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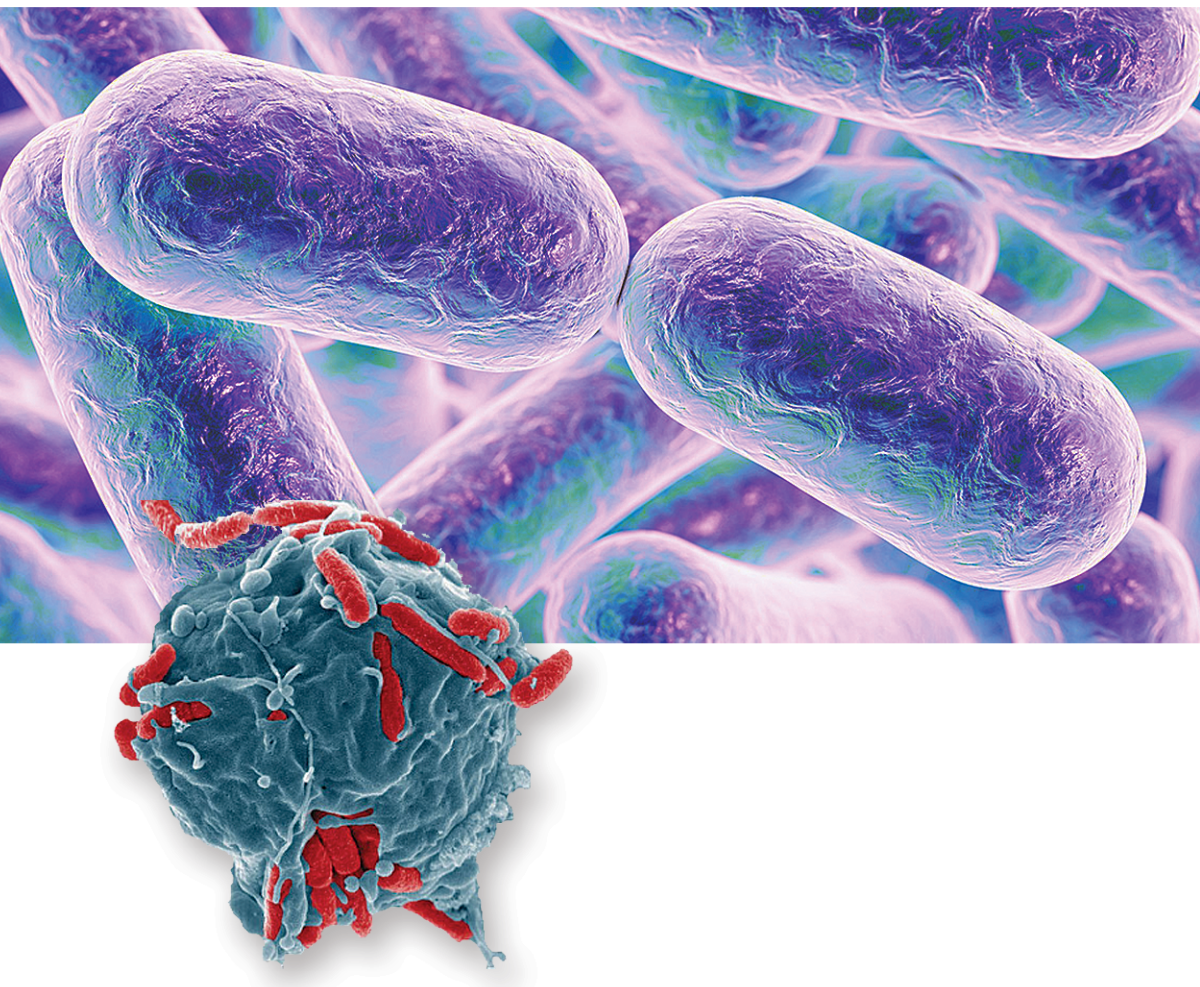
HU Jianhe • HANG Bolin • XU Yanzhao • SUN Yawei

ANIMAL MICROBIOLOGY



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Preface

With the rapid development of modern science and technology, achievements in scientific research are changing with each passing day, and knowledge information is increasing with explosive growth. Given this trend, animal microbiology develops continuously with a lot of new results in theories, knowledge and technology, of which many important results were published in English in international journals. In order to meet the requirement of social and discipline development, meet the needs of teaching and learning, improve the teaching quality and talent training quality of agriculture and forestry colleges in our country, and promote and facilitate professional academic exchanges internationally, the textbook of *Animal Microbiology* in English as a reference book for bilingual or English-only teaching in agriculture and forestry colleges was written and edited with advanced and innovative writing ideas.

This English textbook basically in accord with Chinese textbooks on general framework focused on basic theories, basic knowledge, basic skills, and took into account of scientificity, systematization and practicability. This English textbook had plenty of contents with comprehensive, systematic and complete body of knowledge. And review questions in each chapter are convenient for students to preview and review. When using this textbook, teachers could select teaching contents and methods according to their respective syllabus and local animal epidemic.

In the process of writing and editing, selected key content of professional teaching were collated with references of English books, journal articles and authoritative websites. But many references can't be listed one by one because of the limited space in this textbook. Therefore, we make sincerest apologies and thanks to those authors and the copyright holders. At the same time, this textbook must have deficiencies, not only because the English level and professional level of writers are limited, but also new methods and technology are developing constantly, and new concepts and ideas are springing up constantly. We would genuinely thank you for your criticism and corrections.

Hu Jianhe
Xinxiang, China
June 2017

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Introduction

Synopsis

Microorganisms are microscopic creatures that are invisible with naked eyes and need to be seen via a microscope. On the species, microorganisms are divided into prokaryotic cell type (including bacteria, spirochaeta, mycoplasma, rickettsia and chlamydia), eukaryotic cell type microorganisms (including yeast and mold) and non-cell type (including virus and subviral agent). Microbiology is a branch of life science, which researches on morphology, structure, physiology, biochemistry, heredity, variation, classification, distribution and relationship with nature, etc. The history of microbiology has experienced the period of germination, morphology, physiology and immunology, and modern microbiology. Microbiology has achieved theoretical and technological milestones in different periods. This chapter also introduces the branch of microbiology and the effect of microorganism on human beings.

Microbiology is one branch of the biology, which primarily researches on the microorganisms or microbes that are unicellular or cell-cluster microscopic organisms and too small to be seen with naked eyes. Microorganisms include eukaryotes, prokaryotes and viruses. Prokaryotic cell type microbes include bacteria, spirochaeta, mycoplasma, rickettsia and chlamydia. Eukaryotic cell type microorganisms include yeast and mold. Non-cellular microorganisms include viruses and subviral agents. Microbiology is mainly a study of microbial morphology and structure, physiology and biochemistry, heredity and variation, classification, distribution and its relationship with the nature.

Microbiology is advancing continually. We have only studied about one percent of all microbe species on the Earth. Compared with older biological disciplines such as zoology and botany, microbiology could be considered in its infancy, because microbes were first observation of microorganisms occurred about three hundred years ago.

1 History of Microbiology

1.1 Ancient Stage

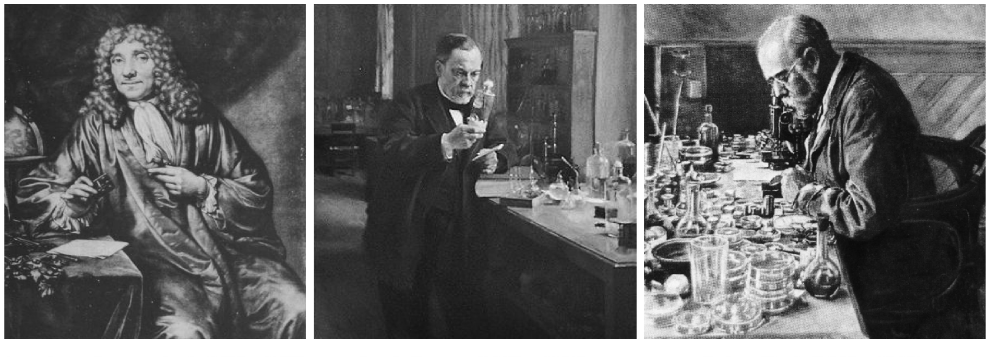
Microorganisms were deemed to be in existence before they were actually discovered

in the 17th century. In 600 BCE, Susruta, a surgeon of ancient Indian, thought that microorganisms were answered for several diseases that could be spread via contact, air or water, which were recorded in *Susruta Samhita*. Roman scholar Marcus Terentius Varro recorded his theories concerning microorganisms in *On Agriculture* (the title of a book), and he proposed that people should be aware of a homestead near to swamps, "…and because there are bred certain minute creatures which cannot be seen by the eyes, which float in the air and enter the body through the mouth and nose and there cause serious diseases." This indicates that the ancient man had known diseases might be transmitted by organisms which couldn't be seen with naked eyes. In *The Canon of Medicine* (1020), Avicenna supposed the contagious features of tuberculosis and other infectious diseases, and applied quarantine as a method to limit the transmission of infectious diseases. During the occurrence of the Black Death in the 14th century in Al-Andalus, Ibn Khatima said that "minute bodies" might cause infectious diseases when they entered the human body. In 1546, Girolamo Fracastoro deemed that transferable seed like entities could cause epidemic diseases, which could be transmitted by direct or indirect contact.

Early narrations of the existence of microorganisms were not confirmed, because there were no any data or science to verify their existence. The main reason might be the lacking of the application of fundamental tools (e.g., microscope) for researching microbiology.

1.2 Modern Stage

In 1676, Antonie van Leeuwenhoek (Fig. 0-1) firstly observed bacteria with a single-lens microscope designed by himself. He named them "animalcules", and reported his findings to the Royal Society. He opened the door of the microbiology. Antonie van Leeuwenhoek is considered as the first microbiologist to observe and



Antonie van Leeuwenhoek (1632—1723) Louis Pasteur (1822—1895) Robert Koch (1843—1910)

Fig. 0-1 Several famous microbiologists (贾文祥, 2008)

describe microorganisms using a microscope, and regarded as "the Father of Microbiology", though Robert Hook observed the fruiting bodies of molds earlier in 1665. Ferdinand Cohn (1828—1898) who once described several bacteria (e.g., *Bacillus* and *Beggiatoa*) is the founder of the bacteriology (an offshoot of microbiology), and he firstly drew up a system for the taxonomy of bacteria. Louis Pasteur and Robert Koch, contemporaries of Ferdinand Cohn, are often deemed the creators of medical microbiology.

Louis Pasteur (Fig. 0-1) is a most famous microbiologist, because he disproved the theory of spontaneous generation that is widely popular with famous flask experiment (Fig. 0-2). Thereby, this event affirmed that microbiology is a kind of biological sciences. Pasteur also set up pasteurization for food preservation and methods for vaccines to prevent several diseases (e.g., fowl cholera, anthrax and rabies).

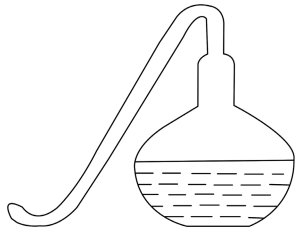


Fig. 0-2 Flask used by Pasteur(陆承平, 2013)

Robert Koch (Fig. 0-1) is best known for his achievements to the germ theory of disease that specific pathogenic microorganisms caused specific diseases. In 1905, Koch was awarded the Nobel Prize, because he attested the germ theory when he researched on tuberculosis. Koch focused on the bacterial isolation and pure culture which led him to find several novel bacteria (e.g., *Mycobacterium tuberculosis*, the pathogen of tuberculosis). He proposed a criterion, namely the Koch's postulates, for affirming that a kind of microorganism is the reason for a unique infectious disease. The contents of Koch's postulates are following: ①the microorganism should exist abundantly in all cases, but not in healthy animals; ②the microorganism should be isolated from sick cases and grow in culture medium; ③the cultured microorganism should cause the same disease after entering into a healthy animal; ④the microorganism should be re-isolated from the experimental animal and identified as being same with the original microorganism. Though these postulates have some shortcomings, its soul idea is still used nowadays.

Though Pasteur and Koch are considered as the founders of microbiology, their achievements did not wholly reflect the true microbial world. When Martinus Beijerinck (1851—1931) and Sergei Winogradsky (1856—1953) founded general microbiology, the true microbial world was revealed. Martinus Beijerinck discovered

viruses (tobacco mosaic virus), proposed several basic principles of virology, and developed enrichment culture techniques which had important impact on microbiology, thereby much more microbes could be cultured. Sergei Winogradsky developed the conception of chemolithotrophy and revealed the role of microbes in geochemical processes. He firstly isolated and described nitrobacteria and azotobacteria.

1.3 Recent Stage

DNA or RNA as genetic materials were revealed, which pushed the study of microbiology to the molecular level, and now microbiology is in the era of genetic engineering. Antibiotics play vital roles in curing the infectious diseases of human and animal, and people also realize the hazards of antibiotics to the health of humans and animals, such as drug residue, drug tolerance and environmental damage. The concept of antigen breaks through the range of microorganisms, and spreads to the whole biology. Since then immunology has become an independent discipline. In 1977, Carl Woese (1928—2012) proposed the three-domain system according to the sequence of 16S ribosomal RNA (rRNA). In this system, the prokaryotes are divided into two evolutionary domains, the archaea and the eubacteria.

With the electron microscope, we can observe the subcellular and/or molecular structure of microbes. Labeled-antigen or -antibody become an important tool for the diagnose of infectious disease. Cell culture, plague method and the pure methods of protein and nucleic acids accelerate the development of microbiology. Many techniques including molecular cloning, polymerase chain reaction (PCR), sequencing technique of protein and nucleic acids, genomics, proteomics, metabolomics, computer technique are pushing the wheel of microbiology forward.

2 Fields of Microbiology

The fields of microbiology include: microbial physiology, which studies the biochemical functions of microbial cells, such as growth, metabolism and cell structure; microbial genetics, which studies the organization and functions of microbes, is closely associated with molecular biology; cellular microbiology, which is a bridge between microbiology and cell biology; medical microbiology, which studies pathogenic microbes and its role in humans; animal microbiology, which studies the role of microbes related to animals; environmental microbiology, which studies the functions and diversities of microorganisms in natural environments; evolutionary microbiology, which studies the evolutions of microorganisms, includes the taxonomy of bacteria and viruses; industrial microbiology, which applies microbes in industry sector that includes fermentation and the treatment of waste water; aeromicrobiology, which

studies the airborne microorganisms; food microbiology, which studies the microorganisms in food spoilage, foodborne diseases, and food production using microbes; pharmaceutical microbiology, which studies the microorganisms in pharmaceutical contamination and spoilage, drug production using microbes; etc.

3 Benefits of Microbiology

Though some microbes could cause various infectious diseases in humans, animals and plants, many microorganisms have numerous beneficial roles, such as fermentation, antibiotic production. Scientists have applied microorganisms to prepare important materials (e.g., *Taq* polymerase), and develop novel molecular biology techniques (e.g., the yeast two-hybrid system).

Microorganisms are applied for producing more materials used in many realms (e.g., industrial or food sectors). *Corynebacterium glutamicum* can produce amino acids (mainly L-glutamate and L-lysine). Some kinds of microorganisms can produce various bio-polymers (such as polysaccharides, polyesters and polyamides), especially tailored bio-polymers with biotechnology used in tissure engineering or drug delivery. Non-pathogenic clostridia can enter and grow in solid tumors. Therefore, these avirulent clostridia can be used as vectors to deliver therapeutic proteins after entering bodies via administration. Microorganisms are applied in the field of biodegradation or bioremediation. The ability to degrade toxic waste is reliant on the features of contaminant contacted by microorganisms.

Bacteria which are helpful to the digestive system are called as probiotics, while substances which can facilitate the growth of probiotic microorganisms are called as prebiotics. Both are beneficial to human and animal health.

Review Questions

1. Term explanation: microorganism, microbiology.
2. Try to describe the contribution of microbiology to the development of human society.
3. What are the characteristics of microorganisms?
4. Please briefly describe the major contributions made by two prominent microbiologists in the development course of microbiology.

Ⅰ. BACTERIOLOGY

Chapter 1 Morphology and Structure of Bacteria

Synopsis

Bacteria are observed with a microscope, because they are very small. According to the results of Gram staining, bacteria are divided into two groups, namely Gram positive and Gram negative. The basic form of bacteria is mainly spherical, rod-shaped and helix. The unit for the size of individual bacteria is usually micron (μm). Bacteria have a certain of arrangement ways. The basic structure of bacteria includes cell wall, cell membrane, cytoplasm and nucleoid. The Gram positive bacteria have thicker peptides and unique teichoic acids. The Gram negative bacteria have the outer membrane which has specific lipid polysaccharide, while peptide polysaccharide under the outer membrane is thin. The special structures of bacteria include capsule, flagella, pili and spores. These special structures have special construction and physiological functions, of which are related to pathogenicity and immunity.

The bacterium (plural: bacteria) is a large category of small unicellular microorganisms. Bacteria have characteristic shapes that are used for identification and classification, including round, rods, vibrio, spirilla and spirochete. Moreover, the diversity of bacterial size largely relies on their shapes. The study of bacteria is named bacteriology, a branch of microbiology.

Bacteria are now classified as prokaryotes. Unlike animals and other eukaryotes cells, bacterial cells do not contain a nucleus and rarely harbour membrane-bound organelles. Although all prokaryotes traditionally were belonging to the bacteria, the scientific classification changed after the discovery in the 1990s that prokaryotes consisted of two very different groups of organisms evolved independently from an ancient common ancestor. These evolutionary fields are called bacteria and archaea.

Bacteria, despite their simpleness, contain a well-developed cell structure which is carried out many of their unique biological properties. Many structural features are special to bacteria and are not discovered among archaea or eukaryotes. Because of the simplicity of bacteria in comparison to larger organisms and the ease with which they can be performed experimentally, the cell structures of bacteria have been well researched, showing

that many biochemical principles have been subsequently as same as other organisms.

1 Cell Morphology

Perhaps the most basic structural feature of bacteria is cell morphology (shape). Bacteria show a wide diversity of shapes and sizes, which is called morphologies.

Cell shape is generally determined by a given bacterial species, but can vary depending on growth conditions. Bacteria generally develop distinctive cell morphologies with light microscope and distinct colony morphologies when grown on plates. These are often the first characteristics observed by a microbiologist to identify and classify an unknown bacterial culture.

Typical figures include: round (cocci), rod (*Bacillus*), vibrio (a curved rod), spirilla (corkscrew shaped), and spirochete (a flexible wavy shape) (Fig. 1-1). The majority of bacterial species are either spherical, called cocci (singular: coccus) or rod-shaped, called bacilli (singular: *Bacillus*). Some *Bacillus* are slightly curved or comma-shaped; others, can be spiral-shaped, called spirilla, or tightly twisted, called spirochetes. A minority of species even have tetrahedral or cuboid shapes. Recently, bacteria were discovered deeply under the earth's crust that developed as long rods with a star-shaped cross-section. The large surface area to volume ratio of the morphology may give these bacteria an advantage in nutrient-poor environments. This wide variety of shapes is decided by the bacterial cell wall and cytoskeleton, and is important because it can affect the ability of bacteria to gain nutrients, attach to surfaces, swim through liquids and avoid predators.

Many bacterial species present simply as single cell, while others connect with characteristic patterns: *Streptococcus* form chains, and *Staphylococcus* group associate in "bunch of grapes" clusters. Bacteria can also be elongated to produce filaments, e.g., the actinomyces. Filamentous bacteria are often surrounded by a sheath in which have many individual cells. Certain types, such as species of the genus *Nocardia*, even create complex, branched filaments, similar to the appearance of fungal mycelia.

Bacteria often attach to surfaces and create dense aggregations called biofilms or bacterial mats. These films can vary from a few micrometers in thickness to up to half a meter in depth, and may include multiple species of bacteria, protists and archaea. Bacteria living in the biofilms show a complex arrangement of cells and extracellular components, producing secondary structures such as microcolonies, through which there are networks of channels to diffuse better nutrients. In natural environments, such as soil or the surfaces of plants, most of bacteria are bound to surfaces in biofilms. Biofilms are also significant in medicine, as these structures are often occurred during chronic bacterial infections or in infections of implanted medical devices, and bacteria protected within biofilms are much harder to eliminate than individual isolated bacteria.

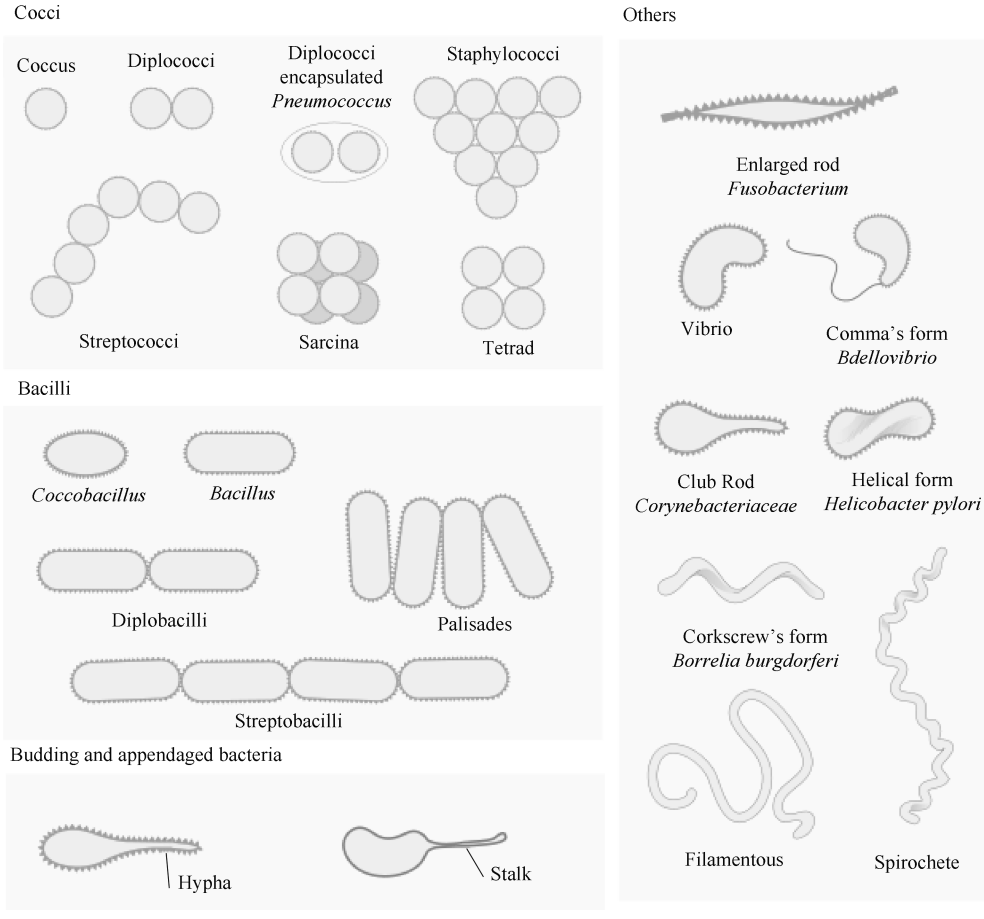


Fig. 1-1 Morphology of bacteria (https://en.wikipedia.org)

2 Cell Size

Perhaps the most remarkable structural characteristic of bacteria is (with some exceptions) their small size (Fig. 1-2). Take the example of *Escherichia coli*, an "average" sized bacterium, that is length of between 2 micrometres (μm) and 0.5μm in diameter, with a bacterial cell volume of 0.6-0.7μm^3. Bacterial cells are about one-tenth the sizes of eukaryotic cells and are typically 0.5-5.0μm length. However, a few species, such as *Thiomargarita namibiensis*, are up to half a millimetre (mm) long and are visible to the unaided eye. Some bacteria may be even smaller, like nanobacteria, but these ultramicrobacteria are not well-researched.

Small size is very important as it allows for a large surface area-to-volume ratio, which is responsible for rapid absorption and intracellular distribution of nutrients and

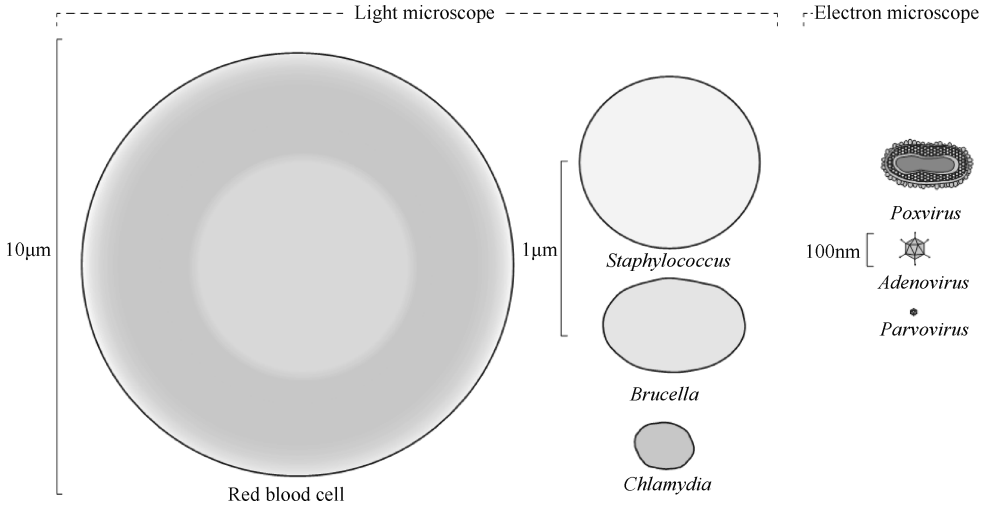


Fig. 1-2 Relative sizes of a red blood cell, bacteria and viruses (Markey *et al.*, 2013)

excretion of waste matter. At low surface area-to-volume ratios, the diffusion of nutrients and waste matter across the bacterial cell membrane affect the rate at which microbial metabolism can happen, making the cell less evolutionarily fit. The cause of the existence of large cells is unknown, although it is assumed that the increased cell volume is used mainly for storage of excess nutrients.

3 Structures of Bacteria

Bacteria contain extracellular structures and intracellular structures (Fig. 1-3). Extracellular structures include fimbriae, pili, S-layer, capsules and flagella. The assembly of extracellular structures is decided by bacterial secretion systems. These transfer proteins from the cytoplasm enter into the periplasm or into the environment around the cell. Many types of secretion systems are already known and these structures are often necessary for the virulence of pathogens, so they are intensively studied. However, we just talk a lot about the basic structure and special structure of the bacterial in this chapter.

3.1 Basic Structure of Bacteria

3.1.1 The Bacterial Cell Wall

Around the outside of the cell membrane is the bacterial cell wall. Bacterial cell wall is made of peptidoglycan (or called murein), which is composed of polysaccharide chains cross-linked by unusual peptides containing D-amino acids. Bacterial cell wall is distinguished from the cell walls of plant and fungus, which are made of cellulose

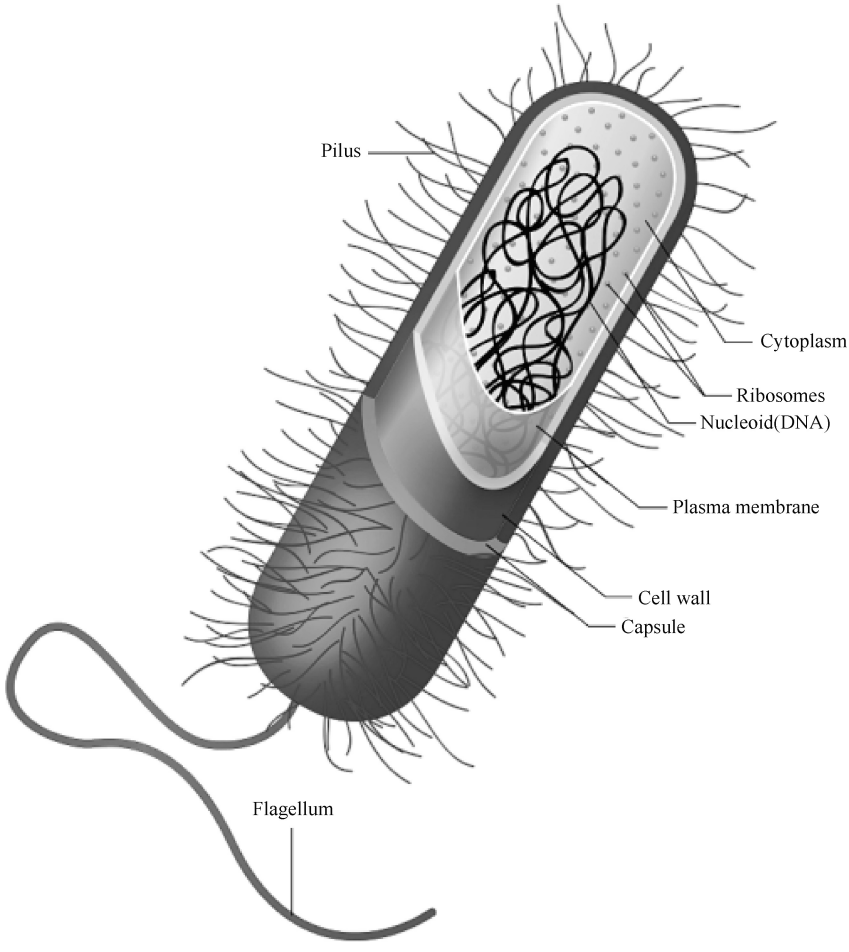


Fig. 1-3 Structure and contents of a Gram positive bacterium (https: //en.wikipedia.org)

and chitin, respectively. The cell wall of bacteria is also distinguished from that of archaea which does not contain peptidoglycan. The cell wall is important to the survival of many bacteria, and the antibiotic penicillin can kill bacteria by hindering a step in the synthesis of peptidoglycan.

As in other organisms, the cell wall of bacteria provides structural integrity to the cell. In prokaryotes, the main function of the cell wall is to defend the cell from internal turgor pressure resulted from the much higher concentrations of proteins and other molecules inside the cell compared to its outside environment. The bacterial cell wall is different from that of all other organisms in the presence of peptidoglycan [*N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM)], which is located directly outside of the cytoplasmic membrane. The function of peptidoglycan is to enhance the rigidity of the bacterial cell wall and the determination of cell shape. It is

relatively porous and is not regarded as a permeable barrier for small substrates. Although all bacterial cell walls (exception of *Mycoplasma*) contain peptidoglycan, not all cell walls have the same cell structure.

Based on cell wall structure, bacteria are either Gram positive or Gram negative, which are distinguished by their Gram staining characteristics respectively. For both Gram positive and Gram negative bacteria, about 2nm particles can pass through the peptidoglycan. The cell wall of Gram positive bacteria has many layers thick and consists of several diagnostic molecules such as teichoic, teichuronic acid. In contrast, the cell wall of Gram negative bacteria is much thinner, which is only one to three layers of peptidoglycan surrounded by a lipid-based outer membrane consisting of lipopolysaccharide and lipoproteins. These differences in cell wall structure can result in differences in antibiotic susceptibility. For example, vancomycin can kill only Gram positive bacteria and is ineffective against Gram negative bacteria, such as *Haemophilus influenzae* or *Pseudomonas aeruginosa*.

