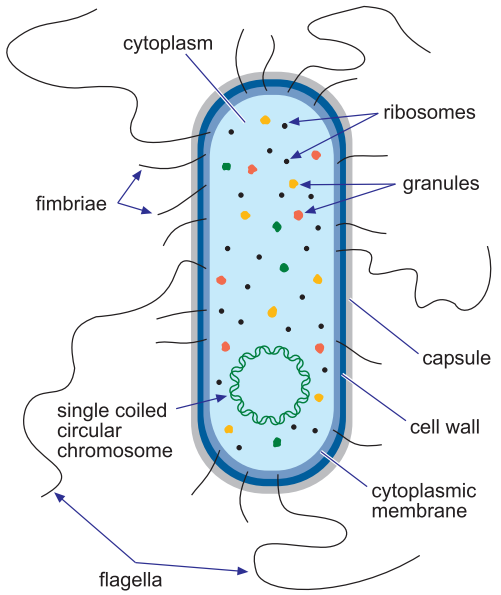


Section I

Introductory Bacteriology

1 Structure of bacterial cells

Structure of a typical bacterium



Common morphological forms of bacteria

Using bright-field microscopy, a magnification of 1,000x is required to observe most bacterial cells

Bacterium		Comments
Coccus	1 μm	Spherical cells; often occurring in chains or in grape-like clusters, usually stained by the Gram method
Bacillus	5 μm	Rod-shaped cells; usually stained by the Gram method
Spirochaete	10 μm	Thin, helical bacteria. Dark-field microscopy (without staining) or special staining methods are required for demonstration of these unusual bacteria

Bacteria are unicellular organisms and usually occur in simple shapes such as rods, cocci, spiral forms and occasionally as branching filaments. They typically have rigid cell walls containing a peptidoglycan layer and multiply by binary fission. Bacteria are smaller and less complex than eukaryotic cells and do not contain membrane-bound organelles. Genetic information essential for organism survival, the core genome, is usually contained in a single circular chromosome; a nuclear membrane and a nucleolus are absent. Some bacteria have more than one chromosome and chromosomes in certain bacteria are linear. The accessory genome encodes non-essential cell functions and may include plasmids and bacteriophages (see Chapter 3). Despite their morphological diversity, most bacteria are between 0.5 and 5 μm in length. Motile bacteria possess flagella by which they can move through liquids *in vivo* and *in vitro*.

Most bacteria found in nature are not harmful to humans, animals or plants. Some bacteria make an important contribution to the utilization of nutrients in soil, in water and in the digestive tracts of animals. Bacteria which cause disease in animals or humans are referred to as pathogenic bacteria.

A typical bacterial cell is composed of a capsule, cell wall, cell membrane, cytoplasm (containing nuclear material) and

Cell walls and associated structures of Gram-positive and Gram-negative bacteria

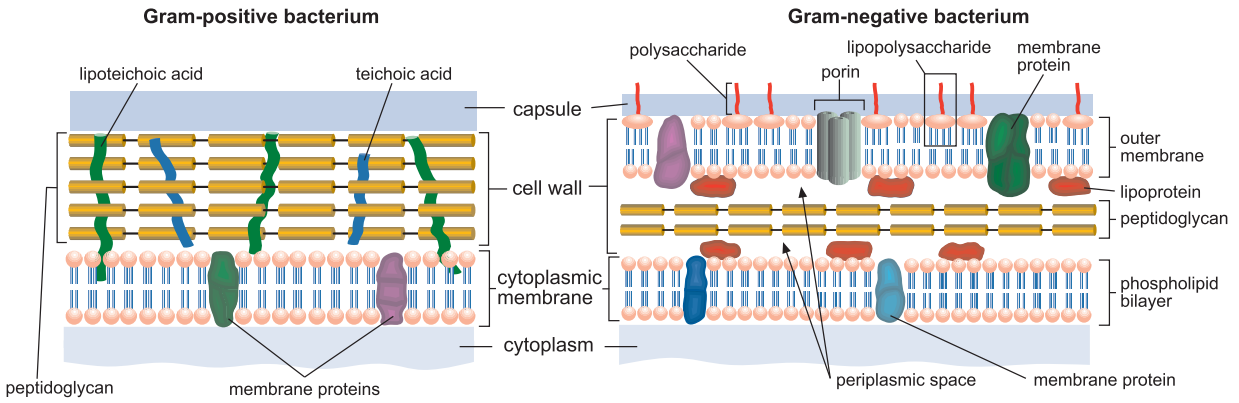


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Table 1.1 Structural components of bacterial cells.