3.1.1.1 The Gram Positive Cell Wall

The Gram positive cell wall is characterized by the presence of a very thick peptidoglycan layer (Fig. 1-4), which is used for the retention of the crystal violet dyes during the Gram staining procedure. Embedded in the Gram positive cell wall are polyalcohols called teichoic acids, some of which are lipid-linked to produce lipoteichoic acids. Because lipoteichoic acids are covalently linked to lipids within the

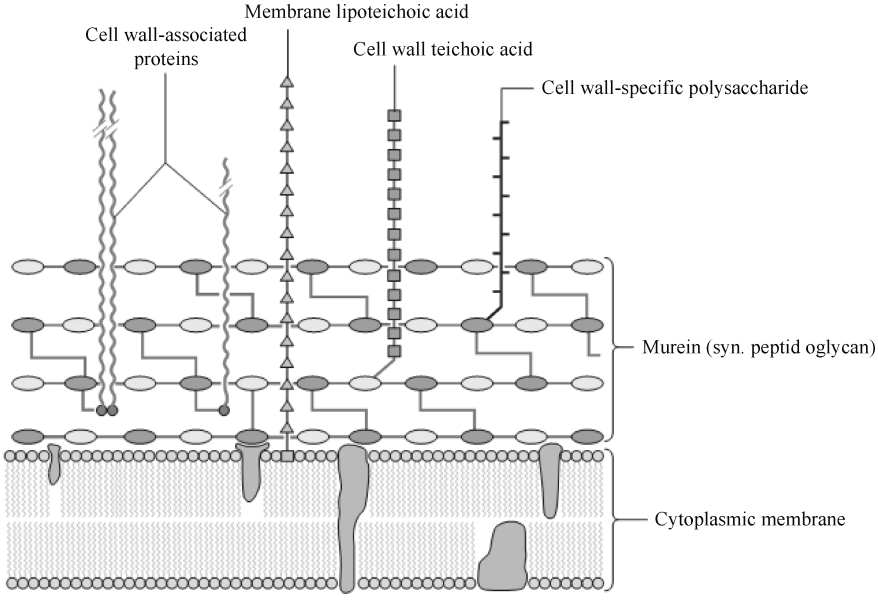


Fig. 1-4 Gram positive bacterial cell wall (Kayser *et al.*, 2005)

cytoplasmic membrane and they are responsible for linking the peptidoglycan to the cytoplasmic membrane. Teichoic acids provide the Gram positive cell wall general negative charge because of the presence of phosphodiester bonds between teichoic acid monomers.

3.1.1.2 The Gram Negative Cell Wall

In contrast to the Gram positive cell wall, the Gram negative cell wall contains a thin peptidoglycan layer next to the cytoplasmic membrane (Fig. 1-5). This is contributed to the cell wall's inability to hold the crystal violet stain with decolourisation using ethanol during Gram staining. Besides the peptidoglycan layer (Fig. 1-6), the Gram negative cell wall also includes an outer membrane composed by phospholipids and

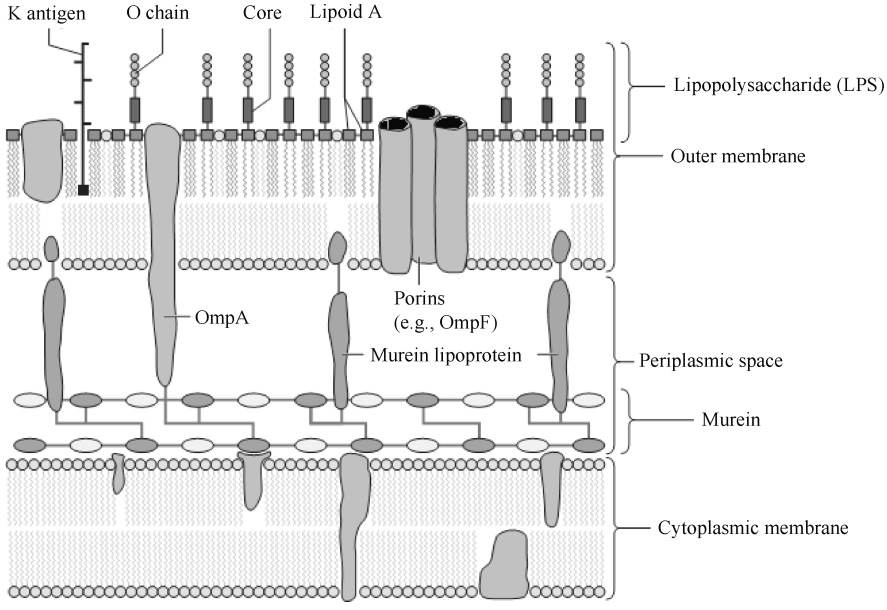


Fig. 1-5 Gram negative bacterial cell wall (Kayser *et al.*, 2005)

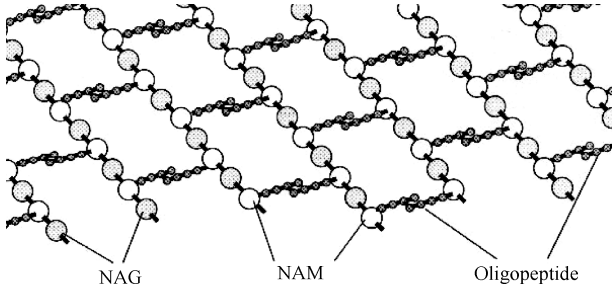


Fig. 1-6 The structure of peptidoglycan of Gram negative bacterial (https://en.wikipedia.org)

lipopolysaccharides, which face the external environment. Because the lipopolysaccharides are highly-charged, the Gram negative cell wall has general negative charge. For the chemical structure of the outer membrane, lipopolysaccharides are often unique to particular bacterial strains and carry out many of the antigenic properties of these strains.

3.1.2 The Bacterial Cytoplasmic Membrane

The bacterial cytoplasmic membrane (also called the cell membrane, plasma membrane or plasma lemma) is the biological membrane separating the interior of a cell from the exterior environment. It is consisted of a phospholipid bilayer and thus has all of the usual functions of a cell membrane, such as performing a permeability barrier for most molecules and locating for the transport of molecules into the cell.

In addition to these functions, prokaryotic membranes also have function in energy conservation as the position about which a proton motive force is produced. Unlike eukaryotes, bacterial membranes usually do not have sterols. However, many microbes do contain structurally related substances called hopanoids which probably fulfill the same function. In contrast to eukaryotes, bacteria can contain a wide variety of fatty acids inside their membranes. In addition to typical saturated and unsaturated fatty acids, bacteria can also contain fatty acids with additional methyl, hydroxy or even cyclic groups. The relative portions of these fatty acids can be moderated by the bacterium to stay the optimum fluidity of the membrane.

As a phospholipid bilayer, the lipid portion of the outer membrane is impermeable to charged molecules. However, channels called porins are present in the outer membrane, which permit passive transport of ions, sugars and amino acids across the outer membrane. These molecules are therefore found in the periplasm, the region between the cytoplasmic and outer membranes. The periplasm consists of the peptidoglycan layer and in which many proteins serve as substrate binding or hydrolysis and receiving of extracellular signals. The periplasm is considered as a gel-like state rather than a liquid, because the high concentration of proteins and peptidoglycan found within it. Because of its position between the cytoplasmic and outer membranes, signals received and substrates bound are applied to be transported across the cytoplasmic membrane using transport and signaling proteins embedded there.

Specific proteins embedded in the cell membrane can serve as molecular signals that let cells communicate with each other. Protein receptors are discovered ubiquitously and worked to transmit information about the extracellular environment to the interior of the cell. The information (signals) are transduced and passed in a different form into the cell. For example, a hormone binding to a receptor could open an ion channel in the receptor and let calcium ions to flow into the cell. Other proteins on the surface of the cell membrane act as “markers” that differentiate a cell from other cells. The interaction of these markers with their respective receptors develops the basis of

cell-cell communication in the immune system.

3.1.3 Intracellular Bacterial Cell Structures

In comparison to eukaryotes, the intracellular features of the bacterial cell are highly simple. Bacteria do not contain organelles in the same sense as eukaryotes. On the contrary, the chromosome and perhaps ribosomes are the only easily obvious intracellular structures found in all bacteria.

As prokaryote, bacteria do not incline to have membrane-bound organelles in their cytoplasm and thus few large intracellular structures within them. Namely, they are absence of a nucleus, mitochondria, chloroplasts and the other organelles showed in eukaryotic cells, such as the Golgi apparatus and endoplasmic reticulum. Bacteria were once regarded as simple bags of cytoplasm, but factors such as prokaryotic cytoskeleton and the specific localization of proteins within the cytoplasm have been found to reveal degree of complexity. These subcellular compartments have been known as “bacterial hyper structures”.

3.1.3.1 The Bacterial Chromosome and Plasmids

In contrast to eukaryotes, the bacterial chromosome is not enclosed inside of a membrane-bound nucleus but resided inside the bacterial cytoplasm. Their genetic material is typically a single circular chromosome located in an irregularly shaped body called the nucleoid. This means that the transduction of cellular information through the processes of translation, transcription and DNA replication all happen within the same compartment and can interact with other cytoplasmic structures, most obviously ribosomes. The bacterial chromosome is not packaged using histones to produce chromatin as in eukaryotes but exists as a highly compact supercoiled structure, the accurately nature of which remains unknown. Most bacterial chromosomes are circular although some species of linear chromosomes existed (e.g., *Borrelia burgdorferi*).

In addition to chromosomal DNA, most bacteria also contain a small DNA molecular, which can replicate independently in the cell, called plasmid. Plasmids always carry many genes, and many genes often encode for traits that are advantageous for the host cell. However, some plasmids have no remarkable effect on the phenotype of the host cell or its benefit to the host cells cannot be determined. Plasmids can be easily gained or lost by a bacterium and can provide a method of horizontal gene transfer between within population of microbes and given environments.

3.1.3.2 Ribosome and Other Multiprotein Complexes

In most bacteria, the most numerous intracellular multiprotein complex is the ribosome, meanwhile, it serves as the site of protein synthesis within all living organisms. However, the structure of the bacterial ribosome is different from those of eukaryotes

and archaea. All prokaryotes contain 70S (where S=Svedberg units) ribosome while eukaryotes contain larger ribosomes which is 80S in their cytosol. The 70S ribosome is composed of 50S and 30S subunits. The 50S subunit have the 23S and 5S rRNA, while the 30S subunit contains the 16S rRNA. These rRNA molecules are different in size in eukaryotes and are connected with a large number of ribosomal proteins, the number and type of which can vary slightly among organisms.

3.1.3.3 Intracellular Membranes

While not typical of all bacteria, some microbes not only contain intracellular membranes but also have their cytoplasmic membranes. An early thought was that bacteria might contain membrane folds termed mesosomes, while they were later shown to be artifacts generated by the chemical materials used to prepare the cells for electron microscopy.

3.1.3.4 Cytoskeleton

In prokaryotes, its cytoskeleton is the collective name for all structural filaments. It was once believed that prokaryotic cells did not have cytoskeletons. However, recent progression visualization technology and structure determination have revealed that filaments indeed exist in these cells. In fact, homologues for most cytoskeletal proteins in eukaryotes have been occurred in prokaryotes. Cytoskeletal elements play important roles in different prokaryotes, such as cell division and protection, shape determination, and polarity determination, and so on.

3.1.3.5 Nutrient Storage Structures

Usually most bacterial do not live in environments which contain large number of indispensible nutrients at all times. To supply these transient levels of nutrients, bacteria contain several different ways of nutrient storage in order to use in time. For example, many bacteria store superfluous carbon in the form of polyhydroxyalkanoates or glycogen for later use. In addition, some microbes also store soluble nutrients such as nitrate in vacuoles. Sulfur is often stored as basic granules which can be stored either intra- or extracellularly. Sulfur granules are particularly common in bacteria which use hydrogen sulfide as an electron source. The above mentioned usually can be viewed using a microscope and are surrounded by a thin non-unit membrane to separate them from the cytoplasm.

3.1.3.6 Gas Vesicles

Gas vesicles are spindle-shaped structures that found in some planktonic bacteria, which provide buoyancy to these organisms by decreasing their overall cell density. They are made up of a capsid, and which is very impermeable to solvents such as water

while permeable to most gases. By changing the amount of gas presented in their gas vesicles, bacteria can increase or decrease their general cell density and therefore move up or down within the water column in order to maintain their position in an environment optimal for growth.

3.1.3.7 Carboxysomes

Carboxysomes are intracellular structures discovered in many autotrophic bacteria. They that are similar to phage heads in their morphology possess protein structures and contain the enzymes of carbon dioxide located in these organisms (especially ribulose bisphosphate carboxylase/oxygenase, rubisco, and carbonic anhydrase). It is believed that the high local concentration of the enzymes together with the rapid conversion of bicarbonate to carbon dioxide by carbonic anhydrase allows faster and more efficient carbon dioxide fixation than possible inside the cytoplasm. Other similar structures are known to harbor the co-enzyme B12-containing glycerol dehydratase, the key enzyme of glycerol fermentation to 1,3-propanediol, which is in some Enterobacteriaceae (e.g., *Salmonella*).

3.2 Special Structure of Bacteria

3.2.1 Fimbriae and Pili

A pilus (Latin for "hair"; plural: pili) is a hair-like appendage located on the surface of many bacteria. The terms of pilus and fimbria (Latin for "thread" or "fiber"; plural: fimbriae) are often used interoperability, although some researchers retain the term pilus for the sexual appendage required for bacterial conjugation. All pili are mainly made up of oligomeric pilin proteins.

3.2.1.1 Fimbriae

Fimbriae are hollow protein tubes and extend out from the outer membrane in many members of the Proteobacteria (Fig. 1-7). They are generally short in length and show high numbers of them on the entire bacterial cell surface. Fimbriae are only 2-10nm in diameter and up to several micrometers in length. In addition, the composition of fimbriae is pilin. Usually fimbriae are distributed over the surface of the cell, and are similar to fine hairs due to outside vision field of light microscope, so which are viewed under the electron microscope. Fimbriae are most important to the virulence of some bacterial pathogens. The function of fimbriae is to facilitate the attachment of a surface of bacterium (e.g., to form a biofilm) or to other (e.g., animal cells during pathogenesis), which is required for colonization during infection or to initiate development of a biofilm. Mutant bacteria that lack fimbriae cannot adhere to their usual target surfaces and, therefore, cannot cause diseases.

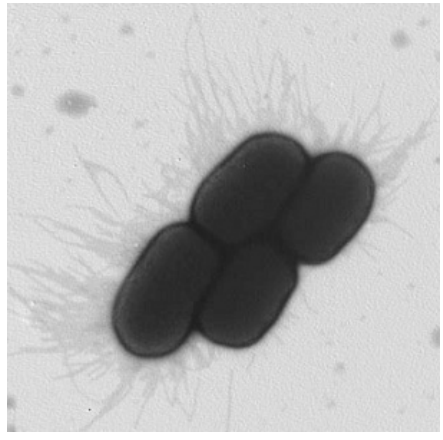


Fig. 1-7 Transmission electron micrographs of uropathogenic *E. coli* with fimbriae (Rosenberg *et al.*, 2013)

Some fimbriae can contain lectins. The lectins are essential to adhere to target cells, because they can identify oligosaccharide units on the surface of these target cells. Other fimbriae can bind to components of the extracellular matrix.

Fimbriae are occured in both Gram negative and Gram positive bacteria. In Gram positive bacteria, the pilin subunits are covalently linked.

3.2.1.2 Pili

The structure of pili is similar to that of fimbriae, which is also cellular appendages, but pili are slightly larger than fimbriae, much longer and distribute on the bacterial cell in low numbers. Pili are related to the process of bacterial conjugation. Non-sex pili also help bacteria grip surfaces.

Pili can connect bacterial among same species and different species and establish a bridge among cytoplasm of the cells. This enables the transfer of plasmids between different bacteria. After exchanged mutually, plasmids can code for new functions, e.g., antibiotic resistance and so on. The pilus is made out of the protein flagellin.

Up to ten of these structures exist on the bacteria. Some bacteria, viruses or bacteriophages attach to receptors on sex pili and initiate their reproductive cycle.

(1) Sex Pili

Despite its name, the sex pilus is not used for sexual reproduction, and cannot be just equated with a penis, although such image is often used to ease understanding.

A pilus is typically 6-7nm in diameter. During bacterial conjugation, a sex pilus of one bacterium ensnares the recipient bacterium, draws it in, and ultimately causes the formation of a mating bridge, which can merge the cytoplasm of two bacteria via a controlled pore so as to contact directly. This pore is responsible for the transfer of

bacterial DNA (plasmid) from the bacteria with the pilus (donor) to the recipient bacteria. Through this method of genetic transformation, advantageous genetic characteristics can be distributed amongst a population of bacteria. However, not all bacteria have the ability to create sex pili. Furthermore, sex pili can produce between bacteria of different species, and the fertility factor is required to produce sex pili.

(2) Ⅳ Pili

Some pili, designated type Ⅳ pili, generate motile forces. The external termini of the pili bind to solid substrate, either the surface to which the bacteria are attached or to other bacteria, and later pilus constriction pulls the bacteria forward, just like a grappling hook. As type Ⅳ pilus-mediated movement is typically jerky, it is called twitching motility, which is distinct from other forms of bacterial motility, such as is generated by flagella. Bacterial type Ⅳ pilus resembles flagellins of archaea flagella in structural component.

3.2.2 S-layers

An S-layer (surface layer) is a cell surface protein layer discovered in many different bacteria and in some archaea, in where it functions as the cell wall. Furthermore, it covers the outside of the cell. All S-layers are composed of a two-dimensional array of proteins and have a crystalline appearance, the symmetry of which differs between species.

Although the precise functions of S-layers are unknown, they have been suggested that they work as a partial permeability barrier for large substrates. These functions are very diverse and different from species to species. For example, an S-layer could conceivably retain extracellular proteins close to the cell membrane by preventing spread away from the cell. In some pathogenic species, an S-layer may be used to facilitate survival within the host by conferring protection against host defence system. Additional functions related to S-layers include: protection against bacteriophages and phagocytosis, resistance against low pH, barrier against high molecular weight substances (e.g., lytic enzymes), adhesion (for glycosylated S-layers), stabilisation of the membrane, provide adhesion sites for exoproteins, supply a periplasmic compartment in Gram positive bacterial associated with the peptidoglycan and the cytoplasmic membrane.

3.2.3 Capsules and Slime Layers

Many bacteria produce capsules that composed of a mucoid or mucilaginous substance cover the cell membrane. The substance of capsules or slime layers are chemical different for the various bacteria. Some capsules are composed of mucin, a slimy material made up of a protein-like substance united with carbohydrate. And other capsules are composed of pure carbohydrate allied to certain of vegetable gums.

Still others are made of lipoid or polysaccharides while polypeptide in *Bacillus anthracis* in certain case.

Capsule is a layer that locates outside the cell wall of bacteria with which the capsule combines firmly. In addition, it is not easily washed off, and is largely related to virulence of bacteria. In comparison to capsule, slime layer is loosely adhered to the cell wall and is easily eluted. The capsule may be easily demonstrated on certain species of bacteria by suitable staining methods, such as foil staining procedure. When viewed under the microscope, bacterial cells as well as the surfaces are stained dark, while the capsule remains pale or colorless and appears as a ring around the cell. Furthermore, capsules are relatively impermeable structures that cannot be stained with dyes such as India ink. The structure of capsule can produce resistance to phagocytosis and the capsulized organism is not as virulent when devoid of its capsule. Therefore, a relationship between capsular substance and virulence apparently exists. Capsules also contain water which protects bacteria against desiccation. Besides, capsules not only exclude bacterial viruses and most hydrophobic toxic materials such as detergents but also allow bacteria to adhere to surfaces and other cells. Vaccination using capsular material is effective against some organisms (such as *Haemophilus influenzae* and *Streptococcus pneumoniae*). It is said that the capsule is found among Gram negative bacteria, such as *Escherichia coli*, *Haemophilus influenzae*. However, some Gram positive bacteria may also have a capsule. For example, *Bacillus megaterium* can synthesizes a capsule composed of polypeptide and polysaccharides; *Streptococcus pyogenes* have a hyaluronic acid capsule. Capsule which is too small to be seen with ordinary microscope is called microcapsule. For example, M protein of *Streptococcus pyogenes* is a microcapsule. Although the yeast *Cryptococcus neoformans* is not a bacterium, it has a similar capsule.

Slime layers are somewhat looser, fibrous structure generally relate to attachment of bacteria to other cells or inanimate surfaces to create biofilms. Slime layers can be acted as a food reserve for the cell. They can also serve as antigens and be involved in cell recognition. The slime layer is not to be confused with the S-layers, a separate and highly organized glycoprotein layer surrounding many bacterial cells. The function of the slime layer is to protect the bacteria cells against environmental dangers such as antibiotics and desiccation. In addition, the slime layer also allows bacteria to adhere to smooth surfaces. What's important, it may allow bacterial colonies to survive in chemical sterilization with chlorine, iodine, and other chemicals, leaving autoclaving or flushing with boiling water as the only certain methods of decontamination. For example, *Streptococcus mutans* attaches to the teeth with a slime layer and forms a sticky film that catches food particles and other bacteria on the teeth (dental plaque). The bacteria then metabolize the trapped food particles and release acids, which can result in tooth decay possibly.

3.2.4 Flagella

Perhaps the most recognizable extracellular bacterial cell structure is flagellum (plural: flagella). Flagella are whip-like structures protruding from the bacterial cell wall and are responsible for bacterial motility. Flagella are rigid protein structures, about 20nm in diameter and up to 20μm in length. The arrangement of flagella about the bacterial cell is unique to the species observed. Common forms include: peritrichous, multiple flagella found at several locations about the cell; polar, single flagellum found at one of the cell poles; lophotrichous, a tuft of flagella found at one cell pole (Fig. 1-8).

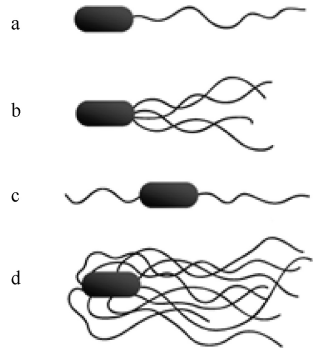


Fig. 1-8 Examples of bacterial flagella arrangement schemes (https://en.wikipedia.org)
a. Monotrichous; b. Lophotrichous; c. Amphitrichous; d. Peritrichous

Flagella are complex structures (Fig. 1-9) that are composed of many different proteins. These proteins include flagellin, which makes up the whip-like tube and a protein complex that spans the cell wall and cell membrane to form a motor that causes the flagellum to rotate. Flagella are driven by the energy released by the transfer of ions down an electrochemical gradient across the cell membrane.

3.2.5 Endospores

Perhaps the most well-known bacterial adaptation to stress is the formation of endospores (Fig. 1-10). Endospores are bacterial survival structures that are highly resistant to many different types of extreme physical, chemical and environmental stresses, such as high levels of ultraviolet (UV) light, γ-rays, detergents, disinfectants, heat, vacuum, pressure and desiccation, and therefore enable the survival of bacteria in environments that would be lethal for these cells in their normal vegetative form. In this dormant state, it has been proposed that endospore formation has allowed for the survival of some bacteria for hundreds of millions of years (e.g., in salt crystals). Endospore formation is limited to several genera of Gram positive bacteria such as *Bacillus*, *Clostridium*. It differs from reproductive spores in that only one spore is formed per cell resulting in no net gain in cell number upon endospore germination.

The location of an endospore within a cell is species-specific and can be used to determine the identity of a bacterium.

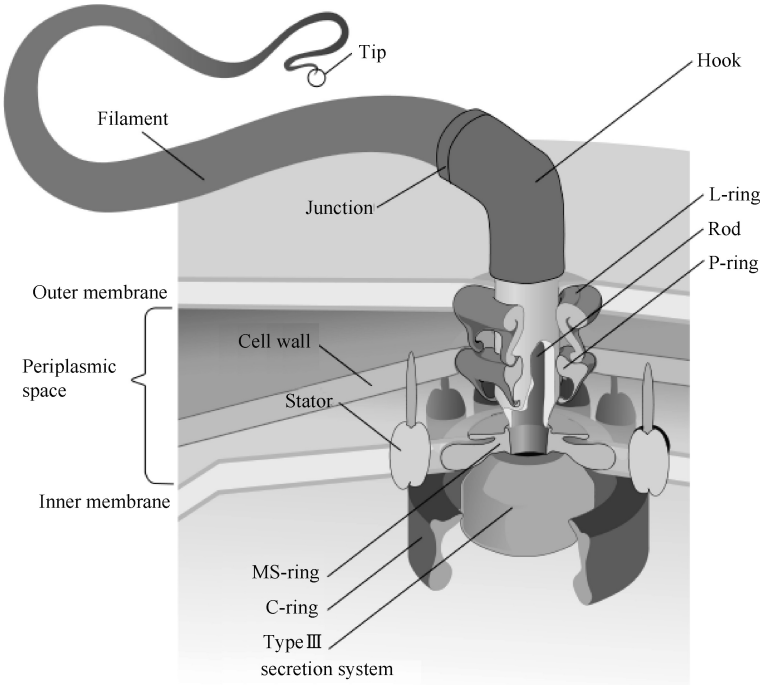


Fig. 1-9 Flagellum base diagram (https://en.wikipedia.org)

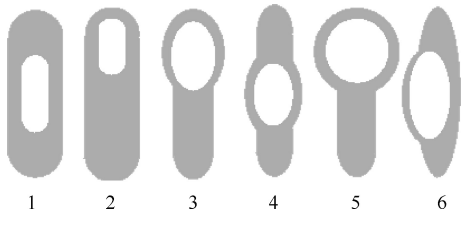


Fig. 1-10 Variations in endospore morphology (https://en.wikipedia.org)
1 and 4. Central endospore; 2, 3 and 5. Terminal endospore; 6. Lateral endospore

The position of the endospore differs among bacterial species and is useful in identification. The main types within the cell are terminal, subterminal and centrally placed endospores. Terminal endospores are seen at the poles of cells, whereas central endospores are more or less in the middle. Subterminal endospores are those between these two extremes, usually seen far enough towards the poles but close enough to the center so as not to be considered either terminal or central. Lateral endospores are seen occasionally. Examples of bacteria having terminal endospores include *Clostridium tetani*. Bacteria having a centrally placed endospore include *Bacillus*

cereus, and those having a subterminal endospore as *Bacillus subtilis*. Sometimes the endospore can be so large that the cell can be distended around the endospore, which is typical of *Clostridium tetani*. Visualising endospores under the light microscope can be difficult due to the impermeability of the endospore wall to dyes and stains. While the rest of a bacterial cell may stain, the endospore is left colourless. To overcome this, a special stain technique called Moeller stain is used. That allows the endospore to show up as red, while the rest of the cell stains blue. Another staining technique for endospores is the Schaeffer-Fulton stain, which stains endospores green and bacterial bodies red.

In contrast to eukaryotic spores, which are produced by many eukaryotes for reproductive purposes, bacteria will produce a single endospore internally. The spore is sometimes surrounded by a thin covering known as the exosporium, which overlies the spore coat. The spore coat, which acts like a sieve that excludes large toxic molecules like lysozyme, is resistant to many toxic molecules and may also contain enzymes that are involved in germination. The cortex lies beneath the spore coat and consists of peptidoglycan. The core wall lies beneath the cortex and surrounds the protoplast or core of the endospore. The core contains the spore chromosomal DNA which is encased in chromatin-like proteins known as small acid-soluble spore proteins (SASPs), that protect the spore DNA from UV radiation and heat. The core also contains normal cell structures, such as ribosomes and other enzymes, but is not metabolically active. Up to 15% of the dry weight of the endospore consists of calcium dipicolinate within the core, which is thought to stabilize the DNA. Dipicolinic acid (DPA) could be responsible for the heat resistance of the spore, and calcium may aid in resistance to heat and oxidizing agents. However, mutants resistant to heat but lacking DPA have been isolated, suggesting other mechanisms contributing to heat resistance are at work. Endospore-forming bacteria can also cause disease.

When a bacterium detects that environmental conditions are becoming unfavorable, it may start the process of sporulation, which takes about 8h. The DNA is replicated and a membrane wall known as a spore septum begins to form between it and the rest of the cell. The plasma membrane of the cell surrounds this wall and pinches off to leave a double membrane around the DNA, and the developing structure is now known as a forespore. Calcium dipicolinate is incorporated into the forespore during this time. Next the peptidoglycan cortex forms between the two layers and the bacterium add a spore coat to the outside of the forespore. Sporulation is now complete, and the mature endospore will be released when the surrounding vegetative cell is degraded. Endospores are resistant to most agents which would normally kill the vegetative cells. Household cleaning products generally have no effect, nor do most alcohols, quaternary ammonium compounds or detergents. However, alkylating agents such as ethylene oxide are effective against endospores. While resistant to

extreme heat and radiation, endospores can be destroyed by burning or autoclaving. Exposure to extreme heat for a long enough time will generally have some effect, though many endospores can survive hours of boiling or cooking. Prolonged exposure to high energy radiation, such as X-rays and γ-rays, will also kill most endospores.

Reactivation of the endospore occurs when conditions are more favourable and involves activation, germination, and outgrowth. Even if an endospore is located in plentiful nutrients, it may fail to germinate unless activation has taken place. This may be triggered by heating the endospore. Germination involves the dormant endospore starting metabolic activity and thus breaking hibernation. It is commonly characterised by rupture or absorption of the spore coat, swelling of the endospore, an increase in metabolic activity, and loss of resistance to environmental stress. Outgrowth follows germination and involves the core of the endospore manufacturing new chemical components and exiting the old spore coat to develop into a fully functional vegetative bacterial cell, which can split to produce more cells.

As a simplified model for cellular differentiation, the molecular details of endospore formation have been extensively studied, specifically in the model organism *Bacillus subtilis*. These studies have contributed much to our understanding of the regulation of gene expression, transcription factors, and the sigma factor subunits of RNA polymerase. Endospores of the bacterium *Bacillus anthracis* were used in the 2001 anthrax attacks. The powder found in contaminated postal letters was composed of extracellular anthrax endospores. Inhalation, ingestion or skin contamination of these endospores, which were technically incorrectly labelled as "spores", led to a number of deaths. *Geobacillus stearothermophilus* endospores are used as biological indicators when autoclave is used in sterilization procedures.

4 Gram Staining

Gram staining (or Grams method) is an empirical method of dividing bacterial species into two large groups (Gram positive and Gram negative) based on the chemical and physical properties of their cell walls. Gram staining is a common procedure in the traditional bacteriological laboratory. The technique is a first step to determine the identity of a particular bacterial sample. Gram staining is performed on body fluid or biopsy when infection is suspected. It yields results much more quickly than culture, and is especially important when infection could make an important difference in the patient's treatment and prognosis.

The method is named after its inventor, the Danish scientist Hans Christian Gram (1853—1938), who developed the technique in 1884 to discriminate between two types of bacteria with similar clinical symptoms: *Streptococcus pneumoniae* and *Klebsiella*

pneumoniae. The word Gram is always spelled with a capital, referring to the name of the inventor of the Gram staining.

4.1 Staining Mechanism

Gram positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stains purple while Gram negative bacteria have a thinner layer (10% of cell wall), which stains pink. Gram negative bacteria also have an additional outer membrane which contains lipids and is separated from the cell wall by the periplasmic space. There are four basic steps of the Gram staining, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), rapid decolorization with alcohol or acetone, and counterstaining with safranin or basic fuchsin.

Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride ions (Cl^-). These ions penetrate through the cell wall and cell membrane of both Gram positive and Gram negative cells. The CV^+ interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine (I^- or I^{3-}) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV-I) within the inner and outer layers of the cell. When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. The CV-I complexes are washed from the Gram negative cell along with the outer membrane. In contrast, a Gram positive cell becomes dehydrated from an ethanol treatment. The large CV-I complexes become trapped within the Gram positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram positive and negative cells if the decolorizing agent is left on too long (a matter of seconds).