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
Cell 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, pili)	Protein called pilin	Also known as fimbria (plural, fimbriae). Thin, straight, thread-like structures present on many Gram-negative bacteria. Mediate attachment to host cells. Specialized pili are involved in conjugation
Chromosome	DNA	Single circular structure with no nuclear membrane
Ribosome	RNA and protein	Involved in protein synthesis
Storage granules or inclusions	Variable chemical composition	Present in some bacterial cells; may be composed of polyphosphate (volutin or metachromatic granules), poly-β-hydroxybutyrate (reserve energy source), glycogen

appendages such as flagella and pili. Some species of bacteria can produce dormant forms termed spores or endospores, structures which are resistant to environmental influences. The principal structural components of bacterial cells are presented in Table 1.1. Some bacteria can synthesize extracellular polymeric material, termed a capsule, which forms a well-defined structure, closely adherent to the cell wall. In the body, capsules of pathogenic bacteria interfere with phagocytosis. The tough, rigid cell walls of bacteria protect them from mechanical damage and osmotic lysis. 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. Mycoplasmas, an important group of bacteria, lack rigid cell walls but have a flexible triple-layered outer membrane.

On the basis of 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.

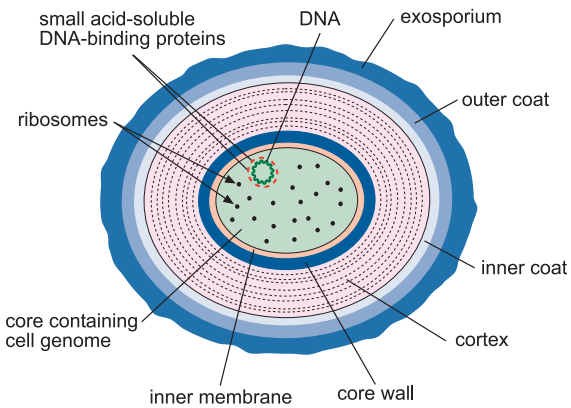
The cell membranes of bacterial cells are flexible structures composed of phospholipids and proteins. Active transport of nutrients into the cell and elimination of waste metabolites are functions of the cell membrane and it is also the site of electron transport for bacterial respiration. The cytoplasm, which is enclosed by the cell membrane, is essentially an aqueous fluid containing the nuclear material, ribosomes, nutrients, enzymes and other molecules involved in synthesis, cell maintenance and metabolism.

In most bacteria, the bacterial genome is composed of a single haploid circular chromosome containing double-stranded DNA. Bacterial genomes differ in size depending on the species. Plasmids, small circular pieces of DNA which are separate from the core genome, are capable of autonomous replication. Plasmid DNA may encode characteristics such as antibiotic resistance and exotoxin production. All protein synthesis takes place on ribosomes, structures composed of ribonucleoproteins.

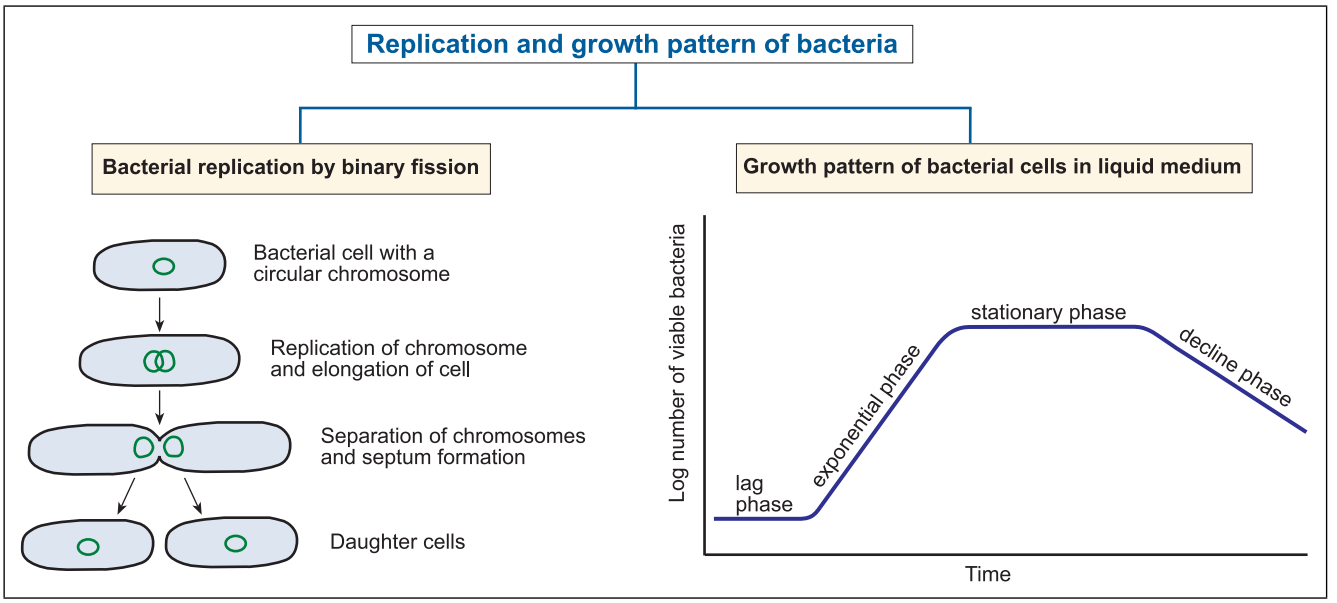
Motile bacteria possess flagella, attached to the cell wall, which are usually several times longer than the bacterial cell and are composed of a protein called flagellin. Fine, straight, hair-like structures called pili or fimbriae, composed of the protein pilin, are attached to the cell wall of many bacteria. In many pathogenic Gram-negative bacteria, adhesins present at the tips of pili function as attachment structures for mammalian cells.

Dormant, highly resistant structures, termed endospores, are formed by some bacteria to ensure survival during adverse environmental conditions. The only genera of pathogenic bacteria which contain endospore-forming species are *Bacillus* and *Clostridium*. The resistance of endospores is attributed to their layered structure, their dehydrated state, their negligible metabolic activity and their high content of dipicolinic acid. Because endospores are thermostable, moist heat at 121°C for 15 minutes is required for their inactivation.

Structural features of a mature bacterial endospore



2 Cultivation, preservation and inactivation of bacteria



Appropriate conditions relating to moisture, pH, temperature, osmotic pressure, atmosphere and nutrients are required for bacterial growth. Bacteria replicate by binary fission. The generation time, the length of time required for a single bacterial cell to yield two daughter cells, ranges from 30 minutes to 20 hours. Long-term preservation of microorganisms usually involves freezing procedures. Heat treatment or chemicals can be used for inactivation of bacteria.

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. During the lag phase, bacterial cells are metabolically active but not dividing; binary fission of cells results in an exponential increase in numbers. Procedures which can be used for total cell counting include direct microscopy, electronic methods and real-time quantitative polymerase chain reaction (PCR)-based methods. Viable bacterial numbers can be determined by colony counting and by membrane filtration. Accurate cell counts may be required for specific purposes such as vaccine preparation and for bacteriological testing of water.

Bacteria acquire nutrients from their immediate environment. Nutrient media for the isolation of pathogenic bacteria are formulated to supply particular growth factors for particular groups of organisms. Most bacteria require carbon and nitrogen in relatively large amounts. Trace elements and certain growth factors such as vitamins are also essential for bacterial growth.