After decolorization, the Gram positive cell remains purple and the Gram negative cell loses its purple color. Counterstain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram negative bacteria a pink or red color.

Some bacteria, after staining with the Gram stain, yield a Gram-variable pattern: mixes of pink and purple cells are seen. The genera *Actinomyces*, *Arthrobacter*, *Corynebacterium* and *Mycobacterium* have cell walls particularly sensitive to breakage during cell division, resulting in Gram negative staining of these Gram positive cells. In cultures of *Bacillus*, *Butyrivibrio*, and *Clostridium*, a decrease in peptidoglycan thickness during growth coincides with an increase in the number of cells that stain Gram negative. In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

4.2 Flaw of Gram Staining

Gram staining is not used to classify archaea, since these microorganisms yield widely varying responses that do not follow their phylogenetic groups. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique, thus forming Gram variable and Gram indeterminant groups as well. The Gram stain is not an infallible tool for diagnosis, identification, or phylogeny, however. It is of extremely limited use in environmental microbiology, and has been largely superseded by molecular techniques even in the veterinary microbiology lab. Some organisms are Gram-variable (that means, they may stain either negative or positive); some organisms are not susceptible to either stain, used by the Gram technique. In a modern environmental or molecular microbiology lab, most identification is done using genetic sequences and other molecular techniques, which are far more specific and information-rich than differential staining.

Review Questions

1. Term explanation: bacterium, capsule, pili, flagella, spore, peptide, lipid polysaccharide, outer membrane protein, plasmid, nucleoid, Gram staining, colony, lawn.
2. Try to plot the basic structure and special structure of bacteria.
3. Try to compare the differences of Gram negative bacteria and Gram positive bacteria in cell wall.
4. What are the basic structures of bacteria, and what are their functions?
5. What are the specific structures of bacteria, and what are their functions?
6. Try to describe the basic principles, steps and significance of Gram staining.
7. Which methods can be used to observe bacteria?
8. What are the forms of bacteria, and what is their significance?

Chapter 2 Physiology and Ecology of Bacteria

Synopsis

Bacteria can perform light energy metabolism and chemical energy metabolism. The growth of bacteria requires nutrients such as carbon source, nitrogen source, sulfur source, phosphorus source, minerals and growth factor, etc. individual bacterium takes bisection for reproduce. Under liquid conditions, the relationship between the group growth of bacteria and the culture time can be expressed by the growth curve which can be divided into four stages, namely the delay period, the logarithm period, the stable period and the decay stage. Different bacteria can be cultured in different types of culture medias. Environmental factors affecting bacterial growth include nutrition, pH, temperature, oxygen, ion strength and osmotic pressure. Biofilm is an important form of bacteria in nature and gives some special characteristics to bacteria. There are complex interactions between microbial populations in animals, while the normal microflora is of great significance to animal health.

1 Metabolism

Bacteria exhibit an extremely wide variety of metabolic types. The distribution of metabolic traits within a group of bacteria has traditionally been used to define their taxonomy, but these traits often do not correspond with modern genetic classifications. Bacterial metabolism is classified into nutritional groups on the basis of three major criteria: the kind of energy used for growth, the source of carbon, and the electron donors used for growth (Table 2-1). An additional criterion of respiratory microorganisms is the electron acceptors used for aerobic or anaerobic respiration.

Table 2-1 Nutritional types in bacterial metabolism (https://en.wikipedia.org)

Nutritional type	Source of energy	Source of carbon
Phototrophs	Sunlight	Organic compounds (photoheterotrophs) or carbon fixation (photoautotrophs)
Lithotrophs	Inorganic compounds	Organic compounds (lithoheterotrophs) or carbon fixation (lithoautotrophs)
Organotrophs	Organic compounds	Organic compounds (chemoheterotrophs) or carbon fixation (chemoautotrophs)

1.1 Carbon Metabolism

Carbon metabolism in bacteria is either heterotrophic, where organic carbon compounds are used as carbon sources, or autotrophic, meaning that cellular carbon is obtained by fixing carbon dioxide. Heterotrophic bacteria include parasitic types. Typical autotrophic bacteria are phototrophic cyanobacteria, green sulfur-bacteria and some purple bacteria, but also many chemolithotrophic species, such as nitrifying or sulfur-oxidising bacteria.

1.2 Energy Metabolism

Energy metabolism of bacteria is either based on phototrophy, the use of light through photosynthesis, or on chemotrophy, the use of chemical substances for energy, which are mostly oxidized at the expense of oxygen or alternative electron acceptors (aerobic/anaerobic respiration).

1.3 Electron Donor

Bacteria are further divided into lithotrophs that use inorganic electron donors and organotrophs that use organic compounds as electron donors. Chemotrophic organisms use the respective electron donors for energy conservation (by aerobic/anaerobic respiration or fermentation) and biosynthetic reactions (e.g., carbon dioxide fixation), whereas phototrophic organisms use them only for biosynthetic purposes. Respiratory organisms use chemical compounds as a source of energy by taking electrons from the reduced substrate and transferring them to a terminal electron acceptor in a redox reaction. This reaction releases energy that can be used to synthesize adenosine triphosphate (ATP) and drive metabolism. In aerobic organisms, oxygen is used as the electron acceptor. In anaerobic organisms other inorganic compounds, such as nitrate, sulfate or carbon dioxide are used as electron acceptors. This leads to the ecologically important processes of denitrification, sulfate reduction and acetogenesis respectively.

1.4 Sources of Metabolic Energy

The three major mechanisms for generating metabolic energy are fermentation, respiration, and photosynthesis. At least one of these mechanisms must be employed if an organism is to grow.

1.4.1 Fermentation

The formation of ATP in fermentation is not coupled to the transfer of electrons. Fermentation is characterized by substrate phosphorylation, an enzymatic process in which a pyrophosphate bond is donated directly to adenosine diphosphate (ADP) by a

phosphorylated metabolic intermediate. The phosphorylated intermediates are formed by metabolic rearrangement of a fermentable substrate such as glucose, lactose, or arginine. Because fermentations are not accompanied by a change in the overall oxidation-reduction state of the fermentable substrate, the elemental composition of the products of fermentation must be identical to those of the substrates. For example, fermentation of a molecule of glucose ($C_6H_{12}O_6$) by the Embden-Meyerhof pathway yields a net gain of two pyrophosphate bonds in ATP and produces two molecules of lactic acid ($C_3H_6O_3$).

1.4.2 Respiration

Respiration is analogous to the coupling of an energy-dependent process to the discharge of a battery. Chemical reduction of an oxidant (electron acceptor) through a specific series of electron carriers in the membrane establishes the proton motive force across the bacterial membrane. The reductant (electron donor) may be organic or inorganic. For example, lactic acid serves as a reductant for some organisms, and hydrogen gas is a reductant for other organisms. Gaseous oxygen (O_2) often is employed as an oxidant, but alternative oxidants that are employed by some organisms include carbon dioxide (CO_2), sulfate (SO_4^{2-}) and nitrate (NO_3^-).

1.4.3 Photosynthesis

Photosynthesis is similar to respiration in that the reduction of an oxidant via a specific series of electron carriers establishes the proton motive force. The difference in the two processes is that in photosynthesis the reductant and oxidant are created photochemically by light energy absorbed by pigments in the membrane; thus, photosynthesis can continue only as long as there is a source of light energy. Some bacteria are able to invest a substantial amount of light energy in making water a reductant for carbon dioxide. Oxygen is evolved in this process, and organic matter is produced. Respiration, the energetically favorable oxidation of organic matter by an electron acceptor such as oxygen, can provide photosynthetic organisms with energy in the absence of light.

2 Source of Nutrition for Growth

Nutrients in growth media must contain all the elements necessary for the biologic synthesis of new organisms. In the following discussion, nutrients are classified according to the elements they supply.

2.1 Carbon Source

Some bacteria are able to use photosynthetic energy to reduce carbon dioxide at the expense of water. These organisms belong to the group of autotrophs, creatures that do

not require organic nutrients for growth. Other autotrophs are the chemolithotrophs, organisms that use an inorganic substrate such as hydrogen or thiosulfate as a reductant and carbon dioxide as a carbon source.

Heterotrophs require organic carbon for growth, and the organic carbon must be in a form that can be assimilated. For example, naphthalene can provide all the carbon and energy required for respiratory heterotrophic growth, but very few organisms possess the metabolic pathway necessary for naphthalene assimilation. On the other hand, glucose can support the fermentative or respiratory growth of many organisms. It is important that growth substrates be supplied at levels appropriate for the microbial strain that is being grown. Levels that will support the growth of one organism may inhibit the growth of another organism.

Carbon dioxide is required for a number of biosynthetic reactions. Many respiratory organisms produce more than enough carbon dioxide to meet this requirement, but others require a source of carbon dioxide in their growth medium.

2.2 Nitrogen Source

Nitrogen is a major component of proteins, nucleic acids, and other compounds, accounting for approximately 5% of the dry weight of a typical bacterial cell. Nitrogen may be supplied in a number of different forms, and microorganisms vary in their abilities to assimilate nitrogen. The end product of all pathways for nitrogen assimilation is the most reduced form of the element, ammonia (NH_3). When NH_3 is available, it diffuses into most bacteria through transmembrane channels as dissolved gaseous NH_3 rather than ionic ammonium ion (NH_4^+).

The ability to assimilate N_2 reductively via NH_3, which is called nitrogen fixation, is a property unique to prokaryotes. But few bacteria in veterinary microbiology have this process. Most microorganisms can use NH_3 as a sole nitrogen source, and many organisms possess the ability to produce NH_3 from amines ($R\text{-}NH_2$) or amino acids ($RCHNH_2COOH$), generally intracellularly. Production of NH_3 from the deamination of amino acids is called ammonification. Ammonia is introduced into organic matter by biochemical pathways involving glutamate and glutamine.

Many microorganisms possess the ability to assimilate nitrate (NO_3^-) and nitrite (NO_2^-) reductively by conversion of these ions into NH_3. These processes are termed assimilatory nitrate reduction and assimilatory nitrite reduction respectively. These pathways for assimilation differ from pathways used for dissimilation of nitrate and nitrite. The dissimilatory pathways are used by organisms that employ these ions as terminal electron acceptors in respiration.

2.3 Sulfur Source

Like nitrogen, sulfur is a component of many organic cell substances. It forms part of

the structure of several co-enzymes and is found in the cysteinyl and methionyl side chains of proteins. Some autotrophic bacteria can oxidize sulfur to sulfate (SO_4^{2-}). Most microorganisms can use sulfate as a sulfur source, reducing the sulfate to the level of hydrogen sulfide (H_2S). Some microorganisms can assimilate H_2S directly from the growth medium, but this compound can be toxic to many organisms. Some bacteria can produce H_2S as an important trait.

2.4 Phosphorus Source

Phosphate (PO_4^{3-}) is required as a component of ATP, nucleic acids, and such co-enzymes as NAD, NADP, and flavins. In addition, many metabolites, lipids (phospholipids, lipid A), cell wall components (teichoic acid), some capsular polysaccharides, and some proteins are phosphorylated. Phosphate is always assimilated as free inorganic phosphate.

2.5 Mineral Source

Numerous minerals are required for enzyme function. Magnesium ion (Mg^{2+}) and ferrous ion (Fe^{2+}) are also found in porphyrin derivatives: magnesium in the chlorophyll molecule, and iron as part of the co-enzymes of the cytochromes and peroxidases. Mg^{2+} and potassium ion (K^+) are both essential for the function and integrity of ribosomes. Calcium ion (Ca^{2+}) is required as a constituent of Gram positive cell walls, but it is dispensable for Gram negative bacteria. In formulating a medium for the cultivation of most microorganisms, it is necessary to provide sources of potassium, magnesium, calcium, and iron, usually as their ions (K^+, Mg^{2+}, Ca^{2+}, and Fe^{2+}). Many other minerals (e.g., Mn^{2+}, Mo^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+}) are required. These frequently can be provided in tap water or as contaminants of other medium ingredients.

The uptake of iron, which forms insoluble hydroxides at neutral pH, is facilitated in many bacteria and fungi by their production of siderophores-compounds that chelate iron and promote its transport as a soluble complex. These include hydroxamates (—$CONH_2OH$) called sideramine, and derivatives of catechol (e.g., 2,3-dihydroxy-benzoylserine). Plasmid-determined siderophores play a major role in the invasiveness of some bacterial pathogens.

2.6 Growth Factor

A growth factor is an organic compound which a cell must contain in order to grow but it is unable to be synthesized. Many microorganisms, when provided with the nutrients, are able to synthesize all of the building blocks for macromolecules: amino acids;

purines, pyrimidines, and pentoses (the metabolic precursors of nucleic acids); additional carbohydrates (precursors of polysaccharides); fatty acids and isoprenoid compounds. In addition, free-living organisms must be able to synthesize the complex vitamins that serve as precursors of co-enzymes.

Different microbial species vary widely in their growth factor requirements. The compounds involved are found and are essential to all organisms. The differences in requirements reflect differences in synthetic abilities. Some species require no growth factors, while others, like some of lactobacilli, have lost (during evolution) the ability to synthesize as many as 30 to 40 essential compounds and hence require them in the medium.

3 Growth and Reproduction

3.1 Binary Fission

Unlike multicellular organisms, increases in the size of bacteria (cell growth) and their reproduction by cell division are tightly linked in unicellular organisms. Bacteria grow to a fixed size and then reproduce through binary fission (Fig. 2-1), a form of asexual reproduction. Bacterial growth is the division of one bacterium into two daughter cells in a process called binary fission.

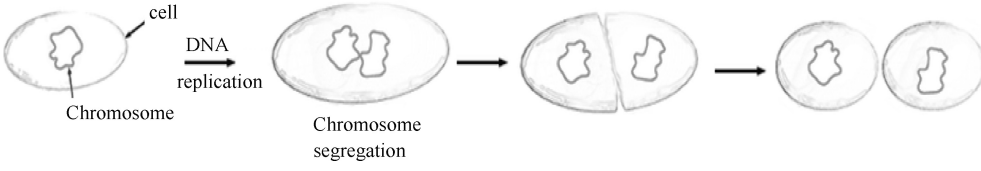


Fig. 2-1 Binary fission of bacterial (https://en.wikipedia.org)

Under optimal conditions, bacteria can grow and divide extremely rapidly, and bacterial populations can double as quickly as every 9.8min. In cell division, two identical clone daughter cells are produced.

Tetrads are a cell arrangement that is a consequence of binary fission not resulting in complete separation of cells, and that occurs in two planes, thus producing a square consisting of four cocci, one at each corner. Sarcina are a cell arrangement that is a consequence of binary fission that does not result in complete separation of cells, and that occurs in three planes, thus producing cubes consisting of eight cocci, one coccus at each corner.

3.2 The Growth Curve

Because individual cells grow larger only to divide into new individuals, microbial

growth is defined not in terms of cell size but as the increase in the number of cells, which occurs by cell division. This emphasis has practical application since it is typically far easier to measure increases in cell number than it is to measure increases in cell size.

In autecological studies, bacterial growth in batch culture can be modeled with four different phases (Fig. 2-2): lag phase, exponential phase, stationary phase, and death phase.

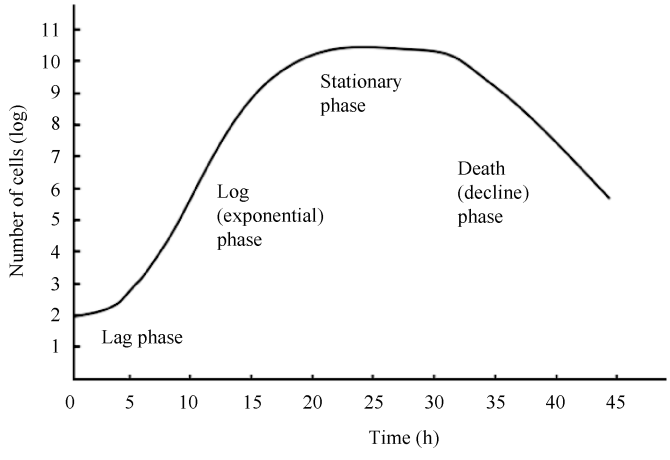


Fig. 2-2 Bacterial growth curve (改绘自黄青云, 2009)

During lag phase, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs.

Exponential phase (sometimes called the log phase) is a period characterized by cell doubling. The number of new bacteria appearing per unit time is proportional to the present population. If growth is not limited, doubling will continue at a constant rate, so both the number of cells and the rate of population increase doubles with each consecutive time period. For this type of exponential growth, plotting the natural logarithm of cell number against time produces a straight line. The slope of this line is the specific growth rate of the organism, which is a measure of the number of divisions per cell per unit time. The actual rate of this growth depends upon the growth conditions, which affect the frequency of cell division events and the probability of both daughter cells surviving. Exponential growth cannot continue indefinitely, however, because the medium is soon depleted of nutrients and enriched with wastes.

During stationary phase, the growth rate slows as a result of nutrient depletion and accumulation of toxic products. This phase is reached as the bacteria begin to exhaust

the resources that are available to them. This phase is a constant value as the rate of bacterial growth is equal to the rate of bacterial death.

At death phase (sometimes called the logarithmic decline phase), bacteria run out of nutrients and die.

4 Medium

A growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms. A growth medium (plural: media) is a mixture of nutrients, moisture and other chemicals that bacteria need for growth in a laboratory environment. There are different types of media for growing different types of cells. The most common growth media for microorganisms are nutrient broths and agar plates; specialized media are sometimes required for microorganism culture growth. Some organisms, termed fastidious organisms, require specialized environments due to complex nutritional requirements.

The most common growth media for microorganisms are nutrient broths (liquid nutrient medium) or LB medium. Liquid media are often mixed with agar (concentration of 1-3%) and poured into petri dishes to solidify. These agar plates provide a solid medium on which microbes may be cultured. They remain solid, as very few bacteria are able to decompose agar. Bacteria grew in liquid cultures often form colloidal suspensions. Bacteria such as *Escherichia coli* may be grown on solid media or in liquid media. Reducing the amount of agar to 0.2-0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains. Certain transport media are semi-solid media.

An important distinction between above two growth media types is that of defined versus undefined media. A defined medium will have known quantities of all ingredients. For microorganisms, they consist of providing trace elements and vitamins required by the microbe and especially a defined carbon source and nitrogen source. An undefined medium has some complex ingredients, such as yeast extract or casein hydrolysate, which consist of a mixture of many chemical species in unknown proportions. Undefined media are chosen, as some microorganisms have never been cultured on defined media.

Minimal media are those that contain the minimum nutrients possible for colony growth, generally without amino acids, and are often used to grow “wild type” microorganisms. Supplementary minimal media are a type of minimal media that also contains a single selected agent, usually an amino acid or a sugar. This supplementation allows for the culturing of specific lines of auxotrophic recombinants.

Differential media or indicator media are those minimal media which are added

some sort of indicator that allows for the differentiation of particular chemical reactions occurring during growth for distinguish one microorganism type from another growing on the same media. This type of media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as neutral red, phenol red, eosin or methylene blue) added to the medium to visibly indicate the defining characteristics of a microorganism. This type of media is used for the detection of microorganisms and by molecular biologists to detect recombinant strains of bacteria. Some examples of differential media include eosin methylene blue (EMB), MacConkey (MCK) agar, mannitol salt agar (MSA), X-gal plates and so on.

Selective media are used for the growth of only select microorganisms. For example, if a microorganism is resistant to a certain antibiotic, such as ampicillin or tetracycline, then that antibiotic can be added to the medium in order to prevent other cells, which do not possess the resistance, from growing. Media lacking an amino acid such as proline in conjunction with *E. coli* unable to synthesize it were commonly used by geneticists before the emergence of genomics to map bacterial chromosomes. Some examples of selective media include EMB, blood agar, MCK agar, Hektoen enteric agar (HE), MSA, terrific broth (TB), xylose lysine desoxyscholate (XLD), buffered charcoal yeast extract agar and so on.

Enriched media contain the nutrients required to support the growth of a wide variety of organisms, including some of the more fastidious ones. They are commonly used to harvest as many different types of microbes as are present in the specimen. Blood agar is an enriched medium in which nutritionally rich whole blood supplements the basic nutrients. Chocolate agar is enriched with heat-treated blood (40-45℃), which turns brown and gives the medium the color for which it is named.

Anaerobic media is for anaerobic bacteria which need special media for growth because they need low oxygen content, reduced oxidation-reduction potential and extra nutrients. Those media may have to be supplemented with nutrients like hemin and vitamin K. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine can render a medium reduced.

5 Environmental Factors Affecting Growth

A suitable growth medium must contain all the nutrients required by the organism to be cultivated, and such factors as pH, temperature, and aeration must be carefully controlled.

5.1 Nutrient

In general, each type of nutrient in media must be provided: hydrogen donors and

acceptors about 2g/L, carbon source about 1g/L, nitrogen source about 1g/L, sulfur and phosphorus about 50mg/L of each, trace elements 0.1-1mg/L of each, amino acids and purines and pyrimidines about 50mg/L of each, vitamins 0.1-1mg/L of each.

5.2 Hydrogen Ion Concentration (pH)

Most organisms have a fairly narrow optimal pH range. The optimal pH must be empirically determined for each species. Most organisms (neutrophiles) grow best at a pH of 6.0-8.0, although some forms (acidophiles) have optima as low as pH 3.0 and others (alkaliphiles) have optima as high as pH 10.5.

5.3 Temperature

Different microbial species vary widely in their optimal temperature ranges for growth. Psychrophilic forms grow best at low temperatures (15-20℃), mesophilic forms grow best at 30-37℃, and most thermophilic forms grow best at 50-60℃. Some are hyperthermophilic and can grow at well above the temperature of boiling water, which exists under high pressure in the depths of the ocean. Most organisms are mesophilic. 30℃ is optimal for many free-living forms. The body temperature of the host is optimal for symbionts of warm-blooded animals. Extremes of temperature kill microorganisms. Extreme heat is used to sterilize preparations; extreme cold also kills microbial cells, although it cannot be used safely for sterilization.

5.4 Aeration

The role of oxygen is hydrogen acceptor. Many microorganisms are obligate aerobes, specifically requiring oxygen as hydrogen acceptor. Some microorganisms are facultative, able to live aerobically or anaerobically. Other microorganisms are obligate anaerobes, requiring a substance other than oxygen as hydrogen acceptor and being sensitive to oxygen inhibition. The natural by-products of aerobic metabolism are the reactive compounds hydrogen peroxide (H_2O_2) and superoxide (O_2^-). In the presence of iron, these two species can generate hydroxyl radicals (·OH), which can damage any biologic macromolecule.

5.5 Ionic Strength and Osmotic Pressure

To a lesser extent, factors such as osmotic pressure and salt concentration may have to be controlled. For most organisms, the properties of ordinary media are satisfactory; however, a few organisms adapted to growth in strong salt solutions or with high osmotic pressures. Organisms requiring high salt concentrations are called halophilic;

those requiring high osmotic pressures are called osmophilic.

Most bacteria are able to tolerate a wide range of external osmotic pressures and ionic strengths because of their ability to regulate internal osmolality and ion concentration. Osmolality is regulated by the active transport of K^+ into the cell. Internal ionic strength is kept constant by a compensating excretion of the positively charged organic polyamine putrescine. Since putrescine carries several positive charges per molecule, a large drop in ionic strength is affected at only a small cost in osmotic strength.

6 Biofilm

A biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to a surface. These adherent cells are frequently embedded within a self-produced matrix of EPS (an abbreviation for either extracellular polymeric substance or exopolysaccharide). Biofilm EPS, which is also referred to as "slime", is a polymeric jumble of DNA, proteins and polysaccharides. Biofilms may form on living or non-living surfaces, and represent a prevalent mode of microbial life in natural, industrial and hospital settings. The cells of a microorganism growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which by contrast, are single-cells that may float or swim in a liquid medium. Given sufficient resources for growth, a biofilm will quickly grow to be macroscopic. Biofilms can contain many different types of microorganism, e.g., bacteria, archaea, protozoa, fungi and algae; each group performing specialized metabolic functions. However, some organisms will form monospecies films under certain conditions.

Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated.

There are five stages of biofilm development followed: ①initial attachment, ②irreversible attachment, ③maturation Ⅰ, ④maturation Ⅱ, ⑤dispersion.

Formation of a biofilm begins with the attachment of free-floating microorganisms to a surface. These first colonists adhere to the surface initially through weak, reversible van der Waals forces. If the colonists are not immediately separated from the surface, they can anchor themselves more permanently using cell adhesion structures such as pili.

The first colonists facilitate the arrival of other cells by providing more diverse adhesion sites and beginning to build the matrix that holds the biofilm together. Some species are not able to attach to a surface on their own but are often able to anchor

themselves to the matrix or directly to earlier colonists. It is during this colonization that the cells are able to communicate via quorum sensing using such products as acylated homoserine lactone (AHL). Once colonization has begun, the biofilm grows through a combination of cell division and recruitment.

The important stage of biofilm formation is known as development. In this stage, the biofilm is established and may only change in shape and size. This development of biofilm allows for the cells to become more antibiotic resistant.

Dispersal of cells from the biofilm colony is an essential stage of the biofilm lifecycle. Dispersal enables biofilm to spread and colonize new surfaces. Enzymes that degrade the biofilm extracellular matrix, such as dispersin B and deoxyribonuclease, may play a role in biofilm dispersal. Biofilm matrix degrading enzymes may be useful as anti-biofilm agents. Recent evidence has shown that a fatty acid messenger, *cis*-2-decenoic acid, is capable of inducing dispersion and inhibiting growth of biofilm colonies.

7 Animal Microflora

The animal microflora is the assemblage of microorganisms that constantly and consistently inhabit the animal body. They include bacteria, fungi and archaea. Some of these organisms are known to perform tasks that are useful for the animal host, while the majority have no known beneficial or harmful effect. Those that are expected to be present, and that under normal circumstances do not cause disease, are deemed members of the normal flora, or microbiota.

It is estimated that 500 to 1,000 species of bacteria live in the animal gut and a roughly similar number on the skin. Bacterial cells are much smaller than animal cells, and there are at least ten times as many bacteria as animal cells in the body. Though members of the microflora are found on all surfaces exposed to the environment (on the skin and eyes, in the mouth, nose, small intestine), the vast majority of bacteria live in the large intestine.

Many of the bacteria in the digestive tract, collectively referred to as the gut microflora, are able to break down certain nutrients such as carbohydrates that animals could not digest. The majority of these commensal bacteria are anaerobes, meaning they survive in an environment with no oxygen. Bacteria of the normal flora can act as opportunistic pathogens at times of lowered immunity.

Bacteria are vital for the maintenance of animal health, but some pathogenic bacteria also pose a significant health threat by causing diseases. Large numbers of bacteria live on the skin and in the digestive tract. Their growth can be increased by warmth and sweat. Large populations of these organisms on animals are the cause of body odor and thought to play a part in acne. There are more than 500 bacterial species present in the

normal animal gut and are generally beneficial. They synthesize vitamins such as folic acid, vitamin K and biotin, and they ferment complex indigestible carbohydrates. Other beneficial bacteria in the normal flora include *Lactobacillus* species, which convert lactose and other sugars to lactic acid in the gut. The presence of such bacterial colonies also inhibits the growth of potentially pathogenic bacteria (usually through competitive exclusion) and some beneficial bacteria are consequently sold as probiotic dietary supplements.

Bacteria make up most of the flora in the colon and 60% of the dry mass of feces. This fact makes feces an ideal source to test for gut flora for any tests and experiments by extracting the nucleic acid from fecal specimens, and bacterial 16S rRNA gene sequences are generated with bacterial primers. This form of testing is also often preferable to more invasive techniques, such as biopsies. Somewhere between 300 and 1,000 different species live in the gut, with most estimates at about 500. However, it is probable that 99% of the bacteria come from about 30 or 40 species. Fungi and protozoa also make up a part of the gut flora, but little is known about their activities.

The relationship between gut microflora and animals is not merely commensal (a non-harmful coexistence), but rather is a mutualistic, symbiotic relationship. The microorganisms perform a host of useful functions, such as fermenting unused energy substrates, training the immune system, preventing growth of harmful species, regulating the development of the gut, producing vitamins for the host (such as biotin and vitamin K), and producing hormones to direct the host to store fats. However, in certain conditions, some species are thought to be capable of causing disease by causing infection or increasing cancer risk for the host.

Review Questions

1. Term explanation: autotrophic bacteria, heterotrophic bacteria, light energy nutritional type, chemical energy nutritional type, fermentation, respiration, culture medium, anaerobe, aerobe, growth curve, biofilm, normal microflora, prebiotics, probiotics.
2. What kinds of nutrients are needed for bacterial growth?
3. How do bacterial individuals reproduce? How do bacterial populations reproduce?
4. What are the characteristics of the growth curve of bacteria, and What's the significance?
5. Why don't the anaerobes grow in aerobic conditions?
6. What are the characteristics of biofilms?
7. What are the physiological functions of normal bacteria?
8. What are the environmental factors that affect the growth of bacteria?

Chapter 3 Disinfection and Sterilization

Synopsis

Sterilization and disinfection are often used in practice. Aseptic technology is a key skill in microbiology experiments. Antisepsis can be used in medicine and food preservation. Flame sterilization, hot air sterilization, high pressure steam sterilization, boil sterilization, pasteurization, ultraviolet rays, and gamma irradiation, filtration and other physical methods have different features and use range. There are many chemical disinfectants, such as ether, aldehydes, alcohols, phenols, halogens, oxidants, surfactants, etc., but they are susceptible to environmental factors. Antibiotics are mainly used to treat bacterial infections. Bacteriocin can inhibit or kill bacteria that are close to kin. Microbiological laboratory operators should pay more attention to the biological safety of the laboratory, protect themselves and prevent the pathogens from spreading out of the laboratory. According to the microbial hazard differences, the biosafety level (BSL) of the microbiology laboratory is usually divided into 1-4, i.e., BSL1-BSL4, of which BSL4 has the highest security level. It is necessary to carry out the examination and approval system for the study of high pathogenic microorganisms.

1 Term

1.1 Sterilization

Sterilization is defined as the killing or removal of all microorganisms and viruses from an object or product. The standard sterilization methods are capable of causing irreversible damage to relevant microorganisms such as bacteria, fungi and parasite (protozoans and helminths) including worm eggs/oocyst.

1.2 Disinfection

Disinfection means rendering an object, the hands or skin free of pathogens. Disinfection is a specifically targeted antimicrobial treatment with the objective of

preventing transmission of certain microorganisms. The purpose of the disinfection procedure is to render an object incapable of spreading infection. Disinfection is usually done with chemical agents, the most important of which are aldehydes, alcohols, phenols, halogens, and surfactants.

1.3 Asepsis

The term asepsis covers all measures aiming to prevent contamination of objects or wounds.

1.4 Antisepsis

Preservation is a general term for measures taken to prevent microbe caused spoilage of susceptible products (pharmaceuticals, foods). Decontamination is the removal or count reduction of microorganisms contaminating an object. In antiseptic measures, chemical agents are used to fight pathogens in or on living tissue, for example in a wound.

2 Physical Methods of Sterilization and Disinfection

2.1 Heat

The application of heat is a simple, cheap and effective method of killing pathogens. When microorganisms are killed by heat, their proteins (enzymes) are irreversibly denatured. Methods of heat application vary according to the specific application.