In addition to nutritional factors, growth of bacteria is influenced by genetic factors and by chemical, physical and other environmental influences. Growth of bacteria in culture is influenced by temperature, hydrogen ion concentration, availability of moisture, atmospheric composition and osmotic pressure. Most bacteria grow optimally at neutral pH. The majority of pathogenic bacteria can be grown aerobically on a nutrient agar medium at 37°C, close to the body temperature of humans and most domestic species. Bacteria with an optimal incubation temperature of 37°C are termed mesophiles and most pathogenic bacteria belong to this category. Based on their preference for particular levels of oxygen, bacteria can be assigned to four main groups, namely aerobes, anaerobes, facultative anaerobes and microaerophiles. A fifth group, capnophiles, are aerobic bacteria with a requirement for carbon dioxide. Anaerobic bacteria are unable to grow in an atmosphere containing oxygen. Strict anaerobes are cultured in tightly sealed jars in an atmosphere from which free oxygen has been removed.

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), freezing at –70°C and ultra-freezing in liquid nitrogen at –196°C. Freezing organisms in vials containing 20–30 porous polypropylene beads which can be removed and cultured singly is a convenient method of avoiding the need for repeated freezing and thawing of cultures. If properly used, these preservation methods can maintain organisms in a hypobiotic state for more than 30 years and ensure that the organisms remain unchanged and uncontaminated.

Sterilization is the method employed for the destruction of microorganisms on equipment used in microbiological and

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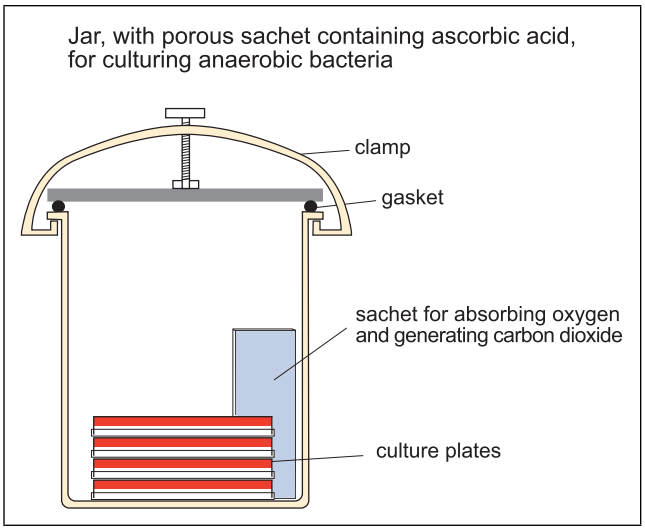


surgical procedures. Physical and chemical methods can be used for inactivation of microorganisms. Chemicals which inactivate bacteria include disinfectants and other compounds with bactericidal activity. Methods for preventing spoilage or limiting microbial growth in food are presented in Table 2.1. Physical methods for sterilizing equipment or fluids are presented in Table 2.2. Sterilization procedures are effective for the destruction of bacterial, fungal and viral agents. When dealing with bacterial endospores, such as those of *Clostridium* species, heating at a temperature of 121°C for 15 minutes in moist heat is required for their inactivation.

Table 2.2 Physical methods for sterilizing equipment or fluids; some can be used for disposing of contaminated material.

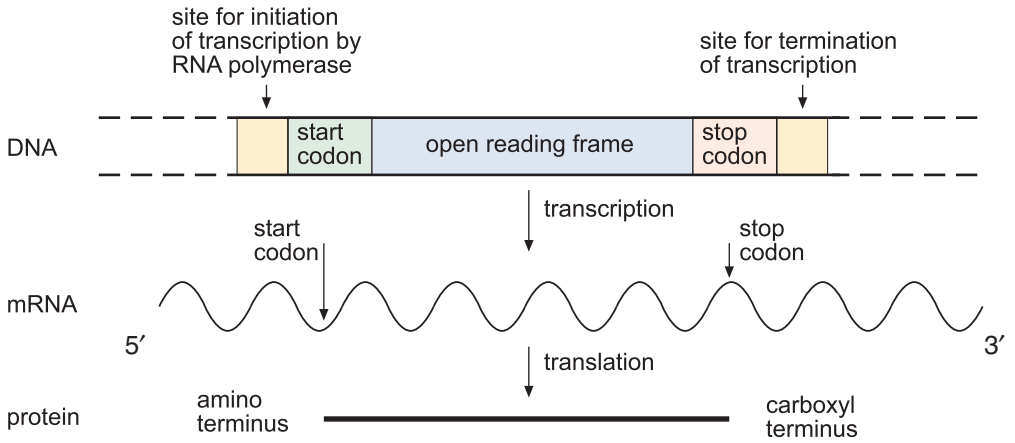
Method	Comments
Moist heat (autoclaving) employing steam under pressure to generate 121°C for 15 minutes or 115°C for 45 minutes	Used for sterilizing culture media, laboratory items and surgical equipment. Inappropriate for heat-sensitive plastics or fluids. Prions are not inactivated by this treatment
Dry heat in a hot-air oven at 160°C for 1–2 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 consequence
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
Ultraviolet (UV) light	Non-ionizing rays with poor penetration. Used in biosafety cabinets
Membrane filtration	Used for removing bacteria from heat-sensitive fluids such as serum and tissue culture media. Pore size of filter should be 0.22 μm or less