2.1.1 Dry Heat Sterilization

The guideline values for hot-air sterilizers are as follows: 180℃ for 30min, 160℃ for 120min, whereby the objects to be sterilized must themselves reach these temperatures for the entire prescribed period.

2.1.2 Moist Heat Sterilization

Pasteurization is the antimicrobial treatment used for foods in liquid form (milk). ①Low-temperature pasteurization: 61.5℃, 30min; 71℃, 15s. ②High-temperature pasteurization: brief (seconds) of exposure to 80-85℃ in continuous operation. ③Uperization: heating to 150℃ for 2.5s in a pressurized container using steam injection.

Autoclaves charged with saturated, pressurized steam are used for this purpose: 121℃ for 15min; 134℃ for 3min. In addition, the proteins of microbes are much more readily denatured in a moist environment than under dry conditions. Much more extreme processes are required to inactivate prions, such as autoclaving at 121℃ for 4.5h or at 134℃ for 30min.

2.2 Radiation

2.2.1 Nonionizing Radiation

Ultraviolet (UV) rays (200-280nm) are a type of nonionizing radiation that is rapidly absorbed by a variety of materials. Exposure to UV light results in structural changes in DNA (thymine dimers) that prevent it from replicating. This damage can be repaired to a certain extent by light (photoreactivation). UV rays are therefore used only to reduce airborne pathogen counts (surgical theaters, filling equipment) and for disinfection of smooth surfaces.

2.2.2 Ionizing Radiation

Two types are used: ①gamma radiation consists of electromagnetic waves produced by nuclear disintegration (e.g., radioisotope ^{60}Co); ②corpuscular radiation consists of electrons produced in generators and accelerated to raise their energy level. Ionizing radiation results in the formation of reactive groups that contribute to chemical reactions affecting DNA and proteins.

Radiosterilization equipment is expensive. On a large scale, such systems are used only to sterilize bandages, suture material, plastic items, and heat-sensitive pharmaceuticals. The required dose depends on the level of product contamination (bioburden) and on how sensitive the contaminating microbes are to the radiation. As a rule, a dose of 2.5×10^4Gy (Gray) is considered sufficient.

2.3 Filtration

Liquids and gases can also be sterilized by filtration. Most of the available filters catch only bacteria and fungi, but with ultrafine filters viruses and even large molecules can be filtered out as well. With membrane filters, retention takes place through small pores. The best-known type is the membrane filter made of organic colloids (e.g., cellulose ester). These materials can be processed to produce thin filter layers with gauged and calibrated pore sizes. In conventional depth filters, liquids are put through a layer of fibrous material (e.g., asbestos). The effectiveness of this type of filter is mainly depend on the adsorption. Because of possible toxic side effects, they are now practically obsolete.

3 Chemical Methods of Sterilization and Disinfection

Most chemical agents (alcohols, phenols, aldehydes, heavy metals, oxidants) denature proteins irreversibly. Surfactant compounds (amphoteric and cationic) attack the

cytoplasmic membrane. Acridine derivatives bind to DNA to prevent its replication and function (transcription). The following different types of chemical agents for sterilization and disinfection are introduced in brief.

3.1 Ethylene Oxide

Ethylene oxide can be used for sterilization at low temperatures (20-60℃). The gas has a high penetration capacity, but it cannot kill dried microorganisms and require a relative humidity level of 40-90% in the sterilizing chamber.

3.2 Aldehydes

Formaldehyde (HCHO) is the most important aldehyde for gas sterilization. However, its main use is in disinfection. Formalin is a 35% solution of this gas in water. Formaldehyde is a broad-spectrum germicide for bacteria, fungi and viruses. At higher concentrations, spores are killed as well. This substance is used to disinfect surfaces and objects in 0.5-5% solutions. The mechanism of action of formaldehyde is based on protein denaturation.

3.3 Alcohols

Ethanol (70%) is an important alcohol in disinfection. Alcohols are quite effective against bacteria and fungi, less so against viruses. They do not kill bacterial spores. Due to their rapid action and good skin penetration, the main areas of application of alcohols are surgical and hygienic disinfection of the skin and hands. But their effect is not long-lasting (no depot effect).

3.4 Phenols

Today, phenol derivatives substituted with organic groups and/or halogens (alkylated, arylated and halogenated phenols) are widely used. Phenolic substances have weak performance against spores and viruses. Phenols denature proteins. They are suitable for disinfection of excreted materials.

3.5 Halogens

Chlorine, iodine, and derivatives of these halogens are suitable for use as disinfectants. Chlorine and iodine show a generalized microbicidal effect and also kill spores. Chlorine denatures proteins by binding to free amino groups. On the other hand, hypochlorous acid (HOCl), is produced in aqueous solutions, then disintegrates into

HCl and 1/2 O_2 and thus acts as a powerful oxidant. Iodine has qualities similar to those of chlorine. The most important iodine preparations are the solutions of iodine and potassium iodide in alcohol (tincture of iodine) used to disinfect skin and small wounds. Iodophors are complexes of iodine and surfactants (e.g., polyvinyl pyrrolidone).

3.6 Oxidants

This group includes ozone, hydrogen peroxide, potassium permanganate, and peracetic acid. Their relevant chemical activity is based on the splitting off of oxygen. Most are used as mild antiseptics to disinfect mucosa, skin, or wounds.

3.7 Surfactants

These substances (known as surface-active agents, tensides, or detergents) include anionic, cationic, amphoteric, and nonionic detergent compounds, of which the cationic and amphoteric types are the most effective. The bactericidal effect of these substances is only moderate. Their efficacy is good against Gram positive bacteria, but less so against Gram negative rods.

4 Antibiotic and Bacteriocin

4.1 Antibiotic

An antibiotic is a substance or compound that kills bacteria or inhibits their growth. Antibiotics belong to the broader group of antimicrobial compounds, used to treat infections caused by microorganisms, including fungi and protozoa. Many antibiotics are relatively small molecules with a molecular weight less than 2,000Da. Antibiotics may be divided into two broad groups according to their effect on microorganisms: those that kill bacteria are bactericidal agents, whereas those that only impair bacterial growth are known as bacteriostatic agents.

Most antibiotics target bacterial functions or growth processes. Antibiotics that target the bacterial cell wall (penicillins, cephalosporins), or cell membrane (polymixins), or interfere with essential bacterial enzymes (quinolones, sulfonamides) are usually bactericidal in nature. Narrow-spectrum antibiotics target particular types of bacteria, such as Gram negative or Gram positive bacteria, whereas broad-spectrum antibiotics affect a wide range of bacteria.

4.2 Bacteriocin

Bacteriocins are proteinaceous toxins produced by bacteria to inhibit the growth of

similar or closely related bacterial strains. They are typically considered to be narrow spectrum antibiotics, though this has been debated. They are phenomenologically analogous to yeast and paramecium killing factors, and are structurally, functionally, and ecologically diverse.

Bacteriocins are categorized in several ways, including producing strain, common resistance mechanisms, and mechanism of killing. Alternative methods of classification include: method of killing (pore forming, DNase, nuclease, murein production inhibition, etc.), genetics (large plasmids, small plasmids, chromosomal), molecular weight and chemistry (large protein, polypeptide, with/without sugar moiety, containing atypical amino acids like lanthionine) and method of production (ribosomal, post ribosomal modifications, non-ribosomal). One method of classification fits the bacteriocins into Class Ⅰ, Class Ⅱa/b/c and Class Ⅲ.

Class Ⅰ bacteriocins are small peptide inhibitors and include nisin and other lantibiotics. Class Ⅱ bacteriocins are small heat-stable proteins. Class Ⅱa bacteriocins (pediocin-like bacteriocins) are the largest subgroup and contain an N-terminal consensus sequence-Tyr-Gly-Asn-Gly-Val-Xaa-Cys across this group. The C-terminal is responsible for species-specific activity, causing cell-leakage by permeabilizing the target cell wall. Class Ⅱa bacteriocins have a large potential for use in food preservation as well medical applications, due to their strong antilisterial activity, and broad range of activity. Class Ⅱb bacteriocins (two-peptide bacteriocins) require two different peptides for activity. Other bacteriocins can be grouped together as Class Ⅱc (circular bacteriocins). These have a wide range of effects on membrane permeability, cell wall formation and pheromone actions of target cells. Class Ⅲ bacteriocins are large, heat-labile protein bacteriocins.

Bacteriocins are of interest in veterinary medicine because they are made by non-pathogenic bacteria that normally colonize the animal body. Bacteriocins have also been suggested as a cancer treatment.

5 Laboratory Safety

5.1 Precautions in Lab

Precautions to avoid laboratory hazards consist essentially of good laboratory practices and adequate facilities and equipment. Safe practices in veterinary microbiology laboratory include: ① rigorous aseptic technique must be used; ②laboratory clothing, gloves and even masks or respirators are chosen to match the virus or clinical material being worked with; ③various classes of biological safety cabinets providing increasing levels of containment are available for procedures of various degrees of biohazard;

④careful attention must be given to the autoclaving of all potentially infectious waste; ⑤mouth pipetting is banned, instead mechanical pipetting devices are used; ⑥particular care is taken when centrifuging material containing or possibly containing a dangerous virus; ⑦where possible, particularly dangerous microorganisms employed in various laboratory tests are inactivated; ⑧spills are cleaned up with an appropriate chemical disinfectant; ⑨where zoonotic tissues are handled, personnel are immunized against such pathogens as rabies virus, avian influenza virus, and *Mycobacterium bovis*, as well as against exotic agents such as rift valley fever virus in the special laboratories handling them; ⑩in lab, pregnant women or immunosuppressed employees must be very careful.

5.2 Biosafety Level

A biosafety level is the level of the biocontainment precautions required to isolate dangerous biological agents in an enclosed facility. The levels of containment range from the lowest biosafety level 1 to the highest at level 4.

Biocontainment can be classified by the relative danger to the surrounding environment as the biosafety level (BSL). There are four safety levels. These are called BSL1 to BSL4, with one anomalous level BSL3-ag for agricultural hazards between BSL3 and BSL4. Facilities with these designations are also sometimes given as P1 to P4 (for pathogen or protection level), as in the term P3 laboratory. Higher numbers indicate a greater risk to the external environment.

At the lowest level of biocontainment, the containment zone may only be a chemical fume hood. At the highest level, the containment involves isolation of the organism by means of building systems, sealed rooms or sealed containers, personal isolation equipment commonly referred to as “space suits” and elaborate procedures for entering the room, and decontamination procedures for leaving the room. In most cases this also includes high levels of security for access to the facility, ensuring that only authorized personnel may be admitted to any area that may have some effect on the quality of the containment zone. This is considered a hot zone.

5.2.1 Biosafety Level 1

This level is suitable for work involving well-characterized agents not known to consistently cause disease in healthy adult humans, and of minimal potential hazard to laboratory personnel and the environment. It includes several kinds of bacteria and viruses, e.g., non-pathogenic *Escherichia coli*, as well as some cell cultures and non-infectious bacteria. Laboratory personnel have specific training in the procedures conducted in the laboratory and are supervised by a scientist with general training in microbiology or a related science.

5.2.2 Biosafety Level 2

This level is similar to biosafety level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment. It includes various bacteria and viruses that cause only mild disease to humans, or are difficult to contract via aerosol in a lab setting. Genetically modified organisms also have been classified as level 2 organisms, even if they pose no direct threat to humans. BSL2 differs from BSL1 in that: laboratory personnel have specific training in handling pathogenic agents and are directed by scientists with advanced training; accessing to the laboratory is limited when work is being conducted; extreme precautions are taken with contaminated sharp items; and certain procedures in which infectious aerosols or splashes may be created are conducted in biological safety cabinets or other physical containment equipment.

5.2.3 Biosafety Level 3

This level is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease after inhalation. It includes various bacteria, parasites and viruses that can cause severe to fatal disease in humans, but for which vaccines or other treatments exist. Laboratory personnel have specific training in handling pathogenic and potentially lethal agents, and are supervised by competent scientists who are experienced in working with these agents. This is considered a neutral or warm zone. All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets, specially designed hoods, or other physical containment devices, or by personnel wearing appropriate personal protective clothing and equipment. The laboratory has special engineering and design features. It is recognized, however, that some existing facilities may not have all the facility features recommended for biosafety level 3 (e.g., double-door access zone and sealed penetrations). In this circumstance, an acceptable level of safety for the conduct of routine procedures (e.g., diagnostic procedures involving the propagation of an agent for identification, typing, susceptibility testing, etc.), may be achieved in a biosafety level 2 (P2) facility, providing the filtered exhaust air from the laboratory room is discharged to the outdoors, the ventilation to the laboratory is balanced to provide directional airflow into the room, access to the laboratory is restricted when work is in progress, and the recommended *Standard Microbiological Practices*, *Special Practices*, *and Safety Equipment* for biosafety level 3 are rigorously followed. The decision to implement this modification of biosafety level 3 recommendations is made only by the laboratory director.

5.2.4 Biosafety Level 4

This level is required for work with dangerous and exotic agents that pose a high

individual risk of aerosol-transmitted laboratory infections, agents which cause severe to fatal disease in humans for which vaccines or other treatments are not available. When dealing with biological hazards at this level the use of a hazmat suit and a self-contained oxygen supply is mandatory. The entrance and exit of a level 4 biolab will contain multiple showers, a vacuum room, an ultraviolet light room, and other safety precautions designed to destroy all traces of the biohazard. Multiple airlocks are employed and are electronically secured to prevent both doors opening at the same time. All air and water service going to and coming from a biosafety level 4 (P4) lab will undergo similar decontamination procedures to eliminate the possibility of an accidental release. Agents with a close or identical antigenic relationship to biosafety level 4 agents are handled at this level until sufficient data is obtained either to confirm continued work at this level, or to work with them at a lower level. Members of the laboratory staff have specific and thorough training in handling extremely hazardous infectious agents and they understand the primary and secondary containment functions of the standard and special practices, the containment equipment and the laboratory design characteristics. They are supervised by qualified scientists who are trained and experienced in working with these agents. Access to the laboratory is strictly controlled by the laboratory director. The facility is either in a separate building or in a controlled area within a building, which is completely isolated from all other areas of the building. A specific facility operations manual is prepared or adopted. Building protocols for preventing contamination often use negatively pressurized facilities, if compromised, which would severely inhibit the containment of an outbreak of aerosol pathogens.

Within work areas of the facility, all activities are confined to Class Ⅲ biological safety cabinets, or Class Ⅱ biological safety cabinets used with one-piece positive pressure personnel suits ventilated by a life support system. The biosafety level 4 laboratory has special engineering and design features to prevent microorganisms from being disseminated into the environment. The laboratory is kept at negative air pressure, so that air flows into the room if the barrier is penetrated or breached. Furthermore, an airlock is used during personnel entry and exit.

Review Questions

1. Term explanation: disinfection, sterilization, asepsis, antisepsis, antibiotics, bacteriocin, biosafety.
2. What are the physical methods to achieve disinfection purposes?
3. What are the types of chemical disinfectants and their action principles?
4. Try to describe the principle of thermal sterilization, and compare the advantages and disadvantages of the dry heat sterilization and the heat sterilization.

5. Try to describe the principle of radiation and filtration, and their advantages and disadvantages.

6. What are the differences between bacteriocin and antibiotics?

7. What are the main measures of biosecurity in veterinary microbiology laboratories?

8. What are the operations suitable for different levels of biosafety laboratories?

Chapter 4 Infection and Pathogenesis of Bacteria

Synopsis

Bacterial infection and disease have a complex process. Many terms are used to describe this process. These terms are parasitism, symbiosis, pathogenic microorganisms, opportunity pathogenic microorganisms, pathogenicity, virulence, infection, infection spectrum, infection way, infection of the minimum dose, pollution, colonization, recessive infection, apparent infection, endogenous infection, exogenous infection, local infection, systemic infection, septicemia, bacteremia, superinfection and secondary infection, etc. The pathogenicity and virulence of bacteria are mainly determined by the adhesion, invasin, aggressin, impedin, opsonin. After bacteria adhere to host cell, they invade, spread, and resist to the host non-specific and specific immune with different mechanism, then they grow and damage to host with different mechanism such as cytopathy, exotoxin, hydrolytic enzymes, virulence proteins, inflammation. The virulence of bacteria can be regulated by gene alteration, transcriptional regulation, post-transcriptional modification, temperature, ion, and quorum sensing.

1 Basic Terminology of Infectiology

Terms of pathogenicity and virulence are not clearly differentiated microbiologically (Table 4-1 and Table 4-2), and they could be considered as synonyms sometimes. There is a proposal that the term pathogenicity should be used to characterize a particular species while the term virulence should be used to describe the sum of the disease-causing properties of a population (strain) of a pathogenic species. Pathogenicity and virulence of a microorganism correspond to the infectious ability to a specific host species and affinity in a specific host organism. As a result, different individuals and different areas of the body may have different susceptibility to a pathogen, from highly disposed to resistant.

Table 4-1 Basic infectiological terminology Ⅰ (pathogen) (Kayser *et al.*, 2005)

Term	Explanation
Parasites	Unicellular or metazoan organism living in or on an organism of another species (host) on the expense of the host
Commensal	Normal inhabitants of skin and mucosa; the normal flora is thus the total commensal population
Pathogenic microorganisms	Classic disease-causing pathogens
Opportunists or facultative pathogenic microorganisms	Can cause disease in immunocompromised individuals given an "opportune" situation; these are frequently germs of the normal flora or occasionally from the surrounding environment, animals, or other germ carriers
Pathogenicity	Capacity of a pathogen species to cause disease
Virulence	Sum of the disease-causing properties of a strain of a pathogenic species
Infection spectrum	The totality of host species "susceptible" to infection by a given pathogen
Minimum infective dose	Smallest number of pathogens sufficient to cause an infection
Mode of infection	Method or pathway used by pathogen to invade host

Table 4-2 Basic infectiological terminology Ⅱ (host) (Kayser *et al.*, 2005)

Term	Explanation
Contamination	Microbiological presence of microorganisms on objects, in the environment, or in samples for analysis
Colonization	Presence of microorganisms on skin or mucosa; no penetration into tissues; typical of normal flora; pathogenic microorganisms occasionally also show colonization behavior
Infection	Invasion of a host organism by microorganisms, proliferation of the invading organisms, and host reaction
Inapparent (or subclinical) infection	Infection without outbreak of clinical symptoms
Infectious disease (or clinical infection)	Infection with outbreak of clinical symptoms
Endogenous infection	Infection arising from the colonizing flora
Exogenous infection	Infection arising from invasion of host by microorganisms from sources external to it
Local infection	Infection that remains restricted to the portal of entry and surrounding area
Generalized infection	Lymphogenous and/or hematogenous spread of invading pathogen starting from the portal of entry; infection of organs to which pathogen shows a specific affinity (organotropism) ; three stages: incubation, generalization, organ manifestation
Sepsis	Systemic disease caused by microorganisms and/or their toxic products; there is often a localized focus of infection from which pathogens or toxic products enter the bloodstream continuously or in intermittent phases
Bacteremia parasitemia	Brief presence of microorganisms in the bloodstream
Superinfection	Occurrence of a second infection in the course of a first infection
Reinfection	Series of infections by different pathogens

2 Determinants of Bacterial Pathogenicity and Virulence

The complicated relationships between the host and invading organisms, which are

various depending on the pathogens involved, determine the genesis, clinical picture and outcome of an infection. In spite of the variability, some general principles could describe the routine of the interactions between the invading pathogen with its aggressive factors and the host with its defenses. Being thoroughly studied in the area, the following summary is about the host-invader interactions in bacterial infectious diseases.

Bacterial pathogenicity and virulence could be determined by followings: ①adhesion to host cells; ②breaching of host anatomical barriers and colonization of tissues; ③strategies to overcome innate defenses, especially antiphagocytosis mechanisms; ④strategies to overcome specific immunity, or the most important of the productions of IgA proteases, immunogen variability and molecular mimicry; ⑤damage to host tissues and due to bacterial cytotoxicity, exotoxins and exoenzyme; ⑥damages lead to inflammatory reactions in the microorganism, and activation of complement and phagocytosis; induction of cytokines production.

The above bacterial pathogenicity factors are opposed by the following host defense mechanisms: ①Nonspecific defenses including mechanical, cellular systems, and humoral cellular systems. Phagocytosis is the most significant part. ②Specific immune responses based on antibodies and specific reactions of T lymphocytes. The response of these defenses to infection connects with many different mechanisms. Defects of innate defense undermine the restriction of an infection to take hold. Innate immune defects rarely occur while secondary immunity is common, thus some microorganisms called "facultative pathogens" (opportunists) take advantage and thrive.

Five potential bacterial contributors to the pathogenesis of infectious diseases: ①adhesin, which promotes adhesion to specific target cells; ②invasin, which takes part in active invasion of the cells of the macroorganism; ③impedin, which destroys host immune defenses in some cases; ④aggressin, which includes toxins and tissue-damaging enzymes; ⑤modulin, which induces excess cytokine production (e.g., lipopolysaccharides of Gram negative bacteria, murein fragment, superantigens).

2.1 Adhesion

The first step of an infectious process is adhesion. During the adhesion period, as soon as pathogenic contact with animal surface tissues (e.g., mucosa), they contrive to adhere their various surface structures (attachment pili, attachment fimbriae, adhesion proteins in the outer membrane of Gram negative bacteria, cell wall-associated proteins in Gram positive bacteria) to cell-surface receptor. This specific process requires the adhesion structure (or ligand) and the receptor to fit together like a key in a keyhole.

2.2 Invasion and Spread

2.2.1 Invasion

Bacteria may invade a host passively through micro-traumata or macro-traumata in the skin or mucosa. In another word, if bacterium invade through intact mucosa, it first attaches to this anatomical barrier, and then actively breaches it. Different bacterial species deploy a variety of mechanisms to reach this end, which is that producing of tissue-damaging exoenzyme to destroy anatomical barriers, and the other is that parasite-directed endocytosis, induced by invasioning on the surface of the bacterial cells, causes the cytoskeleton of the epithelial cell to form pseudopod that lead to endocytosis.

2.2.2 Spread

With the help of tissue-damaging exoenzyme (hyaluronidase, collagenase, elastase, and other proteases), bacteria spread immediately in the local tissue as they entry. Firstly, bacteria transfer into the intracellular space by endocytosis, concentrate the actin into filaments. The filaments tag on a bottom of the bacterium and push up against the inner side of the cell membrane. Next, the membrane will fuse with another membrane of the neighboring tissue cell so that the bacterium transfers to the new cell (typical of *Listeria* and *Shigella*).

2.3 Strategies Against Innate Immunity

The capacity of the pathogens conquering the host's innate immune defenses is the prerequisite to the establishment of a bacterial infection in a host. The following three points are the most important mechanisms maneuvered by bacteria.

2.3.1 Antiphagocytosis

2.3.1.1 Capsule

Blocked by capsule, the alternative complement pathway could not be activated, leading to the lack of the C3b (ligand for C3b receptor of phagocytes) on the surface of encapsulated bacteria. Microorganisms that apply these strategies include *Haemophilus influenzae* and *Streptococcus pneumoniae*.

2.3.1.2 Phagocyte Toxins

Examples: streptolysin from streptococci, leukocidin from staphylococci.

2.3.1.3 Inhibition of Phagosome-lysosome Fusion

Examples: *Chlamydophila psittaci*, tuberculosis bacteria.

2.3.1.4 Inhibition of the Phagocytic "Oxidative Burst"

No formation of reactive O_2 radicals in phagocytes. Examples: *Salmonella typhimurium*.

2.3.1.5 Others

Type Ⅲ secretion system of certain Gram negative bacteria (for example: *Shigella*, *E. coli*, *Salmonellae* and *Yersinia*). Toxic proteins are injected into the macrophages by this system.

2.3.2 Serum Resistance

Modified lipopolysaccharides in the outer membrane of bacteria inhibit the initiation of alternative complement pathway so that the membrane-attack complex, which would otherwise lyse holes in the outer membrane, is no longer produced.

2.3.3 Siderophores

Siderophores (e.g., enterochelin, aerobactin) as the low-molecular-weight iron-binding molecules, transport Fe^{3+} actively into the intracellular space. Their affinity to iron enable them to steal this element from proteins containing iron (transferrin, lactoferrin). The intricate iron transport system locates in the cytoplasmic membrane as well as in Gram negative bacteria's outer membrane. To thrive, bacteria require 10^{-5}mol/L free iron ions. The free availability of only about 10^{-20}mol/L iron ions in human body fluids, however, presents a challenge to them.

2.4 Strategies Against Specific Immunity

2.4.1 Immunotolerance

In such condition, the immune system is incapable of responding to extraneous bacterial immunogen.

2.4.2 Molecular Mimicry

By means of presenting the molecules on the surface, bacteria disguise themselves as intrinsic cells of their host in order to evade attacks of the immune system.

2.4.3 Antigen Variation

With the variability of the structural genes coding the antigen proteins, some bacteria are characterized by a pronounced variability of their immunogen. Varied antigens barely match with the dated antibodies so that the bacteria can escape from the capture of the immune system and thrive. For instance, gonococci can modify the primary structure of the pilin of their attachment pili in a high frequency.

2.4.4 IgA Proteases

Mucosal secretions contribute secretory antibodies of the sIgA to the specific local immunity of the mucosa. Some pathogens such as *Haemophilus influenzae* and gonococci produce proteases to destroy this immunoglobulin.

3 Clinical Diseases

The clinical symptoms of a bacterial infection can be caused by the damaging bacterial noxae and excessive host immune responses, both specific and nonspecific. Paradoxically, immune response has the potential either to protect or to damage host's health.

3.1 Cytopathic Effect

The infected host cell may die because of the reproduction of the obligate intracellular parasites (e.g., *Rickettsiae*, *Chlamydiae*) inside the host cell.

3.2 Exotoxins

Pathogenic bacteria can produce a variety of toxins that can cause disease, independently (e.g., in diphtheria, cholera, and tetanus) or coupling with other factors. Admittedly, the classification and nomenclature of these toxins must reflect the type of cell affected. Cytotoxins produce toxic effects in a large range of different host cells; neurotoxins affect the neurons; enterotoxins affect enterocytes. The structures and mechanisms of action of the toxins should be also taken into account in their classification.

3.2.1 AB Toxins

AB toxins contain subunit "B" and subunit "A". The subunit "B" is responsible for binding to specific surface receptors on target host cells whereas subunit "A" has catalytic activity representing the active agent. AB toxins only target and damage the cells which present the "B" receptors.

3.2.2 Membrane Toxins

With the attachment and assembly between the toxins and biological membranes, toxins membrane structure by forming phospholipases.

3.2.3 Superantigens

These antigens stimulate T lymphocytes and macrophages to produce excessive amounts of harmful cytokines.

3.3 Hydrolytic Exoenzymes

Proteases (e.g., elastase, collagenase and nonspecific proteases), neuraminidase (synonymous with sialidase), hyaluronidase, DNase and lecithinase contribute at various levels to the pathogenesis of an infection.

3.4 Secretion of Virulence Proteins

The protein synthesis was processed at ribosomes in the cytoplasm. The protein secretion must cross through the cytoplasmic membrane and in Gram negative bacteria through the outer membrane as well. In addition, with the various compositions and pathway, the complex protein secretion systems (Ⅰ-Ⅳ) contribute to the secretion process. The type Ⅲ (virulence-related) secretion system in certain Gram negative bacteria (*Shigella*, *Salmonella*, *Escherichia coli*, *Bordetella*, *Yersinia*, *Chlamydia*) is particularly important.

As soon as specific Gram negative rod bacteria touch the eukaryotic target cells, a molecular sensor interacts with a receptor on the target cells. This interaction induces the cells to open secretion channel of so-called “needle complex” (extending through both the cytoplasmic membrane and outer membrane) and to form a pore in the membrane. Cytotoxic molecules are transported into the cytoplasmic matrix through the pore and channel. In the cytoplasmic matrix, the cytotoxic molecules inhibit cytokine production (in macrophages) and phagocytosis, destroy the cytoskeleton of the target cell, and generally work to induce apoptosis.

3.5 Cell Wall

The endotoxin of Gram negative bacteria (lipopolysaccharide) significantly corresponds to the representation of clinical symptoms. It initiates the complement alternative pathway and, as a result, releases chemotactic components C3a and C5a so that induces an inflammation at infection region. Moreover, it also facilitates the production of endogenous pyrogens (interleukin 1, tumor necrosis factor) by stimulating macrophages, thus inducing fever centrally. The production of endogenous pyrogens and other cytokines are increased, which result in hypotension, stimulation of granulopoiesis, intravasal coagulation, thrombocyte aggregation and intravasal coagulation. Increased production of cytokines by macrophages, and the cytokines were also induced by soluble murein fragments and, by teichoic acids.

3.6 Inflammation

Inflammation can be induced by the integrated effects of the specific and nonspecific

immune responses of the host organism. Complement activate by way of both the classic and alternative pathways stimulates phagocyte to migrate to the infection region. Purulent tissue necrosis follows. Tuberculosis developed typical granulomas and caseous necrosis are the resulted of excessive reaction by the cellular immune system to the immunogen of tuberculosis bacteria.

4 Regulation of Bacterial Virulence

Many pathogenic bacteria have the ability to live outside or inside a host and to attack various host species. Reproduction in various environments demands an effective regulation of virulence which need to be expressed in accordance with the requirement. Some different regulatory mechanisms have been described as followed.

4.1 DNA Changes

The nucleotide sequences of virulence determinants are altered, such as pilin gene variability which is related to intracellular recombination as described above in gonococci and inverting a leader sequence to turn genes on and off in the phase differentiation of H antigens in *Salmonellae*.

4.2 Transcriptional Regulation

The principle of transcriptional control of virulence determinants substantially resembles that applying to the regulation of metabolic genes, in another word repression and activation.

4.3 Simple Regulation

The regulation of diphtheria toxin genes has been thoroughly characterized. For example, a specific cytoplasmic iron concentration motivates the diphtheria toxin regulator (*DtxR*). The production of active repressor combines to the promoter region to block the transcription of the toxin gene.

4.4 Complex Regulation

In many cases, a number of virulence genes are initiated and repressed by the same regulator protein. The pertinent virulence determinants are either components of the same operon or are located at individual genome regions. Several *vir* (virulence) genes with promoter regions are controlled by the same regulator protein, so-called *vir* regulon. Regulation of the virulence regulon of *Bordetella pertussis* gene activation has

been studied precisely. This particular regulon containing over 20 virulence determinants which are all regulated by the same *vir* regulator protein (or *BvgA* coding region).

4.5 Posttranscriptional Regulation

This term refers to regulation by a posttranslational protein or mRNA modification.

4.6 Quorum Sensing

This term describes that bacterial cell density regulates genes expression. This phenomenon is observed in both Gram negative and Gram positive bacteria. It suggests a mode of communication between bacterial cells that enables a bacterial population to react analogously to a multicellular organism.

Accumulation of certain density of a low-molecular-weight pheromone enables a bacterial population to sense when the critical cell density (quorum) has been reached, that will enable bacteria to invade the host successfully, at which point transcription of virulence determinants is initiated.

5 The Genetics of Bacterial Pathogenicity

The virulence genes of pathogenic bacteria are frequently composed of mobile genetic elements such as conjugation transposon, bacteriophage genomes or plasmids, which make the horizontal transfer of these genes between bacterial cells possible. Regions containing numerous virulence genes in a bacterial chromosome are called pathogenicity islands (PIs).

PIs are found in both Gram positive and Gram negative bacteria. These DNA regions, up to 200kb, often bear not only several different virulence genes but specific sequences located at their ends (e.g., IS elements) facilitating horizontal transfer of the PIs between bacterial cells. Furthermore, the GC contents of PIs are different from general DNA of chromosomal that could indicate the translocation of this island in the evolutionary process.

Review Questions

1. Term explanation: parasitism, symbiosis, pathogenic microorganisms, opportunity pathogenic microorganisms, pathogenicity, virulence, infection spectrum, pollution, colonization, infection, infectious disease, endogenous infection, exogenous infection, local infection, systemic infection, septicemia, bacteremia, superinfection, secondary infection, secretory system.
2. Try to describe the Koch's principles at gene level.

3. What are the components of bacterial virulence?

4. Try to describe the mechanism of bacterial escape from host immunity.

5. What are types of bacterial toxins?

6. How is the expression of bacterial virulent factors regulated?

7. What is the bacterial secretory system, and what is the significance of secretory system?

8. Try to describe the main process of bacterial invasion into host cell.

Chapter 5 Bacterial Heredity and Variation

Synopsis

The genome is the material basis of bacterial heredity and variation. Plasmid is genetic material outside the genome, which can self-replicate and control many traits of bacteria. Bacterial heredity and variation can occur through spontaneous mutation and artificial mutagenesis. The transfer ways of DNA between bacteria mainly includes transformation, transduction, conjugation, etc. F plasmid, resistance plasmid and virulence plasmid are often transferred by means of conjugation.

1 The Molecular Basis of Bacterial Genetics

Bacteria possess two genetic structures which are chromosome and the plasmid. Both of these structures consist of a single circular DNA double helix twisted counterclockwise about its helical axis. Replication of DNA always starts at a certain point (the origin of replication) and is a "semiconservative" pattern, that is, one strand in each of the two double strands resulted remain constant. Most bacterial genes code different proteins or polypeptides, but the non-coding interposed sequences (introns), like those genes seen in eukaryotes, are the exception. Certain bacterial genes have a mosaic structure. The phases of DNA transcription are the promoter recognition, elongation, and termination. Many bacterial mRNAs are polycistronic, which mean they contain the genetic information for several polypeptides. DNA translation is taken place on the 70S ribosomes. The start and stop of polypeptide synthesis are marked with special mRNA codons. Many genes that code for functionally related polypeptides are grouped together in plasmid segments or chromosome, which are known as operons. The most important regulatory mechanism is the positive or negative control of transcription initiation. This kind of control function may be exercised by individual localized genes, such as some of the genes of operons or the genes in regulation.

1.1 The Structure of Bacterial DNA

All of bacterium's genetic information are stored in their chromosomes and plasmids. Each of these structures is made of a single DNA double helix twisted to the right, then additionally twisted to the left about its helical axis, which is named supercoiled structure DNA (Fig. 5-1). In general, the plasmid is composed of circular DNA, sometimes, we can also find linear plasmids, although this is rare. This DNA topology structure solves spatial problems and enables such functions as replication, transcription, and recombination. Some genes are composed of a mosaic of minicassettes interconnected by conserved DNA sequences between the cassettes.

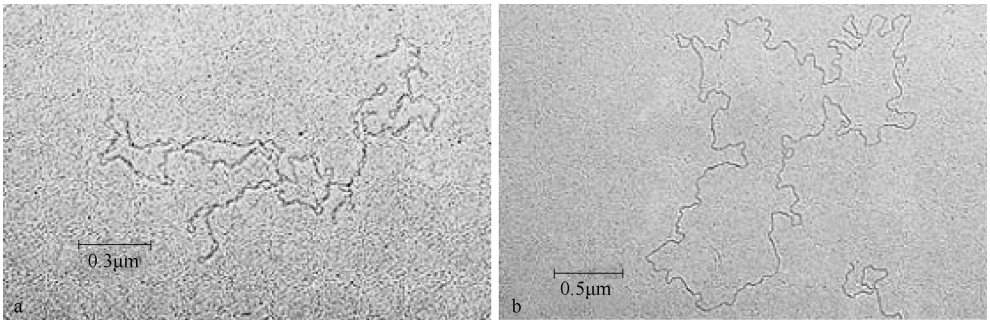


Fig. 5-1 Plasmid in *Escherichia coli* (Kayser *et al.*, 2005)
a. covalently closed circle (CCC); b. open circle

1.1.1 Chromosome

The chromosome corresponds to the nucleoid. For example, the *E. coli* chromosomes are composed of 4.63×10^6 base pairs (bps). It codes for 4,288 proteins at least. The gene sequence is colinear with the expressed genetic products. Non-coding interposed sequences (introns) normally seen in prokaryotic genes are very rare. The chromosomes of *E. coli* and many other pathogenic bacteria have now been completely sequenced.

1.1.2 Plasmids

Plasmids are autonomous DNA molecules of varying size (3×10^3-4.5×10^5bp) localized in the cytoplasm. Large plasmids are usually present one to two copies in per cell, whereas small ones may be present 10, 40, 100 or more copies. Plasmids are not essential for the metabolism of cells.

Many of the genes carried in plasmid that code for certain phenotypic characteristics of the host cell. The following plasmid types are important: ①virulence plasmids, which carry determinants of bacterial virulence, e.g., enterotoxin genes or hemolysin

genes; ②resistance plasmids, which carry genetic information bearing on resistance to anti-infective agents. R plasmids may carry several R genes. Plasmids also have been described that carry both virulence and resistance genes.

1.2 DNA Replication

The identical duplication process of DNA is termed semiconservative, because the double strand of DNA is opened up during replication, whereupon each strand serves as the matrix for synthesis of a complementary strand. Thus, each of the two new double strands "conserves" one old strand. The doubling of each DNA molecule (replicon) begins at a given starting point, the so-called origin of replication. This process continues throughout the entire fission cycle.

1.3 Transcription and Translation

1.3.1 Transcription

The continuous genetic nucleotide sequence is transcribed "colinearly" into mRNA. This principle of colinearity is adapted to very few exceptions. The transcription process can be decomposed into the three stages of promoter recognition, elongation, and termination. The promoter region is the site where the RNA polymerase begins reading the DNA sequence. A sigma factor is necessary for binding to the promoter. Sigma factors are proteins that associate temporarily with the RNA polymerase (core enzyme) to form a holoenzyme, then dissociate themselves once the transcription process has begun, making them available to associate once again. Specific sigma factors can recognize the most genes of standard promoters. Additional sigma factors, their expression depends on the physiological status of the cells, facilitate the transcription of special determinants. Genes that code for functionally related proteins, for example, proteins that act together to catalyze a certain metabolic step, are often arranged sequentially at specific locations on the chromosome or plasmid. Such DNA sequences are named operons (Fig. 5-2). The mRNA synthesized by the transcription of an operon is polycistronic, i.e., it contains the information sequences of several genes. The information sequences are separated by intercistronic regions. Each cistron has its own start codon and stop codon in the mRNA.

1.3.2 Translation

The transformation of the nucleotide sequence carried by the mRNA into the polypeptide amino acid sequence at the 70S ribosomes. In principle, the translation of prokaryote and eukaryotic is the same. The enzymes and other factors involved doing, however, differ structurally and can therefore be selectively blocked by antibiotics.

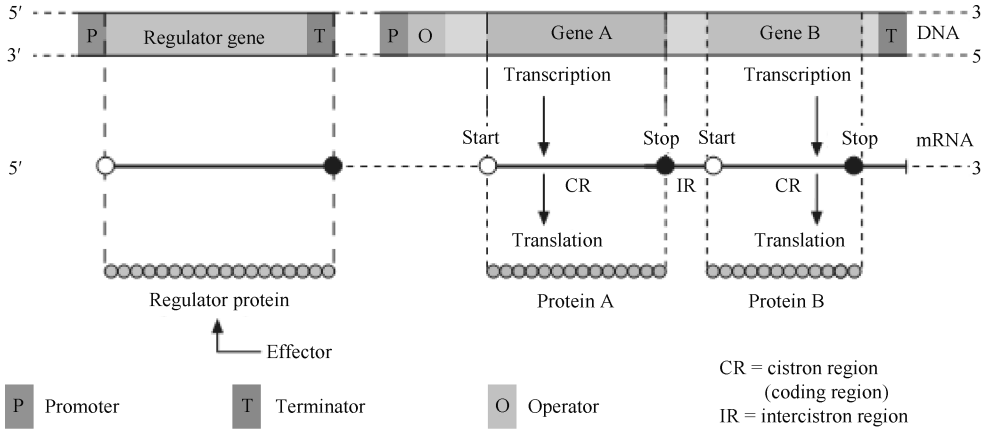


Fig. 5-2 Bacterial operon and regulator gene (Kayser *et al.*, 2005)

1.4 Regulation of Gene Expression

Bacteria present a truly impressive capacity for adapting to their environment. Many of regulatory bacterial mechanisms are known, for example, posttranslational regulation, translational regulation, transcription termination, and quorum sensing. The details of all these mechanisms would exceed the scope of this book. The most important thing is regulation of the initiation of transcription by means of activation or repression, the process is not observed in this form in eukaryotes: a single gene, or several genes in an operon at one DNA location, may be affected (Fig. 5-2). The mechanism that has been investigated most thoroughly is transcriptional regulation of catabolic and anabolic operons by a repressor or activator.

A single regulator protein can also activate or repress several genes not integrated in an operon, i.e., at various locations on the DNA. Such functional gene groups are called regulons. Alternative sigma factors may be involved in transcriptional activation of special genes with special promoters. Physiological cell status determines whether or not these alternative factors are produced.

2 The Genetic Variability of Bacteria

Changes in bacterial DNA are the result of spontaneous mutations in individual genes as well as recombination processes resulting in new genes or genetic combinations. Based on the molecular mechanisms involved, bacterial recombinations are divided into homologous, site-specific, and transpositional. The latter two especially reflect the high mobility of many genes and have made essential contributions to the evolution of bacteria.

Although sexual heredity is unknown in bacteria, they do make use of the mechanisms of intercellular transfer of genomic material known as parasexual processes.

Transformation designates the transfer of DNA that is essentially chemically pure from a donor into a receptor cell. In transduction, bacteriophages can serve as the vehicles for DNA transport. Conjugation is the transfer of DNA by means of cell-to-cell contact. This process made possible by conjugation plasmids and transposons can be a high- frequency one and may even occur among partners of different species, genera, or families. The transfer mainly involves the conjugating elements themselves. Conjugated structures carrying resistance or virulence genes have considerable medical significance.

The processes of restriction and modification are important factors limiting genetic exchange among different taxa. Restriction is based on the effects of restriction endonucleases, which can be capable of specific excision of foreign DNA sequences. These enzymes have become important and invaluable tools in the field of genetic engineering.

2.1 Molecular Mechanisms of Genetic Variability

2.1.1 Spontaneous Mutation

The changes in the characteristics of bacterial populations were confirmed by the so-called fluctuation test, which was the results of rare, random mutations in the genes of individual cells, and then were selected. Such mutations may involve substitution of a single nucleotide, frame-shifts, deletions, inversions, or insertions. The frequency of mutations is expressed as the mutation rate, which is defined as the probability of mutation per gene per cell division. The rate depends on the gene involved and is approximately 10^{-10} to 10^{-6}. Mutation rates may increase drastically due to mutagenic factors, such as radioactivity, UV radiation, alkylating chemicals, etc.

2.1.2 Recombination

The term recombination designates processes that can lead to the restructuring of DNA, formation of new genes or genetic combinations.

2.1.2.1 Homologous (generalized) Recombination

A precise exchange of DNA can happen between corresponding sequences. Several enzymes will contribute to the complex breakage and reunion process involved. The Rec A enzyme and another Rec BC nuclease are the most important.

2.1.2.2 Site-specific Recombination

Integration or excision of a sequence can happen in or from target DNA. Only a single sequence of a few nucleotides of the integrated DNA is homologous with the recombination site on the target DNA. Integration of bacteriophage genomes is an example of what this process facilitates. Integration of several determinants of antibiotic resistance in one integron can also utilize this process (Fig. 5-3). Resistance integrons may be integrated in transposable DNA.

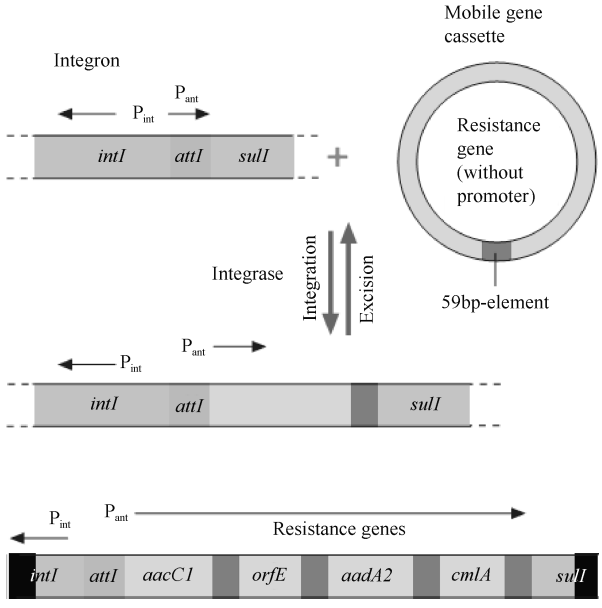


Fig. 5-3 Site-specific recombination (integron) (Kayser *et al.*, 2005)

2.1.2.3 Transposition

It is not necessary to be homologous for the donor and target DNA in the transposition process. DNA sequences can either be transposed to a different locus on the same molecule or to a different replicon. Just as in site specific recombination, transposition has always played an important role in the evolution of multi-resistance plasmids (Fig. 5-4).

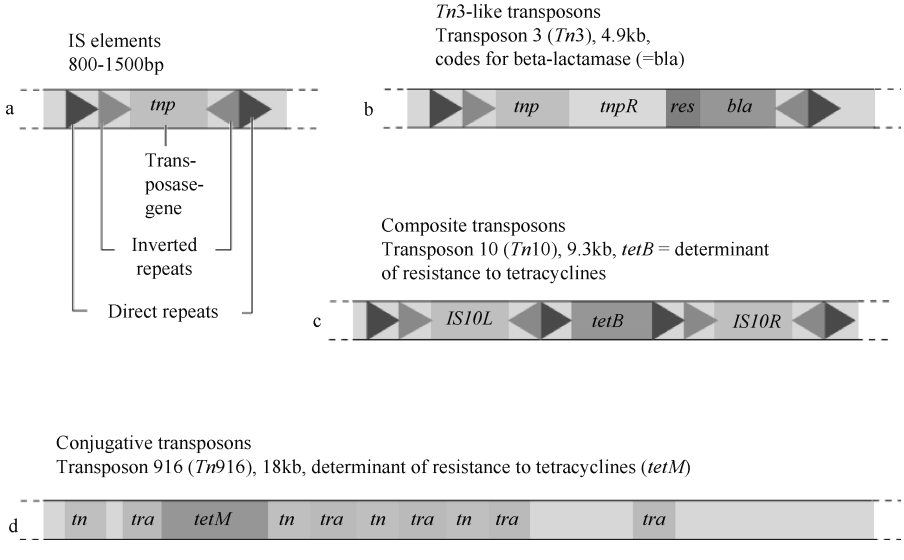


Fig. 5-4 Transposable DNA elements (Kayser *et al.*, 2005)

2.2 Intercellular Mechanisms of Genetic Variability

In the strict sense, although bacteria have no sexual heredity, they have mechanisms that allow for intercellular DNA transfer. These mechanisms, which involve a unilateral transfer of genetic information from a donor cell to a receptor cell, are subsumed under the term parasexuality.

2.2.1 Transformation

Transfer of "naked" DNA. Griffith demonstrated that the ability to produce a certain type of capsule could be transferred between different pneumococci in 1928. Then, Avery showed that the transforming principle at work was DNA in 1944. This transformation process has also been observed in the genera *Streptococcus* and *Haemophilus*.

2.2.2 Transduction

With the help of transport bacteriophages, DNA can transfer from a donor to a receptor (Fig. 5-5). Bacteriophages are viruses which can infect bacteria. During their replication process, DNA sequences from the host bacterial cell may replace all or part of the genome in the phage head. Such phage particles are then defective. They can still enter into receptor cells and inject their DNA, but the infected bacterial cell will then neither produce new phages nor be destroyed.

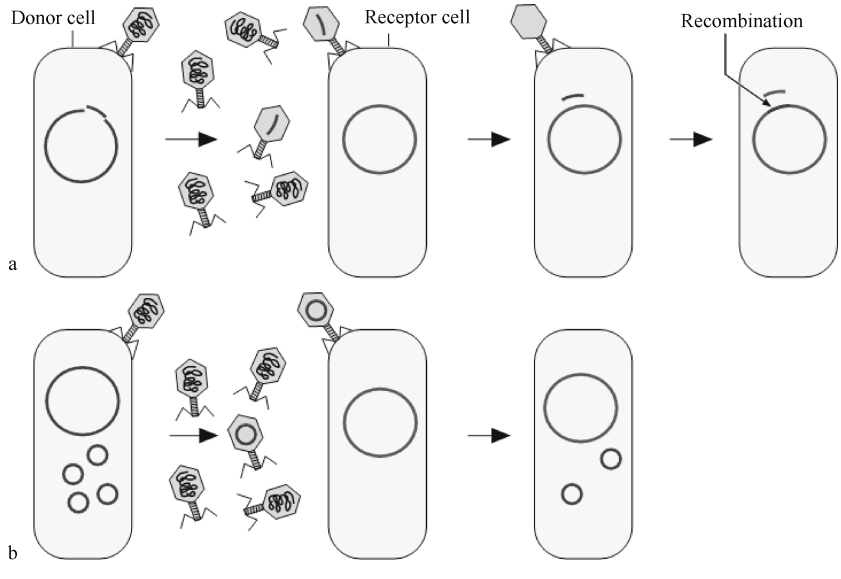


Fig. 5-5 Transduction of a chromosomal DNA sequence (a) and a plasmid (b) (Kayser *et al.*, 2005)

2.2.3 Conjugation

Conjugation is the transfer of DNA from a donor to a receptor in a conjugal process involving cell-to-cell contact. They are two genetic elements, which are the conjugation plasmids and the conjugation transposons, to make conjugation possible (Fig. 5-6).

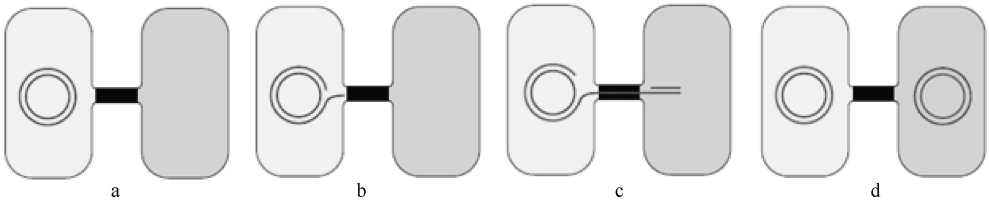


Fig. 5-6 Transfer/replication process of a conjugative plasmid (Kayser *et al.*, 2005)

In the conjugation process, the conjugating elements themselves are what are primarily transferred. However, these elements can also mobilize chromosomal genes or otherwise nontransferable plasmids. Conjugation is seen frequently in Gram negative bacteria, in which the phenomenon has been most thoroughly researched, and the same with enterococci.

2.2.3.1 The F-factor in *E. coli*

This is the prototype of a conjugation plasmid. This factor contains the *tra* (transfer) genes responsible both for the formation of conjugal pili on the surface of F cells and for the transfer process. Transfer of the conjugation plasmid takes place as showed here in schematic steps.

Occasional integration of the F factor into the chromosome can give it the conjugation properties of the F factor. Such integration produces a sort of giant conjugating element, so that chromosomal genes can also be transferred by the same mechanism. Cells with an integrated F factor are therefore called Hfr (high frequency of recombination) cells.

2.2.3.2 Conjugative Resistance and Virulence Plasmids

Conjugation plasmids, which carry determinants coding for antibiotic resistance and/or virulence in addition to the *tra* genes and *repA*, have considerable medical importance. Three characteristics of conjugation plasmids promote a highly efficient level spread of these determinant factors among different bacteria. ①High frequency of transfer, which bases on the "transfer replication" mechanism, each receptor cell which has received a conjugation plasmid automatically can become a donor cell. Each plasmid-positive cell is also capable of multiple plasmid transfers to receptor cells. ②Wide range of hosts, which is that many conjugation plasmids can be transferred between

different taxonomic species, genera, or even families, and they can replicate in these cells, besides the genetic information carrying in plasmid can be transferred into the host cells. ③Multiple determinants, which is that many conjugation plasmids usually carry several genes determining the phenotype of the carrier cell. The evolution of a hypothetical conjugative plasmid carrying several resistance determinants is shown schematically (Fig. 5-7).

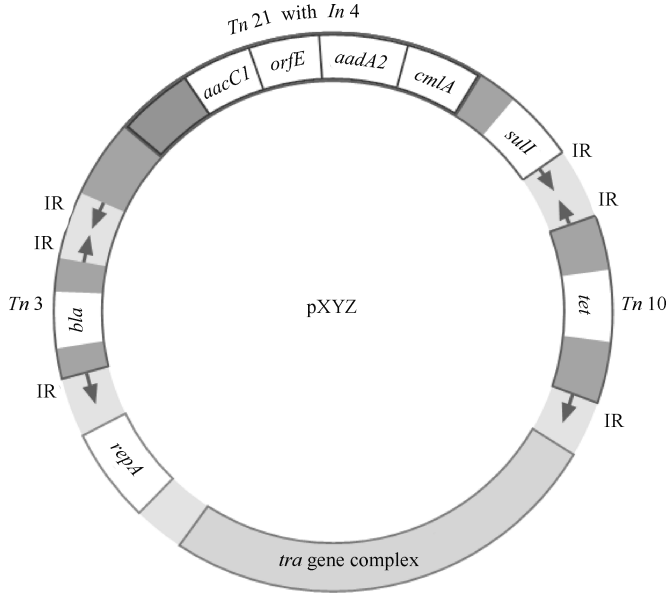


Fig. 5-7 Model of a hypothetical conjugative multiple-resistance plasmid (Kayser *et al.*, 2005)

Multiple resistance plasmids can result from successive integration of transposable resistance DNA or integration of resistance integrons.

*Tn*21, transposon of the *Tn*21 family, codes for resistance to sulfonamides (*sulI*) and contains an R integron (*In*4). *In*4 codes for a chloramphenicol acetyltransferase (=*cmlA*), an aminoglycoside acetyltransferase (=*aacC1*) and an aminoglycoside adenylyltransferase (=*aadA2*); also contains an open reading frame (ORF) of unknown function. *Tn*3, transposon 3, codes for a betalactamase (=*bla*). *Tn*10, transposon 10, codes for resistance to tetracyclines (=*tet*). The *repA* codes for the replication enzyme of the plasmid. The *tra* plasmid DNA region contains 25 *tra* genes, which are responsible for the transfer and replication process.

2.2.3.3 Conjugative Transposons

These DNA elements can usually be integrated into the bacterial chromosome. They happen mainly in Gram positive cocci, but sometimes have also been found in Gram negative bacteria (*Bacteroides*). Conjugation transposons may carry determinants for

antibiotic resistance and thus contribute to horizontal resistance transfer. In the transfer process, the transposon is first excised from the chromosome and circularized. Then a single strand of the double helix is cut and the linearized single strand analogous to the F factors transferred into the receptor cell. Conjugation transposons are also capable of mobilizing non-conjugation plasmids.

3 Restriction, Modification and Gene Cloning

The above descriptions of the mechanisms of genetic variability might make the impression that genes pass freely back and forth among the different bacterial species, rendering the species definitions irrelevant. This is not the case. Many of control mechanisms limit these genetic exchange processes. Among the most important mechanisms are restriction and modification. Restriction endonucleases can destroy foreign DNA that bears no "fingerprint" (modification) signifying "self". These modifications take the form of methylation of the DNA bases by modification enzymes.

The DNA sequence cloned is integrated into a vector DNA (plasmid, bacteriophage genome). This vector is then transferred into a host bacterium, e.g., *E. coli*, for identical replication.

Bacterial restriction endonucleases are invaluable tools in modern gene cloning techniques. The process is termed "gene cloning", because it involves replication of DNA which has been manipulated *in vitro* in a suitable host cell so as to produce identical copies of this DNA: molecular clones or gene clones. The technique simplifies the replication of DNA, and make experimental manipulations easier. On the other hand, the bacteria can also be used to synthesize gene products of different foreign genes. The products, foreign proteins expressed, are called recombinant proteins. Bacterial plasmids often have a function in the role of vectors into which the sequences to be cloned are inserted.

Review Questions

1. Term explanation: heredity, variation, gene, genome, plasmid, virulence island, transposition factor, mutation, gene transfer, gene recombination, transformation, transduction, conjugation, gene engineering.

2. What is the significance of bacterial heredity and variation?

3. What are the types and characteristics of plasmid?

4. What is the difference between the virulence island and the transposition factor? And what functions they have?

5. What are the differences between gene transfer and gene recombination? And what ways they have?

6. What is the difference between transformation and transduction? And what is the features they have?

7. How can we make useful services to the human society through the heredity and mutation of bacteria?

8. What is the difference between natural mutation and artificial mutagenesis?

Chapter 6 Classification and Nomenclature of Bacteria

Synopsis

The name of the bacteria is known as binomial nomenclature, which include generic name and specific name. The presentation of the bacterial name is in Latin, italic, capital and lowercase letters. The classification rank of bacteria adopts the kingdom, the gate, the class, the order, the family, the genus and the species. Species name is the basic unit of bacterial classification. Bacteria can be classified according to different phenotypes or characteristics. According to the results of Gram staining, bacteria can be divided into Gram positive and Gram negative bacteria. According to the differences of cell wall, bacteria can be divided into eubacteria and archaea. At present, classification of bacteria based on phylogenetic relationship (at nucleic acid levels, e.g., 16S rRNA sequencing) is the most common method. *Bergey's Manual of Systematic Bacteriology* and *Bergey's Manual of Determinative Bacteriology* are the authoritative system of bacterial classification, and prokaryotes were supplemented. There are many methods for the identification of bacteria, of which PCR method is widely used.

1 Definition

Classification, nomenclature and identification are the three separate but interrelated areas of taxonomy in microbiology. Classification is the arrangement of organisms into taxonomic groups (taxa) based on similarities or relationships. Classification of prokaryotic organisms such as bacteria requires knowledge obtained by experimental and observational techniques, because biochemical, physiologic, genetic and morphologic properties are necessary for an adequate description of a taxon. Nomenclature is naming an organism by international rules according to its characteristics. Identification refers to the practical use of a classification scheme: ①to isolate and distinguish desirable organisms from undesirable ones; ②to check the reliable or special properties of a culture or which are in a clinical setting; ③to isolate and identify the causative

agent of a disease. Identification schemes are not classification schemes, though there may be a superficial similarity. An identification scheme for a group of organisms can be devised only after that group has first been classified, namely, recognized as being different from other organisms.

2 Criteria for Classification of Bacteria

Suitable criteria for bacterial classification include many properties of bacteria. The value of a taxonomic criterion depends upon the compared groups. Traits shared by all or none cannot be used to distinguish its members, but they may define a group.

Valuable information can be obtained by observing the cell shape and the presence or absence of special structures such as spores or flagella. Staining procedures such as the Gram stain can provide a reliable assessment. Some bacteria produce characteristic pigments, and others can be differentiated on the basis of their extracellular enzymes (the activity of these proteins often can be detected as clearing zones of surrounding colonies grown with insoluble substrates, e.g., zones of hemolysis in agar medium containing red blood cells). The use of specific antibodies can give a rapid indication of similar surface structures carried by independently isolated bacteria. Tests such as the oxidase test can be used to distinguish organisms based on the presence of respiratory enzymes cytochrome C. Simple biochemical tests can know characteristic metabolic functions. Criteria leading to successful grouping of some related organisms include the sensitivity measurement to antibiotics.

Genetic instability can cause some traits to be highly variable within a biologic group or even within a single cell line. Developments in molecular biology now make it possible to investigate the relatedness of genes or genomes by comparing sequences in different bacteria.

Most criteria for classification depend upon growth of the microorganism in the laboratory. However, organisms such as the pathogenic treponema sometimes do not grow in the laboratory, and in these instances techniques that reveal relatedness by measurement of nucleic acid hybridization or by DNA sequence analysis may have particular value.

3 Identification and Classification Systems

3.1 Keys

Keys to organize bacterial traits in a manner permit efficient identification. The ideal system should contain the minimum number of features required for a correct identification. Groups are split into smaller subgroups based on the presence (+) or

absence (–) of a character. Continuation of the process with different characters guides the investigator to the smallest defined subgroup containing the analyzed organism.

In early stages, it would be perfectly reasonable that organisms may be assigned to subgroups based on characteristics which do not reflect genetic relatedness. For example, a key to bacteria includes a group such as "bacteria forming red pigments" even though this would include such unrelated forms as *Serratia marcescens* and purple photosynthetic bacteria. These two bacterial assemblages occupy distinct niches and have entirely different forms of energy metabolism. Preliminary grouping of the assemblages would be useful because which would immediately make it possible to narrow the range of relatively few types for an investigator.

3.2 Numerical Taxonomy

Numerical taxonomy (also called computer taxonomy, phenetics, or taxometrics) had been widely used in the 1960s. Numerical classification schemes use a large number (frequently 100 or more) of unweighted taxonomically useful characteristics. Computer clusters different strains at selected levels of overall similarity (usually >80% at the species level) on the basis of the frequency of shared traits. In addition, numerical classification provides percentage frequencies of positive character states for all strains within each cluster. Such data are used as a basis for the construction of a frequency matrix for identification of unknown strains against the defined taxa. Computerized databases had been applied to develop diagnostic tests that identify clinically relevant isolates through numerical codes or probabilistic systems.

3.3 Phylogenetic Classification

Phylogenetic classification is a measure which can present the genetic divergence of different phyla (biologic divisions). Close phylogenetic relatedness of two organisms implies that they share a recent ancestor, and the fossil record has made such inferences relatively easy to draw.

The genetic properties of bacteria may allow some genes to be exchanged among related organisms. Furthermore, multiplication of bacteria is almost entirely vegetative, and their mechanisms of genetic exchange rarely involve recombination among large portions of genomes. Therefore, the concept of a species, the fundamental unit of eukaryotic phylogenies, has an entirely different meaning when applied to bacteria. Eukaryotic species are a biologic group which can produce viable offspring. The current definition of species of bacteria is pragmatic, operational and universally applicable. A species is a category that circumscribes a genomically coherent group of individual isolates or strains sharing a high degree of similarity in many independent features which are comparably tested under highly standardized conditions. The

decision to circumscribe clusters of organisms within a bacterial species is presented by the taxonomist, who may choose to subdivide the group into biotypes and to cluster species with genera. Broader groupings such as families may be proposed.

The formal ranks used in the taxonomy of bacteria are kingdom, division, class, order, family, genus, species, for example, Prokaryotae, Gracilicutes, Scotobacteria, Eubacteriales, Enterobacteriaceae, *Escherichia coli*. For practical purposes, only the ranks of the family, genus, and species are commonly used.

Genetic diversity exists among bacteria. Chemical characterization of bacterial DNA revealed a wide range of nucleotide base compositions when DNA from different bacterial sources was compared. The G (guanine) and C (cytosine) compositions of DNA from a single source were always equal, as were the A (adenine) and T (thymine) compositions. These data provided an important clue concerning the base pairing of complementary strands in the physical structure of DNA. The evidence also showed that the G+C content of closely related bacteria was similar. This was the first indication that the chemical properties of DNA from different organisms could give an indication of their genetic relatedness. Physical studies revealed that the relatedness of DNA from similar organisms could be discerned by a measurement of the ability of their chromosomal DNA to cross-hybridize. The parameter DNA-DNA similarity and the difference in thermal denaturation midpoint (ΔT_m) are the standards for species delineation.