Table 2.1 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 temperatures
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 or located intracellularly, 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
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

3 Bacterial genetics and genetic variation

Sequence of events, from the initiation of transcription to protein formation, within a bacterial cell



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 and express characteristic traits or phenotypes.

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. A typical bacterium consists of a core genome, mainly composed of genes located on the chromosome consisting of double-stranded DNA, and an accessory genome comprising plasmid and bacteriophage 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 open reading frames (ORFs) coding for 4,126 bacterial proteins. Bacterial chromosomes typically contain sufficient DNA to encode between 1,000 and 4,000 different genes. Individual genes consist of a starting point, referred to as the start codon and composed of the nucleotides ATG, an ORF and a stop codon (TTA, TAG or TGA).

Although the bacterial chromosome exists free in the cytoplasm, it is compacted through supercoiling 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. As the DNA is located in the bacterial cytoplasm, this facilitates the simultaneous transcription and translation of bacterial genes. The gene sequence and its subsequent expression through diverse biochemical pathways accounts for the phenotypic variation observed among bacteria. Recently, these specialized topics have given rise to defined areas of research, referred to as genomics, functional genomics or transcriptomics, and proteomics.

Bacteria replicate by binary fission and 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*. The two parental strands of the helical DNA unwind under the influence of the enzyme DNA helicase and two identical helical DNA molecules are formed through the action of the replicating enzyme, DNA polymerase. The ends of the newly synthesized strands are joined by DNA ligase, resulting in circular chromosomes.

Transcription and translation, the expression of genetic information

Transcription is an enzyme-mediated process that involves DNA being copied from the positive strand, forming an mRNA molecule. This process 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. The information encoded in the mRNA is translated into protein on a ribosome through the involvement of transfer RNA (tRNA), which delivers specific amino acids to the mRNA on the ribosome where the amino acids are enzymatically joined together, forming a peptide bond and extending the polypeptide chain.

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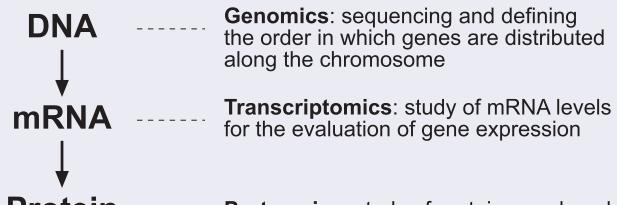
Sequential steps in synthesis of proteins following gene expression in DNA; associated areas of research are indicated



Genetic variation may occur following mutation in which a change occurs in the nucleotide sequence of a gene, or by recombination, whereby new groups of genes are introduced into the genome. 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 that may be beneficial or harmful for the organism. Under defined environmental conditions, selected mutations may provide a 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 which 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.

DNA may become damaged following contact with mutagenic chemicals, exposure to UV irradiation and by other means. Different mechanisms are available within the cell to organize the repair of damaged DNA and the choice of the appropriate method depends on the type of damage requiring correction.

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.