DNA sequencing has become a routine laboratory procedure. Comparison of the DNA sequences can give a measure of their relatedness. Genes for different functions have diverged at different rates, but in general, the relative rates of divergence are similar. Thus, DNA sequence differences among rapidly diverging genes can be used to ascertain the genetic distance of closely related groups of bacteria, and sequence differences among slowly diverging genes can be used to measure the relatedness of widely divergent groups of bacteria.

Ribosomes have an essential role in the synthesis of protein. Genes encoding ribosomal RNAs and ribosomal proteins have been highly conserved throughout evolution and have diverged more slowly than other chromosomal genes. Comparison of the nucleotide sequence of 16S rRNA from a range of biologic sources can reveal evolutionary relationships among widely divergent organisms and has led to the elucidation of a new kingdom, the archaea.

Recently, hybridization of DNA to high-density oligonucleotide arrays has been applied for species identification.

3.4 *Bergey's Manual of Systematic Bacteriology*

The possibility that one might draw inferences about phylogenetic relationships among

bacteria is reflected in the organization of the latest edition of *Bergey's Manual of Systematic Bacteriology*. First published in 1923, the manual is an effort to classify the known bacteria and to make this information accessible in the form of a key. A companion volume, *Bergey's Manual of Determinative Bacteriology*, serves as an aid in the identification of those bacteria that have been described and cultured.

Because emerging information concerning phylogenetic relationships will lead to further modifications in the organization of bacterial groups within *Bergey's Manual*, its designations must be regarded as provisional.

4 Descriptions of the Major Categories of Bacteria

There are two different groups of prokaryotic organisms: eubacteria and archaea. Eubacteria contain the more common bacteria which most people are familiar. Aarchaea do not produce peptidoglycan, a major difference between them and typical eubacteria, live in extreme environments (e.g., high temperature, high salt, or low pH), and carry out unusual metabolic reactions (e.g., the formation of methane). The four major categories are known based on the character of the cell wall: Gram negative eubacteria that have cell walls, Gram positive eubacteria that have cell walls, eubacteria lacking cell walls, and the archaea.

4.1 Gram Negative Eubacteria That Have Cell Walls

This is a heterogeneous group of bacteria that have a complex (Gram negative type) cell envelope consisting of an outer membrane, an inner, thin peptidoglycan layer (which contains muramic acid and is present in all but a few organisms that have lost this portion of the cell envelope), and a cytoplasmic membrane. The cell shape may be spherical, oval, straight or curved rods, helical, or filamentous. The mode of reproduction is binary fission. Members in this category may be phototrophic or non-phototrophic bacteria, and include aerobic, anaerobic, facultatively anaerobic and microaerophilic species, while some members are obligated to intracellular parasites.

4.2 Gram Positive Eubacteria That Have Cell Walls

These bacteria have a cell wall profile of the Gram positive type. Cells generally, but not always, stain Gram positive. Cells may be spherical, rods, or filaments. The mode of reproduction is binary fission. Some bacteria in this category produce spores as resting forms (endospores). These organisms are generally chemosynthetic heterotrophs, and include aerobic, anaerobic and facultatively anaerobic species.

4.3 Eubacteria Lacking Cell Walls

These are microorganisms that lack cell walls (commonly called mycoplasmas and comprising the class Mollicutes) and do not synthesize the precursors of peptidoglycan. They are enclosed by a unit membrane (the plasma membrane). They resemble the L forms that can be generated from many species of bacteria (most Gram positive eubacteria). However, mycoplasmas never revert to the walled state, and there are no antigenic relationships between mycoplasmas and eubacterial L forms.

4.4 Archaea

These prokaryotic organisms are predominantly inhabitants of extreme terrestrial and aquatic environments (high salt, high temperature, anaerobic). Some archaea are symbionts in the digestive tract of animals. The archaea consist of aerobic, anaerobic, and facultatively anaerobic organisms that are chemolithotrophs, heterotrophs, or facultative heterotrophs. Some species are mesophiles, while other can grow at temperatures above 100℃. These hyperthermophilic archaea are uniquely adapted for growth and multiplication at high temperatures. With few exceptions enzymes isolated from these organisms are intrinsically more thermostable than their counterparts from mesophilic organisms. Some of these thermostable enzymes, such as the DNA polymerase (*Taq* polymerase) from *Thermus aquaticus*, are important components of DNA amplification methods such as the polymerase chain reaction (PCR). Archaea can be distinguished from eubacteria in part by their lack of peptidoglycan in the cell wall, their possession of isoprenoid diether or diglycerol tetraether lipids, and characteristic ribosomal RNA sequences. Archaea also share some molecular features with eukaryotes. Cells may have a diversity of shapes (spherical, spiral, and plate- or rod-shaped), unicellular and multicellular forms in filaments or aggregates. Multiplication occurs by either binary fission, budding, constriction, fragmentation, or by unknown mechanisms.

5 Subtyping

Under certain circumstances (e.g., an epidemic) it is important to distinguish between strains of a given species or to identify a particular strain. This is called subtyping. It is made by examining bacterial characteristics that allow discrimination below the species level. Subtyping system must be effective to differentiate case from non-case isolates. Classically, subtyping has been accomplished by biotyping, serotyping, antimicrobial susceptibility testing, bacteriophage typing and bacteriocin typing. For example, more than 130 serogroups of *Vibrio cholerae* have been identified based on antigenic differences in the O polysaccharide of the LPS. However, only the O1 and

O139 serogroups are associated with epidemic and pandemic cholera. Within these serogroups, only strains that produce cholera toxin are virulent and cause the disease cholera.

Clonality with respect to isolates from a common-source outbreak is an important concept in the epidemiology of infectious diseases. Generally, these infectious microorganisms are clonal. In other words, they are the progeny of a single cell and thus, for all practical purposes, are genetically identical. Thus, subtyping plays an important role in identifying these particular microorganisms. Recent advances in biotechnology have dramatically improved our ability to subtype microorganisms. Hybridoma technology has resulted in the development of monoclonal antibodies against cell surface antigens, which have been used to create highly standardized antibody-based subtyping systems.

6 Nonculture Methods for the Identification of Pathogenic Microorganisms

Attempts to estimate total numbers of bacteria, archaea, and viruses are frustrating because of difficulties such as detection and recovery from the environment, our incomplete knowledge and the problem of the species concept in these groups. Nevertheless, the number of uncultured microbial taxa may greatly exceed those of the cultured organisms. It is estimated that the number of bacterial species in the world range from 10^7 to 10^9. Generally, microbial identification required the isolation of pure cultures (or in some instances defined co-cultures) followed by testing for multiple physiologic and biochemical traits. Clinicians have long been aware of human diseases which are associated with visible but non-culturable microorganisms. Scientists often use a PCR-assisted approach to identify pathogenic microorganisms *in situ*. The first phase of this approach involves the extraction of DNA from a suitable specimen, the use of standard molecular techniques to obtain a clone library, the retrieval of rDNA sequence information, and a comparative analysis of the retrieved sequences. This yields information on the identity or relatedness of the sequences in comparison with the available data base. In the second phase, proof that the sequences are from cells in the original specimen is obtained by *in situ* hybridization using sequence-specific probes. This approach has been used in the identification of pathogenic microorganisms. For example, a previously uncharacterized actinomycete has been identified as the Whipple-disease-associated rod-shaped bacterium, for which the name *Tropheryma whipplei* has been proposed. The rRNA approach has also been used to identify the etiologic agent of bacillary angiomatosis as *Bartonella henselae* and to show that the opportunistic pathogen *Pneumocystis jiroveci* is a member of the fungi.

Review Questions

1. Term explanation: classification, nomenclature, identification, family, genus, species, strain, binomial nomenclature.

2. What is the classification rank of life? What is the basic unit of bacterial classification?

3. How to name bacteria? What is the difference between the Chinese name and the Latin name of the bacteria in the typesetting and read-writing bacteria?

4. What are the methods of bacterial classification?

5. What are the criteria of the classification and determination for bacteria? What are the advantages and disadvantages they have?

6. Briefly describe the authoritative classification system of bacteria in world.

7. Try to describe the classic program of bacterial identification.

8. Why is 16S rRNA considered as the indication of prokaryotic evolution?

Chapter 7 Coccus of Gram Positive

Synopsis

The most representative Gram positive coccus is *Staphylococcus* and *Streptococcus*. *Staphylococcus aureus* can cause infectious diseases and toxic diseases in humans and many kinds of animals, which have many important virulence factors including hemolysin, heat-stable nucleic acid enzyme, enterotoxin, coagulase and so on. The clear of *Staphylococcus* largely depend on phagocytosis, but the role of immunity is uncertain. Many members of *Streptococcus* are associated with the diseases of humans and animals, because they have many important virulence factors such as adhesion, suppurative toxin, hemolysin, streptokinase, hyaluronic acid enzyme, etc. Morphological observation is beneficial to the identification of *Staphylococcus* and *Streptococcus*. After the dye detection of suspicious samples of *Staphylococcus* or *Streptococcus*, biochemical identification can be used after separation. The therapy of *Staphylococcus* disease and *Streptococcus* disease should choose sensitive drugs.

1 *Staphylococcus*

Staphylococci are spherical Gram positive bacteria that divide in several planes to form irregular clusters. They are present in the upper respiratory tract and other epithelial surfaces of all warm-blooded animals. Four of some 20 species are important to veterinary: *Staphylococcus aureus*, *S. intermedius*, *S. hyicus*, and *S. schleiferi* subsp. *coagulans*. As a common pyogenic agent, *S. aureus* is present in humans and several animal species. *Staphylococcus intermedius* is the leading pus-forming bacterium in dogs. *Staphylococcus hyicus*, which is found in several species, causes exudative epidermidis of swine and bovine mastitis. *Staphylococcus schleiferi* subsp. *coagulans* (along with *S. intermedius*) is sometimes associated with otitis externa of dogs. *Staphylococcus sciuri* and *S. xylosus* (and rarely *S. epidermidis*) are universally present on skin and some mucous membranes, but nonpathogenic. The coagulase can be produced by pathogenic staphylococci.

1.1 Descriptive Features

1.1.1 Morphology and Staining

The diameter of Staphylococci is 0.5-1.5μm, and it can be stained Gram positive generally (Fig. 7-1). The exudates can form clusters, pairs, or short chains, but no spores and flagella, encapsulation is variable.

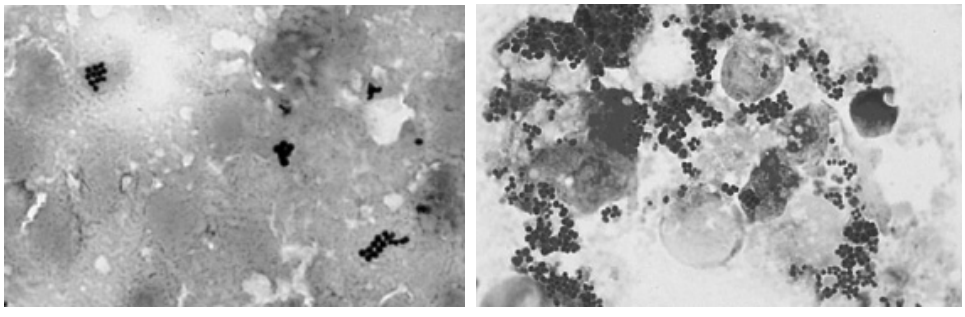


Fig. 7-1 *Staphylococcus aureus* from a bovine mastitis milk (×1,000) (Markey *et al.*, 2013)

1.1.2 Structure and Composition

The cell wall contains proteins and polysaccharides. One protein ("clumping factor" "bound coagulase") is usually present in *S. aureus* and *S. intermedius*. Clumping factor interacts with fibrinogen *in vitro* to produce an aggregation-like reaction. Another, protein A can produce aggregation by combining with the Fc fragment of immunoglobulins. The predominant polysaccharide is teichoic acid linked to peptidoglycan. Its alcohol moiety is ribitol in *S. aureus*, and glycerol in *S. epidermidis*, which is present in the cell membrane, can impart a "golden" (Latin: "aureus") color to colonies of *S. aureus*. A capsule is produced by *S. aureus* sometimes, and often a "pseudocapsule", a loosely associated carbohydrate structure, is produced by strains causing bovine mastitis.

1.1.3 Cellular Products of Medical Interest

Most of what follows has been determined for *S. aureus*, the most intensively studied species of *Staphylococcus*. Presumably, the other species have similar traits and characteristics that make them potentially pathogenic.

1.1.3.1 Adhesins

Staphylococci can produce a number of surface proteins, which can bind to various extracellular matrix proteins of the host (fibronectin, fibrinogen, collagen, vitronectin, laminin). These "adhesins" have been termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Staphylococci can also produce a number of

different adhesins, giving some strains affinity for a certain tissue types (bone, kidney and bladder).

1.1.3.2 Capsule

Staphylococcus aureus produces 11 serologically distinct polysaccharide capsules. The genes encoding capsule production are located on staphylococcal cassette chromosome genetic elements (SCCs). SCCs satisfy the characteristics outlined for defining a pathogenicity island, a cluster of genes encoding virulence determinant(s), an integrase protein, a specific insertion site, and mobility. The capsule is thought to play a role in preventing phagocytosis.

1.1.3.3 Cell Wall

The cell wall of this genus is one typical Gram positive bacteria. The lipoteichoic acids and peptidoglycan of the Gram positive cell wall interact with macrophage cells causing the release of proinflammatory cytokines.

1.1.3.4 Enterotoxins/Pyrogenic Toxin Superantigens

Coagulase-positive staphylococci produce a group of exotoxins that have similar size and three-dimensional shape. These include 11 enterotoxins (SE, for staphylococcal enterotoxin) A-M (there is neither F nor J), and the toxic shock syndrome toxin (TSST-l). The genes encoding these toxins are located on pathogenicity islands (SEB, SEC, SEK-M, TSST-l), prophages (SEA, SEE), or plasmids (SED). All of them are small proteins (20,000 to 30,000 in molecular weight) and dissimilar in primary amino acid sequence, but remarkably similar in shape. They are resistant to heat and digestive enzymes. The SEs, which are usually ingested as a preformed toxin, play a role by bin ding to an undefined receptor in the wall of the intestinal tract triggering reflex stimulation of the vomiting center. Both SEs and TSST-l are superantigens, and part of the systemic symptomatology may be related to the cytokine "storm" that results from the interaction of T lymphocyte receptors, macrophages, and these toxins which are released *in vivo* (TSST-l crosses mucus membranes, the SEs do not).

1.1.3.5 Exfoliative Toxins

Staphylococcus aureus produces two exfoliative toxins (sETA, ETB), and *S. hyicus* produces three different antigenically exfoliative toxins (shETA-shETC). The genes encoding the ETs of *S. aureus* are located either on a prophage (sETA) or plasmid (ETB). The exfoliative toxins are atypical glutamate-specific serine proteases. They target the intercellular adhesion protein, desmoglein (a cadherin), found only in the epidermis. Whether the exfoliative toxins are superantigens, is unsettled.

1.1.3.6 Hemolytic Toxins

There are four hemolytic toxins (alpha, beta, gamma and delta), because of their action on erythrocytes *in vitro*. Hemolysis is not a property observed during the disease process. The hemolytic toxins are expressed singly, in combination, or not at all. They differ in antigenicity, biochemistry, and in their effect on the erythrocytes of various species. The genes encoding the hemolytic toxins are located on the chromosome.

Alpha toxin acts on membrane lipids, is hemolytic *in vitro*, is mitogenic, is lethal to rabbits following intravenous injection, and is necrotizing upon intradermal injection. The major effect *in vivo* is related to its insertion into membranes. The toxin is secreted as monomers, which come together in the host cell membrane to form a cylinder through ions flow. The damnify of membrane integrity in various cell types cause untoward effects on the host. In high concentration of alpha toxin initiates target-cell death by necrosis (ion and ATP depletion). In lower concentrations, apoptosis is triggered. In certain instances, coagulase-positive staphylococci are internalized by nonprofessional phagocytes (endothelial cells, some epithelial cells), but escape the endosome to multiply within the cytoplasm. Endosomal escape is thought to be associated with alpha hemolysin-mediated lysis of the endosomal membrane.

Beta toxin, a phospholipase C is prevalent in animal strains. Beta toxin produces broad zones of "hot-cold lysis" on sheep or cattle blood agar at 37℃, a partial hemolysis ("water-stain" appearance) that occurs and goes to completion by further incubation at lower temperatures. Its role *in vivo* is unclear, but damage host cell membrane is a reason able assumption.

Gamma toxin is a bicomponent toxin composed of two proteins that combine to form an active moiety. This toxin promotes degranulation of phagocytic cells, thereby intensifying inflammatory responses and tissue damage. Gamma toxin is not observed surrounding colonies growing on blood agar plate because of it is inhibited by agar, but in fact, all strains of coagulase-positive staphylococci produce the toxin.

Delta toxin lyses cells of various species by a detergent-like action but is inhibited by serum. Like gamma toxin, almost all strains of coagulase-positive strains produce this toxin. Its role in disease, however, is undefined.

1.1.3.7 Iron Acquisition

Staphylococci with pathogenic potential (i.e., coagulase-positive strains) grow better in iron-restricted conditions (as would occur *in vivo*), as compared to those staphylococci with less potential (coagulas-negative strains). Under iron-limiting conditions, the coagulase-positive strains produce siderophores, aurochelin and staphyloferrin, which are responsible for iron acquisition from extracellular sources (transferrin, lactoferrin). Staphylococci also utilize the siderophores that produced by other bacteria, specifically

enterobactin and aerobactin from Gram negative organisms.

1.1.3.8 Leukocidin

Like gamma hemolysin, leukocidin, also called Panton-Valentine toxin, is a bicomponent toxin. In fact, some of the proteins encoded at the locus containing the genes for gamma hemolysin, form a part of leukocidin. Leukocidin promotes the degranulation of phagocytic cells, thereby it can intensify inflammatory responses and tissue damage.

1.1.3.9 MprF

MprF (multiple peptide resistance factor) is resistant to the action of defensins through the lysinylation of phospholipids in the cell membranes of coagulase-positive staphylococci. Not only does this permit survival within defensin-containing phagolysosomes (this does not protect against oxygen-dependent killing), it allows existence within niches that are bathed in defensins (upper respiratory tract, intestinal tract, genital tract).

1.1.3.10 Miscellaneous Product

Staphylococci produce too many other products that may or may not play a role in disease production. These products include lipases, serine proteases, thiol proteases, metalloproteases (aureolysin), esterases, deoxyribonucleases, staphylokinase (a plasminogen activator), hyaluronidase, and phospholipases. Urease, an enzyme produced by coagulase-positive staphylococci is associated with the production of uroliths in the canine bladder. Coagulase, an enzyme that causes plasma coagulation *in vitro* and aids in the identification of the pathogenic species: *S. aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, and some *S. hyicus*, probably is little, may be have any role *in vivo*.

1.1.3.11 Regulation of the Cellular Products of Medical Interest

The production involved with pathogenesis of staphylococcal disease is regulated by a way of a quorum-sensing, global-regulatory system. Aside from the iron-regulated products, not all of the products of interest are regulated by the agr-sar (for accessory gene regulator, staphylococcal accessory gene regulator, respectively) system. This system consists of a series of genes (*agrA-agrD*, and *sarA*) which products "sense" and respond to a thiolactone-containing pheromone (auto-inducing peptide pheromone or AIP, which is the modified product encoded by *agrD* gene) produced by other staphylococci in the immediate environment. Each staphylococcal cell produces a basal level of AIP, a certain environmental concentration of AIP can be attained (quorum sensing). AIP induces the formation of RNAⅢ (not its translated product) that regulates the transcription of the genes involved with staphylococcal disease (global regulation). Thus, when the numbers of staphylococci increase to a certain mass, the

increased amount of AIP causes up regulation (by way of RNAⅢ) of certain genes (those encoding hemolysins, enterotoxins, exfoliative toxins, leukotoxins, lipases, serine esterase, deoxyribonuclease, staphylokinase, hyaluronidase, phospholipase, and capsule production), while down regulate those gene products that are made when AIP is made in lesser amounts (adhesins).

1.1.4 Growth Characteristics

Staphylococci grow up overnight on common laboratory media, over a wide temperature range, and atmospheric conditions producing on agar smooth opaque colonies over 1mm in diameter.

1.1.5 Biochemical Characterization

Staphylococci are catalase-positive, facultative anaerobes that attack carbohydrates oxidatively and fermentatively.

1.1.6 Resistance

Staphylococci prevent dehydration (especially in exudates) for weeks, heating up to 60℃ for 30min, pH fluctuations from 4.0 to 9.5, and salt concentrations run up to 7.5%, which are used in selective media for isolation of staphylococci. Staphylococci are inhibited by bacteriostatic dyes (e.g., crystal violet), bile salts, disinfectants like chlorhexidine, and a lot of antimicrobial drugs.

1.1.7 Variability

Colonies are different from smooth (S) to rough (R). The production of G (gonidial) and L (well-less) variants cell wall will be reduced in unfavorable environmental conditions, such as antibiotic treatment.

Isolates can be “typed” by their susceptibilities to bacteriophage lysis. Phage-typing sets have been developed for human, bovine, and avian *S. aureus*.

Resistance to beta-lactam antimicrobics is due to possession of a plasmid-encoded penicillinase (beta-lactamase), or the presence of a staphylococcal cassette chromosome genetic element containing the gene that encodes a penicillin-resistant penicillin-binding protein (SCCmec). Tolerance, a rarer form of penicillin resistance, is attributed to failure of autolytic cell wall enzymes. Intrinsic penicillin resistance may be due to changes in penicillin-binding proteins (enzymes responsible for cell synthesis). Resistance to other antimicrobics is common.

1.2 Ecology

1.2.1 Reservoir

Coagulase-positive species *S. aureus* and *S. intermedius* inhabit the distal nasal

passages, external nares, and skin, especially near mucocutaneous borders such as the perineum, external genitalia, and bovine udder. They also occur as transients in the gastrointestinal tract.

Coagulase-negative staphylococci, especially *S. sciuri* and *S. xylosus* (and rarely *S. epidermidis*) are predominant among the resident skin flora but also colonize the upper respiratory tract. In swine, this generalization applies to *S. hyicus*, a species potentially pathogenic, especially for piglets.

Staphylococci are found worldwide in warm-blooded animals. But interspecies spread (e.g., humans to cows, dogs to humans) seems to be limited.

1.2.2 Transmission

Staphylococci spread by direct and indirect contact. A lot of animal infections are probably endogenous, caused by a resident strain.

1.2.3 Pathogenesis

1.2.3.1 Mechanisms

The deposition of staphylococcal cells into a normally sterile site is the first step in factious process. Presumably, the numbers of staphylococci at this stage would be few, and the amount of AIP is low. Thus, expression of adhesins (MSCRAMMs) results in adherence to extracellular matrix proteins. Inflammation is initiated by cell wall constituents (peptidoglycans and lipoteichoic acids), and by deposition of complement proteins on the bacterial cell surface, and leading to the generation of split products that are chemotactic and anaphylotoxic. At this stage, the infecting strain may be eliminated, or not, depending upon bacterial factors (inoculum size, virulence of the strain) and host factors (strength of the innate immune system, underlying disease or detects such as the extent of tissue damage). If the infecting strain is not controlled and eliminated, their numbers will be increased and the concentration of AIP will rise, leading to the down regulation of adhesins, and the up regulation of capsule and toxins adhesins and capsule, toxins will be regulated to down and up, respectively. Siderophore production in aid of the acquisition of iron. The capsule prevents further phagocytosis; the toxins aggravate tissue damage by destroying recruited inflammatory cells (membrane active toxins, and triggering degranulation). The predominant pattern of staphylococcal pathogenesis is suppuration and abscess formation. Superantigen activity maybe cause systemic effects.

Cell-mediated immune phenomena intensify inflammatory responses in some staphylococcal infections while spatially confining them. In some forms of canine pyoderma (juvenile pyoderma, folliculitis), cell- and antibody-mediated hypersensitivity may be induced.

The enterotoxin-induced diseases (food poisoning) are not prominent in animals

(though so-called "garbage can enteritis" may be staphylococcal enterotoxin-induced in dogs). Toxic shock syndrome does not occur commonly (if at all) in the veterinary species.

The exfoliative toxins may play a role in exudative dermatitis of pigs. Staphylococcal scalded-skin syndrome has been described in dogs.

1.2.3.2 Pathology

The typical lesion is abscess, an inflammatory focus in which participating cells have been destroyed by the combined effects of bacterial and inflammatory cell activity. This confrontation between leukocytes and microorganisms can produce pus, which is a mixture of host cell debris and bacteria, living and dead. In an abscess, pus is surrounded by intact leukocytes and fibrin strands. Unless the pus is drained, a fibrous capsule will be formed gradually. In chronic, ulcerative staphylococcal wound infections ("botryomycosis"), fibrous elements are predominant, interspersed with pockets of suppuration.

1.2.4 Disease Patterns

Although all warm-blooded animals can be affected by coagulase-positive staphylococci in clinic, the prevalence and form of such interactions are different among host species.

The more common presentations have been reported, and it should be kept in mind that staphylococci can affect any organ or tissue.

1.2.4.1 Ruminant

S. aureus is a leading cause of bovine mastitis. Infection occurs via the teat canal, and the course of the infectious process varies from subclinical to acute suppurative, gangrenous, or chronic, depending on the infecting strain, infecting dose, and host resistance. Bovine mastitis is caused by coagulase-negative staphylococci sometimes, notably *S. epidermidis*, *S. hyicus*, *S. xylosus*, and *S. sciuri*.

Tick pyemia of lambs, resulting from inoculation of indigenous skin *S. aureus* by tick bites, may be acute with toxemic death, or chrome with disseminated abscess formation. It is often linked with tick-borne fever (caused by the rickettsial agent *Anaplasma phagocytophila*).

1.2.4.2 Porcine

Exudative epidermitis (greasy pig disease) is caused by *S. hyicus* in young pigs (7 weeks). It is often systemic and rapidly fatal, affecting the lungs, lymph nodes, kidneys, and brain. Skin lesions are characterized by a thick, grayish-brown exudate, especially around the face and ears.

1.2.4.3 Avian

"Bumble foot" of gallinaceous birds is a chronic pyogranulomatous precess in the

subcutaneous tissues of the foot, and it can result in thick-walled swellings on one or more joints. This trauma is associated with *S. aureus*.

1.2.5 Epidemiology

Staphylococcal diseases (e.g., pyoderma, otitis externa, urinary tract and wound infections) often arise endogenously. Studies on humans suggest widespread staphylococcal colonization within hours of birth. Clinical infections appear to be decisively determined by host factors.

In bovine mastitis, staphylococci may enter the gland during milking. Management practices and milking hygiene affect prevalence significantly. Transmission of *S. aureus* between animals and humans occurs infrequently. Prolonged survival of staphylococci in natural environment will improve indirect transmission between them.

1.3 Immunologic Aspects

Possible immune mechanisms in pathogenesis have already been research reported.

1.3.1 Recovery and Resistance

Clearance of staphylococci relies on phagocytosis chiefly. Humoral factors are apparently important, the reason is that agammaglobulinemic individuals suffer infections frequently. Cell-mediated factors contribute to localization and resolution of lesions. Recovery from staphylococcal infection confers no lasting resistance.

1.3.2 Artificial Immunization

The benefits of vaccination are doubtful. Commercial or autogenous whole-culture preparations, toxoids plus bacterins, are used prophylactically on dairy cattle and sometimes in small animal dermatology to treat persistent infections. Although successes have been reported, controlled evaluations are scant.

The use of staphylococcal phage lysates and nonspecific stimulants of cell-mediated immunity in cases of non-responsive pyoderma needs support by adequate clinical or experimental evidence.

1.4 Laboratory Diagnosis

1.4.1 Sample Collection

Aspirates from unopened lesions, in sterile syringes or sterile containers, are preferred. Swabs in transport media are acceptable. Milk is collected into containers under sterile precautions. The routine culture of blood and urine is appropriate for staphylococcal isolation.

1.4.2 Direct Examination

On Gram-stained films, staphylococci appear as Gram positive cocci in pairs, clusters, or short chains. In specimens from skin pustules, they may be sparse.

1.4.3 Isolation and Identification

Bovine blood agar is best for the detection of beta toxin ("water stain" appearance), which is diagnostic for coagulase-positive staphylococci (*S. aureus*, *S. intermedius*, and *S. schleiferi* subsp. *coagulans*). Biochemical tests are used to identify staphylococcal isolates. Commercial kits are available.

1.5 Treatment and Control

For the most superficial pyoderma, abscesses can excrete pus by topical application of mild antiseptics (hexachlorophene).

Extensive, inaccessible, and disseminated processes need systemic treatment. Staphylococci are commonly resistant to penicillin G, streptomycin, and tetracycline. Usually effective antimicrobics comprise penicillinase-resistant penicillins, fluoroquinolones, chloramphenicol, cephalosporins (first generation), vancomycin, lincosamides (lincomycin and clindamycin), the macrolides (erythromycin, azithromycin, clarithromycin), and trimethoprim-sulfas. Clavulanic acid inactivates the beta-lactamase by *S. aureus* and *S. intermedius*, therefore cell wall antibiotics containing this substance are protected (e.g., clavulanic acid/amoxicillin). The penicillinase-resistant penicillin cloxacillin is effective in treating staphylococcal mastitis, especially in dry period.

For staphylococcal cystitis, penicillins remain in urine. Cloxacillin is used topically and systemically on exudative epidermitis due to *S. hyicus*. A controversial approach to prevent staphylococcal infections in infants utilizes "bacterial interference": the implantation of a nonvirulent strain to preclude colonization by virulent staphylococci. The method shows promise for controlling staphylococcosis in turkeys.

2 *Streptococcus*

Streptococci are Gram positive cocci occurring in pairs and chains; they show considerable ecologic, physiologic, serologic, and genetic diversity. There are 55 recognized species within the genus, but only a handful is regularly associated with diseases of veterinary importance.

The streptococci are superficially "grouped" by growing patterns on blood agar plates. According the growing effects on sheep or bovine blood agar, streptococci are used to divide into three types. Alpha-hemolytic streptococci (α) do not lyse erythrocytes but produce a zone of green discoloration around the colonies (change the hemoglobin

to methemoglobin). Most commensal streptococci of animals are alpha-hemolytic. Streptococci that do this are sometimes referred to as "viridans streptococci". They are not hemolytic in the true sense of the word. Bete-hemolytic streptococci (β) lyse erythrocytes can produce a "clear" zone around the colonies. Most pathogenic types are beta-hemolytic. Gamma streptococci (γ) are nonhemolytic, and most of them are nonpathogenic.

2.1 Descriptive Features

2.1.1 Morphology and Staining

Streptococci vary from spherical to short bacillary cells, about 1μm in diameter. Division occurs in one plane, appearing in pairs and chains (Fig. 7-2). Chain formation is variable, though some species (e.g., *Streptococcus equi* subsp. *equi*) are consistent chain formers. Brief cultures are Gram positive. But the organisms of exudates and older cultures (>18h) are often Gram negative by staining.

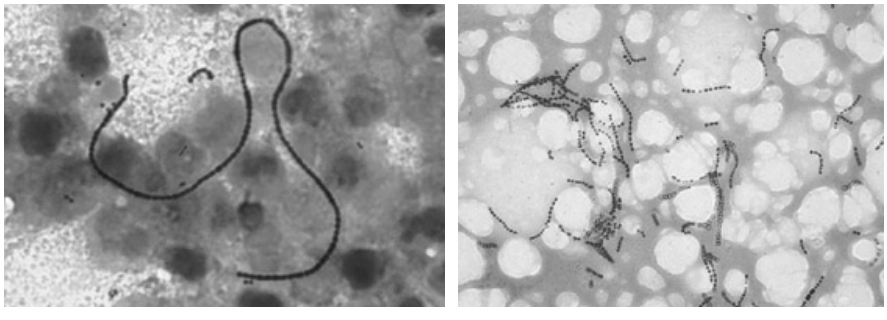


Fig. 7-2 *Streptococcus equi* subsp. *equi* in a smear of pus (left) and streptococci in a bovine mastitic milk (right) (×1,000) (Markey *et al.*, 2013)

2.1.2 Structure and Composition

Streptococci have a typical Gram positive cell wall that consists of proteins and polysaccharides. Some species also contain capsules. Cell wall polysaccharides (C-substance) are sometimes used in streptococcal identification.

2.1.3 Cellular Products and Activities of Medical Interest

2.1.3.1 Adhesins

Streptococci produce lots of surface proteins that bind to a variety of extracellular matrix proteins in the host (fibronectin, fibrinogen, collagen, vitronectin, laminin). These "adhesins" have been termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Some MSCRAMMs, specifically those that bind to fibrinogen (M protein, others), impart an antiphagocytic property to the

streptococcal particle. "Coating" of streptococcal cells with this host protein, is thought to result in the "covering" of sites for complement activation (and thus decrease opsonization) as well as recognized by serum proteins (collectins/ficolins) that opsonize foreign particles. The hyaluronic acid capsule of *Streptococcus pyogenes* is an adhesin (as well as imparting antiphagocytic effects), with affinity for human epithelial cells via CD44, a hyaluronic acid binding glycoprotein. Whether the hyaluronic acid capsules of streptococci of veterinary importance also act as adhesins is unclear. Other adhesins are responsible for binding streptococci to host cells. Psa (for pneumococcal surface adhesin) is a lipoprotein which is found on *S. pneumoniae*, *S. equi* subsp. *equi*, and *S. equi* subsp. *zooepidemicus* and is responsible for adherence to cells lining the upper and lower airways).

2.1.3.2 Capsule

Some species of streptococci can produce a capsule. Streptococcal capsules contain hyaluronic acid. Hyaluronic acid, also a constituent of mammalian connective tissue, is poorly antigenic and does not bind complement components readily (and is thus, antiphagocytic). Hyaluronic acid capsules may also serve as an adhesin.

2.1.3.3 Cell Wall

The Gram positive cell wall contains proteins and polysaccharides that are medical interest. The lipoteichoic acids and peptidoglycan of the Gram positive cell wall interact with macrophage cells leading to the release of proinflammatory cytokines. A fibrillar surface protein, termed the M protein (a MSCRAMM) imparts antiphagocytic properties by binding fibrinogen.

2.1.3.4 Pyrogenic Toxin Superantigens

Most of what is known about the streptococcal pyrogenic exotoxins (SPEs) involves those produced by *Streptococcus pyogenes* (a group A, beta-hemolytic *Streptococcus* affecting people). *S. pyogenes* produces several SPEs: SPEA, C, G-I, J, and Z (SPEF has been shown to be a DNase). Some streptococci have importance in veterinary, *S. equi* have been shown to produce SPEs as well. The genes encoding SPEs are located on chromosome. The SPEs are superantigens, and part of the systemic symptomatology may be related to the cytokine "storm" that results from the interaction of T lymphocyte cell receptors, macrophages, and these toxins.

2.1.3.5 Miscellaneous Products

The streptococci produce lots of proteins *in vitro* by "toxic" activity. These so-called toxins may or may not play a role in disease production and include a peptidase that degrades C5a (Scp for streptococcal cysteine protease), the hemolysins responsible for beta-hemolysis on sheep blood agar plates (streptolysins O and S), hyaluronidase,

DNases (e.g., SPEF which is a DNase), NADases, proteases (e.g., SPEB which is a cysteine protease), and streptokinases (a fibrinolysin). Streptolysin O and streptolysin S are membrane active substances and may play a role *in vivo* to damage cell membranes (they are made *in vivo* since antibodies are produced to these substances by patients that have streptococcal disease). Streptolysin O and suilysin O (produced by *S. suis*) are cholesterol-binding cytolysins (for example, listerial listeriolysin O, clostridial perfringolysin O, and arcano bacterial pyolysin). These toxins bind to cholesterol-containing rafts in the eukaryotic cell membrane, forming a pore, leading to death of the cell.

2.1.3.6 Regulation of the Cellular Products of Medical Interest

Expression of virulence-related genes in *S. pyogenes* is regulated by three global systems at least (whether these systems occur in other streptococci is unclear). The first is a "growth-phase related signal" that is undefined, but certain genes (including those encoding streptolysin S and DNases) are up-regulated during stationary phase, while others (including those encoding capsule, streptokinase. Streptolysin O, and a protein called multigene regulator in group A streptococci or Mga) are up-regulated during late exponential phase of growth. The second system involves regulation by Mga. Mga is regulated in growth phase as well as other undefined environmental cues. Mga up-regulates the genes encoding the M protein, and Scp. Finally, a third system involves the protein Cov (control of virulence). Cov down-regulates all of the genes that were up-regulated in growth phase, except Mga. The gene encoding Cov, like Mga, responds to undefined environmental cues.

2.1.4 Growth Characteristics

The needs of streptococci growth can be satisfied with media containing blood or serum. After overnight incubation at 37℃, streptococci can produce clear colonies, usually less than 1mm in diameter. Encapsulated forms, such as *S. equi* subsp. *equi*, produce larger, mucoid colonies. Pathogenic species grow best at 37℃.

2.1.5 Biochemical Activities

Streptococci are catalase-negative facultative anaerobes, deriving energy from fermentation.

2.1.6 Resistance

Beta-hemolytic streptococci can survive in dry pus for weeks. They are killed at 55-60℃ in 30min, and inhibited by 6.5% sodium chloride and 40% bile (except *S. agalactiae*), 0.1% methylene blue, and low (10℃) and high (45℃) temperatures. Viridans streptococci vary with respect to heat and bile resistance. Only *S. pneumoniae* is bile-soluble. Streptococci can tolerate 0.02% sodium azide, which is used in

streptococcal isolation media.

Pathogenic streptococci are usually susceptible to penicillins, cephalosporins, macrolides, chloramphenicol, and trimethoprim-sulfonamides; they are often resistant to aminoglycosides, fluoroquinolones, and tetracycline.

2.1.7 Variability

Rebecca Lansfield developed the serological method and used to group streptococci according to serologic similarities of cell wall polysaccharide. This method is called Lansfield serologic grouping. Groups are named by capital letters (A to V). Serologic subdivisions exist in most streptococcal species except *S. equi* subsp. *equi*. *S. pyogenes* has about 70 immuno types, based on M and other proteins, while *S. pneumoniae* has over 80 capsular types. In *S. pyogenes*, the change from rough (matte) to smooth accompanies results in loss of M protein and virulence. Cell wall also occur deficient forms (L forms).

2.2 Ecology

2.2.1 Reservoir

Most streptococci of veterinary interest live commensally in the upper respiratory and lower genital tract.

2.2.2 Transmission

Those contagious streptococci (*S. equi* subsp. *equi*, *S. porcinus*, and *S. agalactiae*) are transmitted by inhalation or ingestion, sexually, congenitally, or indirectly via hands and fomites.

2.2.3 Pathogenesis

2.2.3.1 Mechanisms

The relation of streptococcal products to pathogenesis is largely speculative, with the following exceptions. The capsule of *S. pneumoniae* is a proven virulence factor. M protein is an important virulence determinant, and antibody to it is protective. Other antiphagocytic cell constituents and cytotoxins of streptococci are probable virulence factors.

Streptococci trigger inflammatory processes that lead to suppuration and abscess formation.

2.2.3.2 Pathology

The basic pathologic process is similar to staphylococcal infection, i.e., the typical lesion is an abscess, an inflammatory focus in which participating cells have been

destroyed by the combined effects of bacterial and inflammatory cell activity. This confrontation between leukocytes and microorganisms produces pus, a mixture of host cell debris and bacteria, living and dead. In an abscess, pus is surrounded by leukocytes and fibrin strands. Unless the pus is drained, a fibrous capsule will gradually be formed.

2.2.4 Disease Patterns

2.2.4.1 Swine

Cervical lymphadenitis of swine (jowl abscess) is a contagious disease. This condition is associated with *S. porcinus* (previously known as "group E *Streptococcus*"). The disease is analogous to strangles but clinically less drama tic and frequently not diagnosed until slaughter. Its most harm is carcass condemnation.

Secondary pneumonias in swine are sometimes associated with *S. dysgalactiae* subsp. *equisimilis*.

S. suis, *S. dysgalactiae* subsp. *equisimilis* and streptococci belonging to group L and group U which could cause neonatal septicemia, pneumonia, arthritis, and meningitis. The exotoxin, suilysin, cholesterol-binding cytolysin, are produced by *S. suis*. It has been proposed that this cytotoxin is active *in vivo* and may account for some of the tissue damage associated with this disease.

2.2.4.2 Ruminants

The leading agent of streptococcal mastitis is *S. agalactiae*. Less frequent causes are *S. dysgalactiae* subsp. *dysgalactiae* (hereafter referred to as *S. dysgalactiae*) and *S. uberis*.

2.2.4.3 Dogs and Cats

Secondary pneumonias affecting dogs and cats are sometimes associated with *S. canis*. Laboratory cats occasionally experience cervical lymphadenitis from *S. canis*. The condition probably arises endogenously precipitated by an unknown event. *Streptococcus canis* is associated with septicemia in newborn puppies. *Streptococcus canis* has been associated with a toxic shock-like syndrome and necrotizing fasciitis in dogs. No virulence-associated traits have been defined as they have similar conditions seen in humans affected with *S. pyogenes*.

2.2.4.4 Primates

Streptococcus pneumoniae is a primary cause of pneumonia, septicemia, and meningitis in primates. Pneumococcal pneumonia in monkeys runs an acute course with high mortality rates. The lesions are fibrinous pleuropneumonia. Recent shipment and viral infection are common antecedents.

2.2.5 Epidemiology

Healthy individuals may carry all the streptococci discussed, and many infections are probably endogenous and stress-related. Neonatal infections are common vertical transmission.

Strangles and porcine lymphadenitis are contagious disease which affect young animals preferably (past infancy). *Streptococcus equi* and *S. porcinus* are spread by contaminated food, drinking water, or utensils and in recovered animals, which may remain clinically healthy shedders for months. Milking equipment, unskilled attempts at intra-mammary, medication, and unsanitary milking practices often spread *Streptococcus* agalactiae among dairy cows.

Animal streptococci have limited public health. The group B streptococci that cause disease in human infants are apparently distinct from bovine strains, but infections with *S. zooepidemicus* have been traced to infected milk, and *S. suis* (Type 2) has caused serious infections in swine handlers. The group G streptococci affecting dogs (*S. canis*) are apparently different from the group G streptococci affecting human patients.

2.3 Immunologic Aspects

2.3.1 Immune Mechanisms of Disease

Human streptococcal diseases (rheumatic fever, acute glomerulonephritis) are attributed to immuno-pathogenic mechanisms. Similarly, equine purpura hemorrhagica following strangles is probably immune complex-mediated.

2.3.2 Recovery and Resistance

The main defenses against streptococcal infections are phagocytic, and protective antibodies elicited by antiphagocytic M protein. Animals recovered from strangles and cervical lymphadenitis are at least temporarily immune to reinfection.

Polysaccharide capsules of *S. agalactiae* and *S. pneumoniae* cause the formation of opsonizing antibody. In streptococcal pneumonia, their appearance determines recovery from infection. In bovine mastitis, no useful immunity develops. Cows remain infected unless treated. Experimental evidence suggests that anti-capsular IgG_2 type antibody is protective. All immunity is serotype-specific.

2.3.3 Artificial Immunization

A whole-cell bacterin and an M protein vaccine are available for vaccination against strangles. Neither is uniformly effective, and often appears local reactions on the injection site. An intranasal avirulent live vaccine that stimulates essential local antibody responses appears promising. Immunity to porcine jowl abscesses can be produced by feeding live avirulent cultures.

2.4 Laboratory Diagnosis

2.4.1 Sample Collection

Aspirates from unopened lesions, in sterile syringes or sterile containers, are preferred. Swabs in transport media are acceptable. Milk is collected into containers under sterile precautions.

2.4.2 Direct Examination

Smears of exudates or sediments of suspect fluids are fixed and gram stained. Streptococci appear as Gram positive cocci in pairs, short chains, and in some instances very long chains (typically seen in pus aspirated from cervical lymph nodes of horses infected with *S. equi*). Streptococci have a tendency to lose their Gram-positivity and sometimes stain Gram positive or Gram negative weakly.

2.4.3 Culture

Exudates, milk, tissue, urine, transtracheal aspirates, and cerebrospinal fluid are cultured directly on cow or sheep blood agar. Incubation at 37℃ in 3-5% CO_2 is preferable. Streptococcal colonies, smooth or mucoid, will appear in 18-48h. It is sometimes difficult to distinguish alpha from beta hemolysis. Intact erythrocytes remain adjacent to alpha- but not to beta-hemolytic colonies. Beta-hemolytic strains consistently lyse red cells in blood broth; alpha-hemolytic strains of animal origin do not generally.

Identification relies on a combination of classical techniques (determination of Lansfield serum grouping and biochemical tests), and molecular techniques (e.g., determination of the sequence of DNA encoding the 16S ribosomal DNA, or using species specific primers in the polymerase chain reaction). Commercial kits are available for both purposes. Other useful diagnostic tests include the following.

The CAMP phenomenon (named after Christie, Atkins, and Munch-Petersen) reflects hemolytic synergism between staphylococcal beta toxin and a *S. agalactiae* toxin (CAMP protein sometimes referred to as cocytolysin). A beta toxin-producing *Staphylococcus* is inoculated across the equator of a sheep or bovine blood agar plate. On this line, a suspect *S. agalactiae* is inoculated. After incubation, hemolysis by CAMP-positive bacteria will be enhanced in the beta-toxin zone. The combined action of these two toxins on sheep or bovine blood agar produces larger and clearer zones of hemolysis than either agent alone. This reaction has diagnostic value.

Bacitracin disks (0.04 units) inhibit growth of *S. pyogenes* on blood agar. This reaction is not entirely consistent or specific. Bile esculin agar tests the ability of 40% bile-salt-tolerant bacteria to hydrolyze esculin, a characteristic of those belonging to

Lansfield group D. Growth of *S. pneumoniae*, but not other alpha-hemolytic streptococci, is inhibited around disks impregnated with optochin (ethyl-hydrocuprein hydrochloride).

2.5 Treatment and Control

Localized suppurative condtions are drained of pus. For systemic treatment, penicillin G and ampicillin are effective for most beta-hemolytic and viridans streptococci. Cephalosporins, chloramphenicol, and trimethoprim-sulfas are alternatives. Streptococcal endocarditis is treated with combined penicillin and gentamicin. Susceptibility to fluoroquinolones is unpredictable. Streptococcal toxic shock and necrotizing fasciitis are treated with penicillin G and clindamycin (clindamycin decreases toxin production, and penicillin G is bactericidal).

Penicillins (intramammary) are effective for treating mastitis caused by *S. agalactiae* and most other streptococci. For the many available alternatives, a specialty text should be consulted. Important aspects of mastitis control are sanitation and herd management.

For strangles, it is most beneficial to treat exposed and affected animals prior to abscess formation and to continue treatment past the febrile stage. Inappropriate or inadequate therapy of strangles is blamed for prolonging the illness and causing "bastard strangles" (widespread abscess formation with systemic manifestations). Populations at risk must be vaccinated. Affected or suspected horses should be rigorously isolated.

Review Questions

1. Please briefly describe the basic characteristics of *Staphylococcus*.
2. Please describe the colony characteristics of *Staphylococcus aureus*.
3. Please briefly describe the toxins and enzymes produced by *Staphylococcus aureus* and the type of the disease they caused.
4. Describe the biological property of SPA and its application.
5. Please briefly describe the basic characteristics of *Streptococcus*.
6. Please briefly describe the group basis of *Streptococcus* and the pathogenic characteristics of various groups.
7. Please briefly describe the pathogenicity and virulence factors of *Streptococcus*.
8. Try to describe the process of the identification of pathogenic *Staphylococcus* and *Streptococcus*.

Chapter 8 Enterobacteriaceae

Synopsis

The bacteria of Enterobacteriaceae are widely distributed in nature, and some of them have important pathogenesis to humans and animals. The bacteria of Enterobacteriaceae are Gram negative, which are similar in morphology and staining, and have complicated antigens. There are many serotypes in *Escherichia*, and have enterotoxin, shiga toxin, pili adhesin and virulence island, etc. *Escherichia coli* is a typical representative of Enterobacteriaceae and Gram negative bacteria. There are 6 subspecies of *Salmonella enterica* which are more common in *Salmonella*. *Salmonella* have many serotypes, and unique antigens. The types of host tropism include obligate tropism, favouritism tropism and pantropism. *Salmonella enterica* is a pathogenic agent for humans and many kinds of animals, which have enterotoxin, virulence plasmid, virulence island, adhesion, etc. *Escherichia coli* and *Salmonella enteric* are important animal pathogens, which can be treated with sensitive drugs or vaccines. The detection of *Escherichia coli* and *Salmonella enteric* was carried out mainly through many methods, e.g., separation and culture, biochemical characteristics test, serological test and PCR.

Members of the Enterobacteriaceae cause disease in food animals (e.g., neonatal diarrhea and salmonellosis) and companion animals (e.g., urinary tract infections, abscesses). The family contains about 35 genera, but only a few are consistently involved with disease of animals.

1 Features of Enterobacteriaceae

1.1 Descriptive Features

1.1.1 Morphology and Staining

Members of the family are similar in morphology and staining characteristics (Fig. 8-1), being pleomorphic, Gram negative, non-spore-forming rods that measure 2-3μm (for an example of Gram negative rods). It is difficult to distinguish members of one genus from those of another by visual observation.

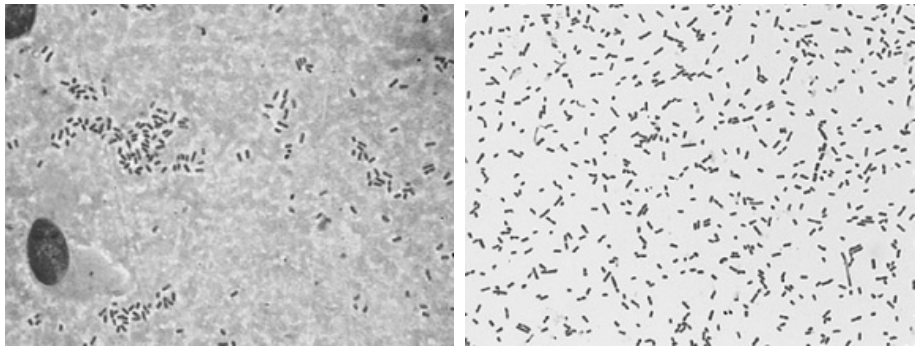


Fig. 8-1 *Escherichia coli* from a tissue (left) and a culture (right) (×1,000) (Markey *et al.*, 2013)

1.1.2 Cellular Anatomy and Composition

The cell wall is typically Gram negative, and it contains inner and outer membranes separated by peptidoglycan. Various proteins are found in each membrane, some traversing both. Capsules, flagella, and various adhesins are present on individuals.

The capsule (K antigen) is the outermost structural component of the bacterial cell. Capsules of enteric organisms are composed of carbohydrates. The various types of carbohydrates, together with the types of linkages between the sugars, are form the antigenic determinants that define capsular antigens. Encapsulated enteric bacteria are relatively hydrophilic, a characteristic imparted by the capsule.

The somatic antigens (O antigens) consist of antigenic determinants formed by the different configurations of sugar types, and the linkages between sugars found in the O repeat portion of the lipopolysaccharide.

Flagellum, which is cellular organelles used for locomotion, consists of protein subunits (flagellin). Different antigenic determinants are formed according to the type of flagellin. These antigenic determinants contain the H antigen. In cells of most *Salmonella* and some other species, one or two sets ("phases") of antigenic determinants are present possibly. In culture, spontaneous phase variation occurs, in other words, a shift from phase 1 to 2 or vice versa. The antigens of both phases, if present, is useful to define the serotypes.

Fimbriae or pill are protein adhesins that consist of subunits-pilin and assembled in various configurations with different pilin molecules, which results in the generation of different types defined by their affinity for various carbohydrates. The most common fimbriae have affinity for mannose-containing compounds. These fimbriae are called type 1 or common fimbriae (also termed F1). Type 1 fimbriae have not been conclusively shown to be virulence determinants. On the other hand, various other virulence-associated fimbriae have been described that agglutinate erythrocytes in the presence of mannose. Examples of such mannose resistant (MR) hemagglutinins are F4

(K88) and F5 (K99), two virulence-associated adhesins that are important in the pathogenesis of enteric disease produced by certain strains of *Escherichia coli*.

Most members of the family collectively possess mucopeptide antigens, the so-called enterobacterial common antigen.

1.1.3 Cellular Products of Medical Interest

Cellular products of medical interest are endotoxins and various siderophores.

1.1.3.1 Endotoxin

Endotoxin also termed lipopolysaccharide (LPS) is located on the outer membrane, it is one constituent of the Gram negative cell wall. The lipid portion of this substance has the toxic properties associated with endotoxin. The most important constituent of LPS as far as the toxic manifestations of the molecule is the lipid portion which is called lipid A. LPS binds to lipopolysaccharide-binding protein (a serum protein), which in turn transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells and leads to the release of proinflammatory cytokines. It is these cytokines that are responsible, in part, for the abnormal signs associated with endotoxin.

1.1.3.2 Siderophores

Siderophores are iron-carrying molecules (catechols or hydroxamates) of bacterial origin. Their function is solubilization and transport of ferric ions. There is very little free iron in hosts: nearly all is associated with the iron-binding proteins (ferritin, transferrin, and lactoferrin). Since almost all bacteria, parasitic strains, must need iron, especially invasive ones, have to compete for iron. Most utilize siderophores that remove iron from the iron-binding proteins of the host.

1.1.4 Growth Characteristics

Members of this microorganisms group are facultative anaerobes. They utilize various simple substrates for growth. Under anaerobic conditions, they depend upon the presence of fermentable carbohydrate. Under aerobic conditions, the range of suitable substrates includes organic acids, amino acids, and carbohydrates.

The end products of sugar fermentation are useful for identification. Almost all members of this ferment glucose are transformed to pyruvic acid via the Embden-Meyerhof pathway. Some, such as *E. coli* and salmonella, produce succinic acid, acetic acid, formic add, and ethanol by the mixed acid fermentation pathway. Others, such as *Klebsiella* and *Enterobacter*, produce butanediol by adding pyruvic, thereby reducing the relative amounts of acidic by-products.

An important biochemical characteristic of all the members of the Enterobacteriaceae is the absence of cytochrome C, making them oxidase negative.

1.1.5 Resistance

The members of the Enterobacteriaceae can be killed by sun light, drying, pasteurization, and the common disinfectants. They can survive for many months in moist, shaded environments, such as pastures, manure, litter, and bedding. Though many are susceptible to broad-spectrum antimicrobial agents, their susceptibility is not accurately predictable and can change rapidly through acquisition of R plasmids, or resistance-encoding DNA cassettes (which may insert into numerous integrons located in the genome and in plasmids).

1.1.6 Variability

Variability of one isolate of enteric as compared to another same species or genus depending upon the genetic basis for the trait under consideration. Differences in the capsular, somatic, or flagellar antigens account for some variation among members of the same genus and species in the family, as well as among members of different genera and species, are accounted for by the presence of genes residing on plasmids encoding certain phenotypic traits. Such traits as resistance to antimicrobial agents, production of toxin, or secretion of hemolysin may be plasmid encoded and will vary depending on the presence or absence of a particular plasmid.

Transition from the smooth to the rough phenotype occurs with all members of the family. Likewise, change in the O antigen has been shown to occur following lysogeny by certain bacteriophages (lysogenic conversion).

Susceptibility to a variety of bacteriophages (phage typing) is sometimes useful in demonstrating differences in isolates (strains) of the same genus and species. Phage typing is a useful epidemiological tool.

1.2 Laboratory Diagnosis

The family consists of a large number of related, facultatively anaerobic, oxidase-negative, nitrate reducing Gram negative rods. No clear divisions exist in the recognized genera. Differentiation within the family is accomplished by a combination of cultural, biochemical, and serologic tests. A number of manuals deal exclusively with this family, according to the extreme clinical importance and prevalence of these organisms, more and more programmed and/or computerized identification schemes are commercially available.

1.2.1 Morphology and Staining

All of the family are Gram negative rods. All of the family look semblable in the Gram-stained smear.

1.2.2 Cultural Characteristics

Methods used to isolate enteric pathogens depending upon the source of the sample is intestinal or extraintestinal. If the source is extraintestinal, sterile sites is significant for isolating. A culture medium with wide appeal is an agar medium containing red blood cells (usually sheep or cow). The samples are cultured at 35-37℃.

If the source is intestinal, two pathogenic genera may occur in fecal samples: *Salmonella* and *Shigella*. Though pathogenic strains of *E. coli* might be present, it is difficult to distinguish a pathogenic strain from normally occurring and nonpathogenic strains of *E. coli*. All enteric media are configured to aid the identification of *Salmonella* and *Shigella*.

2 *Escherichia*

The genus *Escherichia*, one of the Enterobacteriaceae, consists of several species, but only *E. coli* is an important pathogen of animals. The major facultative Gram negative species comprising the normal flora of the gastrointestinal tract can cause septicemic disease in foals, calves, piglets, puppies, and lambs; diarrhea in newborn farm animals; and edema disease in pigs. It may also be opportunistic in almost all animal species (e.g., in urinary tract disease, abscesses, and pneumonia).

2.1 Descriptive Features

2.1.1 Cellular Anatomy and Composition

The anatomy of the genus *Escherichia* is typical for the Enterobacteriaceae. They may possess capsules (K antigen), flagella (H antigen), or adhesins (fimbria or pili), and a typical Gram negative cell wall composed of lipopolysaccharides (O antigen) and proteins.

2.1.2 Cellular Products of Medical Interest

2.1.2.1 Adhesins

Adhesins has two categories, one is pilus or fimbria, the other is afimbrial adhesion. But the adhesins we referred to generally is fimbria or pilus. Adhesins are proteins that mediate adherence to target cells in the gastrointestinal tract and to cells comprising the niche for the strain. Owing to their relative hydrophobicity, adhesins may also promote association with the membrane of phagocytic cells. Adhesins are important virulence factors when the microbe is on mucosal surfaces. *E. coli* produces a lot of different types of adhesin, most being linked with strains associated with a specific disease. Almost all of the adhesins enhance adherence to glycoproteins on the surface of

epithelial cells in intestinal tract.

(1) F4, F5, F6, F41

The fimbrial adhesins F4 (formerly known as K88), F5 (K99), F6 (987P), and F41 are used by enterotoxigenic *E. coli* adhering to target cells in the small intestine. F4, F5 and F6 are usually plasmid-encoded, while F41 is chromosomal.

(2) F17

The protein F17 (like CS31A) is a plasmid-encoded adhesin, it is responsible for adherence of septicemic ("invasive") strains of *E. coli* to their small intestine target cells.

(3) CS31A

The protein CS31A (like F17) is a plasmid-encoded adhesin, it is responsible for adherence of septicemic ("invasive") strains or *E. coli* to the small intestine target cells.

(4) AAF

The adhesin AAF (aggregative adherence fimbriae) is responsible for the adherence of enteroaggregative *E. coli* to their small intestinal epithelial cell targets.

(5) Bfp

The Bfp adhesin (for bundle forming pilus, due to its propensity to tangle together and form "bundles" when viewed under the electron microscope) is responsible for the adherence of enteropathogenic *E. coli* to their small intestinal epithelial cell targets.

(6) Curli

Curli are adhesins that enhance adherence to extracellular matrix proteins.

(7) OmpA

OmpA (outer membrane protein A) is a putative adhesin used by adherence of enterohemorrhagic *E. coli* to large intestinal epithelial cell targets.

2.1.2.2 Capsule

Capsular polysaccharides (K antigens) are important for those microorganisms (such as invasive strains of *E. coli*) that come in contact with elements of the innate immune system of the host. Capsular substances protect the outer membrane against attack, and inhibit the microbe that is from attachment, and ingest by phagocytosing host cells. The capsule is deemed to endow a degree of hydrophilicity relative to the membrane of phagocytic cells. Most capsules are negatively charged, as the same as the membranes or phagocytic cells.

2.1.2.3 Cell Wall

The cell wall of this genus is one typical of Gram negatives. The lipopolysaccharide (LPS) in the outer membrane is an important virulence determinant. Not only is the

lipid A component toxic (endotoxin), but the length of the side chain in the O repeat unit hinders the attachment of the membrane attack complex or the complement system to the outer membrane. LPS binds to lipopolysaccharide-binding protein (a serum protein), which in turn transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells by triggering the release of proinflammatory cytokines.

2.1.2.4 Enterotoxins

Enterotoxins are usually plasmid-encoded proteins. *E. coli* produces at least has three types: labile toxin (LT), stable toxin (ST), and enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST 1). These protein exotoxins affect the control of cyclic nucleotide activity within the "target" cell, which results in deregulation of water and electrolyte secretion by affecting host cell.

(1) Labile Toxin (LT)

LT affects the adenylyl cyclase system. LT consists of two subunits, A and B. The B subunit is a multimer that binds to gangliosides on the surface of the intestinal host cell, followed by translocation of the A subunit across the host cell membrane. The A subunit, after activation, cleaves nicotinamide from nicotinamide adenine dinucleotide (NAD) and then couples the remaining ribosyl adenine diphosphate onto the G regulatory protein of the adenylyl cyclase enzyme system. The result is deregulation of adenylyl cyclase, causing overproduction of cyclic AMP (cAMP) followed by opening of chloride channels in crypt cells (so-called cystic fibrosis transmembrane conductance regulator chloride channels) and the blockage of NaCl absorption in apical tip cells. As a result, water and electrolytes (chloride, sodium, and bicarbonate ions) are lost into the intestinal lumen. These events lead to diarrhea, hypovolemia, metabolic acidosis. There are two distinct subclasses of LT. LT-Ⅰ is plasmid-encoded and neutralized by anticholera toxin antibodies; LT-Ⅱ is neither. LT-Ⅰ could be isolated from *E. coli* affecting humans (LTh-Ⅰ) and swine (LTp-Ⅰ). LT-Ⅱ has been isolated from cattle, water buffalo, human and food.