The transfer of genetic material in the form of plasmids of various sizes during conjugation is a complex process that has been extensively studied in the enteric bacterium *Escherichia coli*. During conjugation, F^+ (male) bacteria synthesize a modified pilus, the F or sex pilus. This pilus allows direct contact to occur between the male (F^+) and a suitable female (F^-) bacterium during the process and provides a conduit through which a plasmid or an F-factor can be transferred. One strand of plasmid DNA is unwound and passed to the recipient female (F^-) bacterium in which a complementary strand is later synthesized. Once a new plasmid is formed, the recipient cell is converted into an F^+ bacterium. Individual bacteria may contain several different types of compatible plasmids.

Plasmid transfer by conjugation has important ecological significance, particularly when antibiotic resistance-encoding genes are involved. A plasmid containing an antibiotic resistance gene in a bacterial cell can, under appropriate conditions, convert the amenable bacterial population into similar plasmid-containing bacterial cells.

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.

Transformation is a process involving 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 only in a few bacterial genera.

Examples of mobile genetic elements

Plasmids

Although most bacteria carry all 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 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 of the bacterium, but they may offer a selective advantage under certain conditions, such as the ability to conjugate and transfer genetic information, encode resistance to antibiotics, produce bacteriocins and synthesize proteins inhibitory to other bacteria (Table 3.1). All plasmids carry the genes required for their stable maintenance. In some pathogenic bacteria, plasmids encode virulence factors and antibiotic resistance.

Plasmids that can coexist in the same host bacterium are referred to as compatible, whereas those that cannot are defined

Table 3.1 Virulence factors of pathogenic bacteria encoded by defined genetic elements.

Pathogen	Virulence factors / Genetic elements
Bacillus anthracis	Toxins, capsule / plasmids
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

as incompatible. Incompatibility (Inc) group typing of plasmids has identified several different incompatibility groups in the *Enterobacteriaceae*.

The number of copies of a plasmid may vary, with some present in high numbers. 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 in the previous section. The broad host range of some plasmids, together with their ability to be transferred, contributes to their 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 of particular significance in veterinary medicine. This correlates with the use of drugs for growth promotion in some instances and treatment of infectious diseases in animals. Importantly, in some circumstances, this may have an impact on human health where resistant zoonotic bacteria such as *Salmonella* and *Campylobacter* may be transferred to humans via the food chain.

Bacteriophages

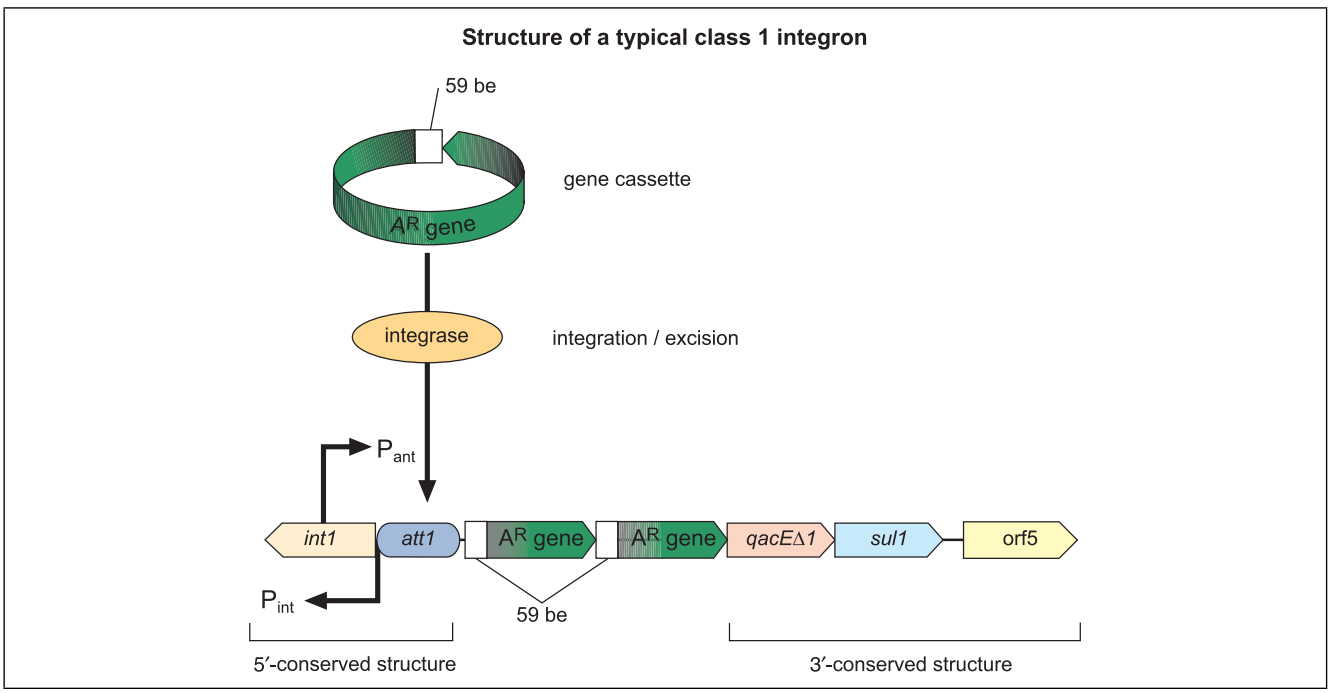
Viruses that infect bacteria are termed bacteriophages or phages. Depending on their mode of replication, phages may be either virulent or temperate. Most phages attack a small number of strains of related bacteria and therefore can be described as having a narrow and specific host range. Virulent phages 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, conferring additional properties on the host cell. The production of neurotoxins by certain types of *Clostridium botulinum* is associated with lysogenic conversion of host cells (Table 3.1).

Insertion sequences and transposons

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 do not possess an origin of replication and consequently replicate as the bacterial host replicates. Transposons encode the necessary features to promote self-mobilization. An example of a simple transposon is an insertion sequence element, denoted as IS, that contains only a transposase-encoding gene required for insertion into new locations. Several IS elements are known and these differ in the numbers of nucleotides they contain. 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, flanked by two IS*50* elements, IS*50L* and IS*50R*, as in Tn*5*. Other transposons such as Tn*3* encode a β-lactamase gene along with other transposase genes (*tnpA* and *tnpR*) required to catalyse the molecular events involved in integration. The complex transposon Tn*1546* encodes genes conferring resistance to the glycopeptide antibiotics vancomycin, teicoplanin and the formerly used growth promoter avoparcin.

Integrons 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 ORFs encoding antibiotic



resistance gene(s) and a 59-base recognition sequence located at their 3′-end.

Integrons capture a variety of genes encoding resistance to antibiotics such as aminoglycosides and β-lactams, among others, and contribute to the mobilization of these integrons in response to environmental selective pressure. Some integrons possess multiple gene cassettes arranged in a classical 'head-to-tail' orientation.

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 cloning vectors, 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 4).

Genetic engineering is currently used for the production of vaccines, hormones and other pharmaceutical products (see Chapter 78). 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 associated with the parent organism. Genetically engineered vaccines may therefore stimulate an effective immune response without the risk of introducing a pathogen capable of replicating in animals which are being vaccinated.

Genetic databases and bioinformatics

In 1977, the entire DNA sequence of the phage ΦX174 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 high-throughput 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 by manual methods these vast amounts of data. This has necessitated the development of specific computational tools to analyse DNA information and identify genes and their corresponding protein 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 ORFs within the genome, leading to the identification of genes encoding virulence factors associated with 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.

4 Molecular diagnostic methods

Summary of molecular methods for detection of diagnostic biomarkers

DNA	RNA	Protein
Molecular hybridization - Southern blotting DNA sequencing PCR DNA fingerprinting Microarrays Whole genome sequencing	Molecular hybridization - northern blotting - *in situ* analysis (FISH) RT-PCR Microarrays	Molecular hybridization - western blotting Protein sequencing Mass spectrum analysis Protein microarrays

Most of the characteristics of living bacteria are associated with the genes present on their bacterial chromosome. This structure consists of a double-stranded DNA helix, with all the properties required to control replication of the bacterium, store its genetic information and express some characteristics unique to the organism. All these properties are controlled by specific enzymes and the genetic message is, in some instances, subsequently decoded in a process involving other enzymes, leading to the synthesis of a bacterial protein (see Chapter 3).

The properties of DNA used for analytical purposes derive from its chemical structure. These have been used to develop many of the modern protocols for detection of bacterial pathogens in clinical specimens. A DNA molecule has three important analytical features that facilitate its utilization as a diagnostic target:

- Recognition properties: base-pairing rules in DNA underpin the analytical approach of molecular techniques including DNA probe hybridization, DNA sequencing, the polymerase chain reaction (PCR) and, more recently, microarrays.
- Stability and robust flexibility: the DNA molecule is inherently stable, which facilitates its recovery from degraded material.
- Sequence features: when the DNA sequence of any cell is closely examined, open reading frames (ORFs) encoding genes and other features can be determined.

Molecular hybridization

Any labelled DNA probe, under suitable experimental conditions, would be capable of binding or hybridizing to its complementary strand in solution (based on the DNA base-pairing rules). It is this binding event that is subsequently detected. Examples of molecular hybridization techniques include Southern and northern blotting.

DNA sequencing is 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 subsequently 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, or the ORF, can be determined using either a chemical- or an enzyme-based approach. The technical principles of the latter method on which modern dideoxy DNA sequencing protocols are based involves the partial replication of a short DNA sequence using all four deoxyribonucleotides (dNTP) and a chemically modified dideoxyribonucleotide (ddNTP) lacking a hydroxyl group at the 2′-carbon on the ribose sugar ring. Like hybridization, this method is based on sequence recognition according to the base-pairing rules and accurate enzymatic synthesis, all of which are features of the naturally occurring replication event. To sequence a DNA molecule the following steps are usually required: primer hybridization, sequence reaction, detection and data analysis.

In a later development of this technology, fluorescent-based automated DNA sequencing was designed to reduce the manual manipulations involved whilst increasing sample throughput. More recent advances in DNA sequencing technology have produced instrumentation capable of sequencing a bacterial genome in a few hours (see Chapter 6).

The PCR assay was developed out of the strategies used for DNA sequencing. Typically a 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, see following paragraphs). In the first of these, the template DNA, which has been recovered 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. This is followed by an annealing step, wherein the reaction temperature is lowered, allowing two synthetic DNA primers or oligonucleotides to bind (hybridize) to the template. These primers are located on opposite DNA strands. Finally, the temperature is increased again (typically, 74°C) and a thermostable DNA polymerase enzyme begins a round of synthesis. These steps constitute one cycle and in a conventional PCR reaction up to 30 such cycles are carried

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out. This repetitive cycling between temperatures facilitates the amplification of a specific DNA target by up to one million-fold.

A programmable thermal cycler controls the rate of temperature change, length of incubation at each temperature and the number of times each cycle is repeated. Multiple cycles produce an amplified PCR product, or amplicon, that can be detected by conventional agarose gel electrophoresis, stained with ethidium bromide and visualized using ultraviolet light.

Conventional PCR-based assays have been developed to detect a broad range of target genes in pathogenic bacteria associated with animals, including food-borne zoonotic pathogens. Commercial kits are also available for these and other pathogenic organisms.

A potential limitation of DNA-based diagnostic methods is that they detect both viable and non-viable bacterial 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. These assays can also be used to detect RNA deriving from viruses such as rotavirus, coronavirus and norovirus.

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 number of a specific DNA target, such as a virulence gene of veterinary importance, relative to a housekeeping gene, such as 16S rRNA, within a living cell. Quantitative real-time PCR (qPCR) can be used to quantify bacteria, other microorganisms and individual genes. 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.

Expression of any gene in a microorganism or other cell can be determined by measuring the mRNA transcription, using RT-PCR. This technique is referred to as quantitative RT-PCR (qRT-PCR). Based on this protocol, commercial kits are now available to detect and quantify a range of pathogenic organisms relevant to veterinary medicine.

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 can be attached. 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 culturing conditions, including aerobic versus anaerobic environmental conditions. In environmental microbiology, DNA microarrays containing 16S rDNA sequences can be used to identify bacteria 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*. 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 environmental niches.