(2) Stable Toxin (ST)

There are two kinds of ST, STa and STb. The genes encoding STa are located on a transposable element. The genes encoding STb are not. STa causes fluid accumulation in the intestines of suckling mice and piglets: STb causes fluid accumulation only in piglets and weaned pigs. The toxins are not related to antigenicity. STa affects the guanylyl cyclase system by deregulating cyclic GMP (cGMP) synthesis, resulting in fluid and electrolyte accumulation in the bowel lumen impede sodium and chloride ion (and thus water) absorption (tip cells) and loss of chloride (crypt cells). The STa receptor is a membrane-bound guanylate cyclase. This receptor will result in the

synthesis of cGMP when it is bound. The increase of intracellular cGMP causes the opening of chloride channels with the resultant flow of chloride and water into the intestinal lumen. The STa receptor is normally target for guanylin, which is produced by goblet cells. Guanylin appears responsible for hydration of mucus that is also produced by goblet cells. STa and guanylin have common C-termini. STb binds to a sulfatide receptor followed by synthesis of cAMP activating a G regulatory protein. This leads to an increase in intracellular calcium concentration, which in turn activates protein kinase C. Protein kinase C belongs to phosphorylate proteins that consist of the chloride channels which can lead to loss of chloride and water into the intestinal lumen, as well as the phosphorylation of the membrane-associated ion transport proteins leads to impede the absorption of NaCl. In wild strains of enterotoxigenic *E. coli*, STa is found more commonly.

(3) Enteroaggregative

Heat-stable enterotoxin 1 (EAST 1) of *E. coli* is an enterotoxin that is similar to STa, and similar to guanylin for function.

2.1.2.5 Other Enteric Toxins

E. coli produce other proteins that affect the cells of the intestinal tract. Though these products have "enterotoxic" activity, the termed "enterotoxin" is reserved in LT, ST, and EAST 1 as discussed above. The other enteric toxins include shiga and shiga-like toxins, cytotoxic necrotizing factor, a plasmid encoding toxin, and a cytolethal-distending toxin.

(1) SLT (shiga and shiga-like toxin)

SLTs (also known as Vero tissue culture toxins because of their characteristic effects on Vero cells) are protein toxins that are similar to shiga toxin produced by *Shigella*. These toxins consist of an A subunit and a B subunit. The B subunit is responsible for binding of the toxin to endothelial cells. The A subunit inhibits protein synthesis of the target cell (an endothelial cell) following interaction with the 60S ribosomal subunit. There are two types of shiga-like toxins, SLT-Ⅰ and SLT-Ⅱ. SLT-Ⅰ is neutralized by antibody specific for the shiga toxin produced by *Shigella* spp. SLT-Ⅱ is not. SLT-Ⅰ is probably identical to shiga toxin, whereas SLT-U is a variant. A family of bacteriophages has been shown to encode the shiga and shiga-like toxins. A variant of SLT-Ⅱ, called SLT-Ⅱe, is responsible for swine edema disease which characteristic is vascular damage. The genes encoding SLT-Ⅱe do not seem to be bacteriophage-associated.

(2) CNF (cytotoxic necrotizing factor)

CNFs are proteins that interact with small GTP-binding protein Rho of epithelial cell, resulting in membrane "ruffles". There are two types of CNF, CNF l and CNF 2, which are related in immunology and similar in size. The gene encoding CNF 1 is located on

the chromosome in a pathogenicity island [a cluster of genes encoding virulence determinant(s), an integrase protein, a specific insertion site, and mobility]. The pathogenicity island that contains the gene encoding CNF 1 and also contains the genes encoding a number of other virulence traits, e.g., hemolysin, serum resistance and the adherence protein Pap, are needed when some strains of *E. coli* to adhere to urinary tract epithelium antecedent to appearing urinary tract disease. The gene encoding CNF 2 is plasmid-based.

(3) Pet (plasmid-encoded toxin)

Pet is a serine protease enteric-acting toxin that affects the cellular cytoskeleton of intestinal epithelial cells, resulting in damage to the cell and stimulating an inflammatory response. Diarrhea is considered to resulting from prostaglandin synthesis by the recruiting PMNs and affected epithelial cells, as well as activation of various inositol-signaling pathways within affected host cells. Result in the secretion of chloride ions and water at last.

(4) Cdt (cytolethal distending toxin)

Cdts are a family of related toxins that affect the mammalian cell cycle. A role in pathogenicity has not been proved.

2.1.2.6 Hemolysins

E. coli produce at least three hemolysins: alpha hemolysin, enterohemolysin (Ehx, enterohemorrhagic *E. coli* toxin), and cytolysin A (Cly).

(1) Alpha Hemolysin

Alpha hemolysin (Hly) as well as enterohemolysin belongs to the RTX (repeats in toxin, so called because of the common feature of repeats in glycine-rich sequences within the protein) family of toxins. Hly is secreted by many virulent strains of *E. coli*. Loss or gain of Hly by genetic manipulation produces corresponding changes in virulence of *E. coli* strains. Hly can damage cell membranes, and is detected *in vitro* on a medium containing red blood cells.

(2) Enterohemolysin

Enterohemolysin as well as Hly belongs to the RTX (repeats in toxin, so called because of the common feature of repeats in glycine-rich sequences within the protein) family of toxins. Evidence suggests that Ehx may be the result of a defect in the secretion system in Hly. Ehx is secreted by many virulent strains of *E. coli* (especially SLT) and is detected on media supplemented with red blood cells and calcium.

(3) Cytolysin A

The gene encoding cytolysin A occurs in strains of *E. coli* commonly. Expression of cytolysin A (Cly) occurs following infection of the bacterial cells with the temperate

bacteriophages Ehy 1 and Ehy 2 (incorrectly termed "enterohemolysin 1 and enterohemolysin 2").

2.1.2.7 Iron Acquisition

Iron is an absolute growth requirement for living things nearly. Siderophores (e.g., aerobactin) that remove iron from host iron-binding proteins are necessary if a microbe possesses invasive capabilities.

2.1.3 Miscellaneous Products

2.1.3.1 Acid Tolerance

RNA polymerase containing RpoS (the sigma factor associated with stationary phase) preferentially transcribes genes responsible for acid tolerance (survival at pH 5), allowing safe transit through the stomach.

2.1.3.2 Intimin

Intimin is a protein encoded by *eae* (for *E. coli* attaching effacing) located within a pathogenicity island [a cluster of genes encoding virulence determinant(s), an integrase protein, a specific insertion site, and mobility] called LEE (locus effacing *E. coli*). Intimin attaches to another secreted bacterial protein, Tir (translocated intimin receptor) inserted into the host cell membrane.

2.1.3.3 Type Ⅲ Secretion System

The genes encoding a Type Ⅲ secretion system (an assemblage of proteins-more than 20-that form a hollow tube-like structure through which effector proteins are "injected" into host "target" cells) also located within LEE.

2.1.3.4 Esp

The gene encoding Esp (for EPEC signaling protein, also located within LEE) activates a tyrosine phosphokinase in infected cell, it causes cytoskeletal rearrangements and then leads to "collapse" of the microvilli (effacement). Secondary diarrhea can increase intracellular calcium ions and activate protein kinase C. Protein kinase C is responsible for phosphorylation of proteins composing the chloride channels, resulting in loss of chloride and entrance of water into the intestinal lumen, as well as the phosphorylation of the membrane associated ion transport proteins causing blockage of NaCl absorption.

2.1.4 Variability

The variability of *E. coli* resides in the antigenic makeup of the O-repeat units (type of sugar subunits, how the subunits are hooked together, and the length of the chain), the composition of the flagellar protein (flagellin), and the composition of the capsule. There are at least 80 distinct K antigens, approximately 165 serologically distinct

O groups, and at least 50 serologically different flagellar (H) antigens. The O antigen, H antigen, and K antigen are used in serotyping a particular isolate. For example, O141: K85: H3 describes an isolate with antigens of the 141 serogroups, capsular antigen number 85, and flagellar antigen number 3.

2.2 Ecology

2.2.1 Reservoir and Transmission

Strains of *E. coli* with pathogenicity reside in the lower gastrointestinal tract and the environments inhabited by animals. The route of transmission is fecal-oral.

2.2.2 Pathogenesis

2.2.2.1 Mechanisms and Disease Patterns

It is very difficult for most strains of *E. coli* to produce disease because they do not have the necessary genes encoding the proteins. If genes are acquired by transduction, conjugation or transformation, the nonpathogenic strain may be changed to one with pathogenic potential. Producing disease would depend upon the genes acquired.

2.2.2.2 Enterotoxigenic Diarrhea

This disease occurs in neonatal pigs, calves, and lambs, and weanling pigs. It also has been reported in dogs and horses. Enterotoxigenic diarrhea is caused by strains of *E. coli* that produce adhesins, the adhesins can enhance attachment to glycoproteins on the surface of epithelial cells of the jejunum and ileum, and an enterotoxin(s) that affects the epithelial cell (to which the enterotoxigenic strain of *E. coli* is adhered), lead to fluid secretion and diarrhea at last. Both traits are necessary for disease, since unless the ingested strain adheres to these cells, peristalsis will move it into the large bowel. The cells of the jejunum and the ileum are susceptible to the action of enterotoxin, but the cells of the large bowel are not.

At least four adhesins have been found on enterotoxigenic *E. coli*, F4, F5, F6, and F41. They possess some host species specificity: F4 and F6 are almost always associated with isolates from swine; F5 associated with isolates from cattle, sheep and swine; and F41 associated with those from cattle. The epithelial cell receptors for these adhesins regulate the age incidence of this disease as well. In calves and lambs, the receptors appear transiently during the first week of life. Analogous receptors are present in pigs throughout the first six weeks of life. There are many uncharacterized adhesins that probably play a role.

Aside from the adhesins outlined above, some enterotoxigenic strains of *E. coli* express curli. So, in addition to adherence to glycoproteins on the surface of epithelial cells, some strains adhere to extracellular matrix proteins. The presence

of curli may explain the increase the susceptibility of age to enterotoxigenic disease in animals concurrently infected with rotavirus or cryptosporidia, two agents that may cause enough tissue damage will result in exposure of extracellular matrix proteins.

In addition to adherence to the target tissues of the small intestine, enterotoxigenic strains must have the genetic capability of synthesizing enterotoxin. Strains producing only ST are the most common, followed by those secreting both ST and LT, and then by those secreting LT only.

Some of the adhesins and enterotoxins are encoded by plasmid DNA. As a consequence, it is difficult to predict which strain of *E. coli* possesses the genetic information that is necessary to produce disease. Some adhesins prefer to be associated with certain serotypes. In particular, the genes encoding the protein for the F41 adhesin are almost always found in the O9 and the O101 serogroups of strains of *E. coli*. As might be expected, the genes encoding the proteins for F41 adhesin are located on chromosomal DNA.

Following ingestion by the host, enterotoxigenic strains of *E. coli* adhere to target cells, multiply, and secrete enterotoxin. Fluid and electrolytes are accumulated in the lumen of the intestine, resulting in diarrhea, dehydration, and electrolyte imbalances. If the infecting strain is moved away from the target cell in time, the disease process will be restrained, the reason may be the cessation of expression of the adhesin, with a decrease in available substrate following the almost explosive multiplication of the strain in the small intestine. Unless steps are taken to correct if the fluid and electrolyte imbalances are not corrected, the disease will have high mortality.

The minimal symptom diarrhea is watery and non-bloody, if any inflammatory changes in the small intestine. Bacteria will be observed histologically coating the villi of the mid to distal portions of the small intestine.

2.2.2.3 Enteroaggregative Diarrheal Disease

Enteroaggregative strains of *E. coli* (EAEC) associated with this disease are isolated from weaned pigs and calves with diarrhea. EAEC adheres to cells lining the small intestine by AAF adhesin. Following adhesion, EAEC secretes a protein (encoded by a gene termed *agg* for aggregation) that promotes the adherence of microorganisms one to another strongly (some have referred to this as a “biofilm”), thus describing it “enteroaggregative”. EAEC produce EAST 1 and Pet, both are possibly produced with diarrhea.

The diarrhea is usually watery (though blood and leukocytes may be observed in some cases). Histologically, sheets of bacteria (entrapped in mucus) will be seen covering the small intestinal epithelia.

2.2.2.4 Invasive Disease

Association of susceptible animals (usually a neonate that has received superfluous colostrum or the poor quality of colostrum) with invasive strains of *E. coli* may occur by the way of the conjunctivae, inadequately treated umbilicus, or ingestion. If the invasive strain associates via ingestion, it first adheres to target cells in the distal small bowel. Adherence is probably related to expression of adhesins, but CS31A is one that is commonly associated with invasive *E. coli*. Likewise, the adhesin F17 originally described on a plasmid termed Vir (so-called because of its association with virulent or invasive *E. coli*) is prevalent on invasive *E. coli*. Following adherence, invasive strains "induce" their own uptake by expression of either CNF l or CNF 2, resulting in the formation of "ruffles" that entrap adhering bacteria and "pull" them into the intestinal epithelial cell. Entry into the lymphatics and subsequently the bloodstream follows. It does not appear extensive multiplication within the intestinal epithelial cell probably. The mechanism that how the invasive strain invade to lymphatics after uptake by the epithelial cell is unknown. Likewise, the mechanism of transferring into lymphatics after association with conjunctivae or the umbilicus is unclear. Once the epithelial surface is traversed, expression of adhesins will be repressed (otherwise adhesin-expressing bacteria could adhere to host phagocytic cells with disastrous consequences for the bacterium).

The infecting strain multiplies in the lymphatics and bloodstream and endotoxemia develops. The host does not die if carrying out antibacterial therapy, but the immune system does not remove the microorganism. Invasive strains have special qualities, e.g., they must avoid phagocytosis, complement-mediated lysis, and have a mechanism to acquire iron. Capsule and various outer membrane proteins possess resistance to complement-mediated lysis (serum resistance). How capsules protect the outer membrane against insertion of the membrane attack complex is not known. Certain capsules (such as K1) are chemically similar to the surface of host cells in that they consist mainly of sialic acid. Complement components associating with sialic acid are shunted to degradative pathways rather than amplification and formation of membrane attack complexes.

The escape from phagocytosis is also related to capsule and certain outer membrane proteins. How outer membrane proteins function as antiphagocytic factors is not known.

The genes encoding the adhesin (e.g., CS31 A, F17) and these genes that are responsible for siderophore production are located on plasmids. As mentioned above, the genes encoding F17 have been associated with the plasmid Vir, as has the gene encoding CNF 2; those that are responsible for siderophore production have been associated with the plasmid pColV. In the latter instance, the siderophore genes are linked closely with the genes for the production of colicine V. The siderophore,

aerobactin, has a high affinity for iron.

A lot of the strains possess invasive capability, except foals, produce a hemolysin (Hly) and are hemolytic on blood agar.

Histopathologically, there are inflammatory changes in liver, spleen, joints, and meninges. There may be hemorrhages on pericardium peritoneal surfaces, and adrenal cortices.

2.2.2.5 Non-enterotoxigenic Diarrheas

Enteropathogenic strains of *E. coli* (EPEC) produce diarrhea in all animal species, including human beings. EPEC do not produce ST, LT, or any other diarrhea associated toxin. They produce a characteristic lesion in the intestinal tract that is described as an attaching and effacing lesion. The reason of occurring characteristic lesion is the "collapse" of the microvilli of the affected cell via giving the histopathologic appearance of "effacement". The lesion sites of are the distal small intestine, and upper large intestine.

EPEC have many attributes to pathogenesis. The first is the production of the adhesion Bfp. Bfp is responsible for "targeting" the particular intestinal epithelial cell that will become involved in the process. After association with an intestinal epithelial cell, a more intimate attachment occurs by way of intimin, which binds to the protein Tir that has been inserted into the "targeted" cell. Esp proteins are produced and are "injected" in to the target cell by way of the Type Ⅲ secretion system. The Esp proteins produce the effacement lesion, and diarrhea. Many EPEC also produce enterohemolysin (Ehx). What role Ehx plays in enteropathogenic disease is unclear.

Some attaching and effacing strains of *E. coli* are lysogenized with the bacteriophage(s) that encode the shiga-like toxins SLT-Ⅰ and/or SLT-Ⅱ. These strains are termed enterohemorrhagie *E. coli* (EHEC), in addition to producing attaching and effacing lesions, they also produce hemorrhagic diarrhea. However, the target cells are located in the large intestine. Thus, the Bfp adhesin is not involved, and the evidence strongly suggests that it is OmpA. The prototype EHEC is a strain of *E. coli* termed serotype O157: H7 that produces disease in human beings, and calves given the strain experiment. Following attachment (the large intestine via OmpA), an attaching and effacing lesion is produced (the intimin produced by EHEC strains is slightly different from that produced by EPEC, reflecting the different target cell), and SLT is produced. The SLTs affect endothelial cells, leading to their injury and loss of integrity. The effects of SLTs are local, i.e., the endothelial cell under the cell to which EHEC is attached, and systemic, i.e., the endothelial cells elsewhere in the body but mainly in the kidney and brain. The local effect is hemorrhage. The systemic effects of SLT, at least in humans, result in the hemolytic uremic syndrome (HUS), characterized by microangiopathic hemolytic anemia, glomerulonephritis, and thrombocytopenia. How

SLT is absorbed locally or systemically is not unknown. HUS does not appear to be a significant sequela of EHEC-based disease in nonhuman animals. Approximately 5-10% of human patients affected with EHEC (almost all are O157: H7) will develop to HUS. All strains of O157: H7 can virtually produce Ehx.

All animals are infected EPEC/EHEC by way of the oral route. It is not clear whether EPEC have zoonotic potential, but animals (including humans) probably acquire the infecting strain by the fecal-oral route. Strain O157: H7 is a part of the normal flora of nonhuman animals, especially bovines. Human beings become infected via ingestion of contaminated food, mainly beef. The surface of the carcass becomes contaminated with fecal microorganisms. The surfaces of cuts of meat derived from an infected carcass are readily sterilized by cooking. But if the meat is ground, the microorganisms on the surface become mixed throughout.

Diarrhea associated with EPEC will be watery, usually without blood and inflammatory cells. The characteristic histopathologic lesion is an "attaching and effacing" one affected cells of the small intestine. Attachment is localized. Diarrhea associated with EHEC will be hemorrhagic. The characteristic histopathologic lesion will also be an "attaching and effacing" one, but the affected site is large intestine.

2.2.2.6 Edema Disease

Edema disease is an acute, fatal "enterotoxemia", especially to weaned pigs. The disease is characterized by subcutaneous and subserosal edema, caused by absorption of SLT-Ⅱ produced by certain serotypes of *E. coli* (e.g., O141: K8S, O138: K81, and O139: K82). The toxin attaches to and affects endothelial cells throughout the pig, resulting in extensive edema. The toxigenic strains inhabit the large bowel of normal pigs, and these strains are thought to increase in numbers under nutritional, social, or physical stress.

The clinical symptom of typical lesion is generalized edema of various organs and tissues (e.g., head, neck, colon, stomach, intestine, brain).

2.2.2.7 Fowl Colibacillosis

Fowl colibacillosis is an economically important disease caused by invasive strains of *E. coli*. The disease has many infection modes in fowl, depending upon the age of the host. The egg surface can be contaminated with potentially pathogenic strains at the time of laying. The bacteria infect the yolk sac via penetrating the shell. If the bacteria grow, the embryo will die, usually late in incubation. Embryos that survive may die shortly after, with losses occurring as late as 3 weeks after hatching.

The respiratory trac of fowl may also be infected by develop respiratory or septicemic disease. The course may be rapidly fatal or chronic, manifested by debilitation, diarrhea, and respiratory distress.

Other clinical syndromes seemingly caused by *E. coli* include cellulitis, synovitis, pericarditis, salpingitis, and panophthalmitis.

The *E. coli* that are responsible for this disease has been shown to possess some of the same virulence determinants as those isolated from mammals, especially adhesins, production of aerobactin and associated iron-regulated outer membrane proteins.

2.3 Immunologic Aspects

Immunologic defense against diseases produced by pathogenic *E. coli* occurs at two levels: one is the site of attachment to the target cell, the other is the destruction of the bacteria or the neutralization of its products.

2.3.1 Enterotoxigenic Diarrhea

Specific antiadhesin antibody (sIgA and sIgM) found in colostrum and milk can prevent the attachment to "target cells". Likewise, specific anti-LT can neutralize LT enterotoxin.

2.3.2 Enteroaggregative Diarrhea

Specific anti-AAF (adhesin) antibody (sIgA and sIgM) found in colostrum and milk prevents attachment to "target cells".

2.3.3 Invasive Disease

The neonate acquires immunity from the dam depending upon the isotype of the immunoglobulin (IgA, IgG, or IgM), the type of protection differs. For the first 36h of life, ingested IgG and IgM will attach to receptors on the surface of epithelial cells of the small intestine. Transfer follows attachment via penetrating the cell into the systemic circulation. If the antibodies are specific for a virulence determinant, the disease may not result in the neonate encounters a pathogenic strain expressing virulence determinant. For example, anti-capsular antibodies acquired from the dam will protect the newborn from fatal invasive disease caused by strains of *E. coli* possessing particular capsule.

2.3.4 Non-enterotoxigenic Diarrhea

Specific anti-Bfp antibody (sIgA and sIgM) found in colostrum and milk prevents attachment to "target cells". Antibody specific for SLT will neutralize this toxin by preventing its activity on endothelial cells.

2.3.5 Edema Disease

Antibody specific for SLT-Ⅱe will prevent the edema associated with this condition. It is imperative that the dam be exposed either naturally or artificially to the microorganism

and its virulence determinants before parturition. Such exposure allows for antibodies to be made for secretion into colostrum and milk.

2.4 Laboratory Diagnosis

2.4.1 Demonstration of Enterotoxigenic Strains of *E. coli*

The numbers of enterotoxigenic strains proliferation approaches 10^8-10^9/ml in luminal contents. If the animal survives the fluid and electrolyte imbalances, large numbers are shed into the environment. Diagnosis is based on the suspicion that the disease is due to enterotoxigenic *E. coli*. The least troublesome and least invasive procedure (and also the least reliable) for verification of this suspicion are needed to demonstrate large numbers of specific adhesin-expressing *E. coli* in the feces. Demonstration entails plating a portion of a fecal sample onto a selective medium (MacConkey agar, for example). As adhesins are expressed poorly on selective media, a number of colonies are subcultured onto media in order to promote the expression of the various adhesins: for F4, E medium; for F5 and F6, Minca medium; and for F41, E or Minca medium. Slide agglutination tests are performed on each colony using antiserum specific for the various adhesins. An enzyme-linked immunosorbent assay has been developed to measure directly the presence of F4 and F5 adhesin-expressing bacteria in feces. Such a method eliminates many of the problems inherent in the analysis of feces for fimbriated bacteria. Gene-specific primers have been developed so that specific genes in isolated colonies can be proved by polymerase chain reaction.

A more reliable method to verify the clinical diagnosis of enterotoxigenic *E. coli*-induced diarrhea is to quantitate the number of *E. coli* in the small intestine. Normally, there should be very few *E. coli* in such sites, especially in the jejunum, and the presence of many bacteria in these locations is highly suggestive of enterotoxigenic *E. coli* disease. Samples are plated onto different media chosen to promote the expression of the various adhesins, and colonies are picked and tested with the monospecific anti-adhesin sera. Stained smear of the contents in the small intestine is another method based upon the increased numbers of enterotoxigenic *E. coli* in this location; finding > 100 per oil immersion field implies $>10^6$/ml of contents. Although this method lacks specificity, it strengthens the diagnosis.

Fluorescent-labeled antibody technique is the easiest method, and it is probably the most reliable except for demonstration of the toxin. Smears of scrapings taken from the small intestine are flooded with antisera that are specific for the various adhesins. After treatment with fluorescent-labeled secondary antiserum, preparations are examined for labeled bacteria adhering to the epithelial cells.

Enterotoxin production of isolated strains of *E. coli* is best specific for ST and LT detected by ELISA test. This test is reputed to detect 140pg/ml of ST (>100 times more

sensitive than the suckling mouse assay) and 290pg/ml of LT.

E. coli containing the genes encoding the various adhesins, as well as the enterotoxins can be detected by DNA probes or polymerase chain reaction (PCR). The primers are specific for the corresponding base sequences encoding a specific trait (e.g., an adhesin or an enterotoxin). Such probes or primers have been used to detect the genes (in bacteria) in feces as well as in culture.

2.4.2 Demonstration of Strains Producing Enteroaggregative Disease

Isolates suspected as being capable of producing enteroaggregative diarrheal disease, can be tested for their ability to associate with HEp-2 tissue culture cells in aggregative pattern (the "gold standard"). However, demonstration of the presence of DNA associated with the genes encoding EAST 1 and/or AAF is certainly easier and more cost effective. Histopathologically, a diagnosis of enteroaggregative disease is supported by the presence of bacteria associated with the intestinal epithelium.

2.4.3 Demonstration of Strains Producing Invasive Disease

The microbiological diagnosis of invasive disease is based upon the demonstration of *E. coli* in normally sterile sites or locations (joint, bone marrow, spleen, or blood). In fowl, the same sites are cultured and affected (lung, air sac). The embryos dead in-shell are cultured. Culture of the liver is to be avoided even though the Kupffer cells remove bacteria from the blood, because retrograde movement of enteric bacteria during the agonal stages of the disease complicates the microbiologic findings.

2.4.4 Demonstration of EPEC/EHEC Strains

The presence of genes encoding shiga-like toxins can be detected by specific DNA probes or PCR. More cumbersome is the demonstration of cytotoxin activity for tissue culture cells (Vero cells).

The demonstration of attaching and effacing strains caused disease in the live animal is more difficult. Aside from biopsy of intestinal mucosa and the finding of attaching and effacing lesions, detections of genes associated with EPEC/EHEC, *eaeA*, *bfp*, or *slt* have been used (specific DNA probes or polymerase chain reaction with sequence-specific primers), or function assays testing for SLT activity for tissue culture cells. Fecal isolates obtained from a selective medium (e.g., MacConkey agar) can be tested for the genes or production of shiga-like toxin in culture supernatants that are tested for cytotoxicity for tissue culture cells. Most of these isolates have been shown to produce urease, an uncommon trait for *E. coli*. *E. coli* O157: H7 does not ferment sorbitol. MacConkey agar containing this sugar instead of lactose is used to examine feces for the presence of sorbitol negative isolates, which are tested for antiserum specific for O157 and/or H7. Since Ehx production is found with considerable number of SLT-producing strains of *E. coli*, demonstration of these strains can be performed on

blood agar plates supplemented with calcium.

2.4.5 Demonstration of Strains Producing Edema Disease

The microbiological diagnosis of edema disease depends upon the isolation and demonstration of certain serotypes that have been shown to play a role in this disease. The characteristic and microscopic tissue changes make this disease relatively easier to diagnose pathologically than microbiologically.

2.5 Treatment, Control, and Prevention

Treatment of an animal that has diarrhea due to an infectious cause centers on correcting fluid and electrolyte imbalances. If the animal is in shock due to cardiovascular collapse, then the fluid and electrolytes (sodium bicarbonate, KCl) are given Ⅳ; if not, oral electrolyte solutions are given. Since the animals are acidotic, sodium bicarbonate is included. Adding glucose to oral electrolytes will enhance the absorption of the sodium ions excreted. The use of antimicrobials is controversial. Because the concentration of antimicrobic achievable (and available) in the lumen of the bowel is not known, the reliability of the results of susceptibility tests to guide therapy *in vitro* are doubtful. Administration of nonabsorbable antimicrobics (such as neomycin) will sufficiently reduce the number of *E. coli* in the upper small bowel to correct of fluid and electrolyte imbalances. Such reduction occurs even though *in vitro* tests show that strains of *E. coli* commonly test "resistant" to neomycin. The fact that *in vitro* tests measure susceptibility to microgram amounts whereas milligram amounts may be available locally accounts for the discrepancy.

Antimicrobial agents, fluid, and electrolyte augmentation are necessary to successfully treat septicemic disease produced by invasive strains of *E. coli*. Invasive disease leads to an endotoxemia progressing to a lactic acidosis because of decreased organ perfusion secondary to hypotension and disseminated intravascular coagulation. This should be taken into account when the electrolyte replacement is chosen. Select antimicrobial agents should according to susceptibility trends in practice. Usually, *E. coli* isolated from farm animals are susceptible to gentamicin, amikacin, trimethoprim-sulfonamides, and ceftiofur. They are usually resistant to tetracyclines, streptomycin, sulfonamides, ampicillin, and kanamycin. The severity of endotoxemia has been reduced experimentally by administering antibodies to lipid A of LPS.

Prevention and control of the enteric diseases produced by pathogenic strains of *E. coli* are the same. The key is sound rearing pattern. It is important that the dam be exposed to the antigenic determinants of the various virulence factors expressed by the infecting strains. Exposure can be provided naturally by placing the dam into the environment where parturition will take place or artificially by vaccinating the dam with preparations containing the antigenic determinants as a threat to the newborn. The

preparations containing monoclonal antibodies to the adhesins (for ETEC) can be given orally to the neonatal animal. Although this practice can't significantly reduce the diarrhea incidence, it will reduce the severity and mortality.

3 *Salmonella*

The genus *Salmonella* is a member of the Enterobacteriaceae, and it consists of two species, *S. bongori* and *S. enterica*. There are six subspecies within *S. enterica*, *enterica* (sometimes designated as subspecies Ⅰ), *salamae* (subspecies Ⅱ), *anzonae* (subspecies Ⅲ a), *diarizonae* (subspecies Ⅲ b), *houtenae* (subspecies Ⅳ and Ⅶ) and *indica* (subspecies Ⅳ) (those belonging to subspecies Ⅴ were placed into *S. bongori*). There are multitudinous serotypes (serovars), more than 2,000, within *S. bongori*, and the subspecies of *S. enterica*. The majority of these serovars have been given names that are capitalized and depicted in roman print. Others are merely denoted by antigenic formulae. Those belonging to *S. bongori* and *S. enterica* subspecies Ⅱ to Ⅶ are mainly associated with cold-blooded vertebrates, while those belonging to *S. enterica* subspecies Ⅰ are more commonly found in mammals and birds. However, each serovar is capable of producing disease regardless of the host.

Follows, the subspecies designation will not be used unless important to the discussion. For example, *Salmonella enterica* subsp. *enterica* serotype typhimurium will first be denoted as *Salmonella enterica* serotype typhimurium, and then, simply *S. typhimurium*.

3.1 Descriptive Features

3.1.1 Cellular Anatomy and Composition

There is one capsular type, Vi (for virulence), though most members of the genus do not produce one. The cell wall is typical of Gram negative bacteria, composed of lipopolysaccharide (LPS), and protein. The antigenic composition of the polysaccharide portion of the LPS in part determines the serotype. The kind and number of sugars together with the linkage between them determine the antigenic determinants comprising the O antigens of the particular isolate. The O antigens, together with the antigenic determinants on the surface of the flagella (H antigens), which are possessed by most salmonellae, help to define an isolate serologically. This classification scheme is called the Kauffman-White schema.

3.1.2 Cellular Products of Medical Interest

3.1.2.1 Adhesins

Adhesins (also known as fimbria or pili) mediate adherence to target cells in the

gastrointestinal tract and to cells comprising the niche for the strain. Owing to their relative hydrophobicity, adhesins may also promote association with the membrane of phagocytic cells. Adhesins are important virulence factors only when the microbe is on mucosal surfaces. There are at least three different adhesins implicated in the interaction between salmonellae and target cells (M cells, intestinal epithelial cells) of the host-Pef, Agf, and Lpf. Pef (plasmid encoded fimbriae) are responsible for attachment to small intestinal epithelial cells. Agf (thin aggregative fimbriae, or curli) are also responsible for attachment to small intestinal epithelial cells. Lpf (long polar fimbriae) are responsible for attachment to M cells.

3.1.2.2 Capsule

The role of the capsule (Vi) is unclear. Since salmonellae are primarily intracellular parasites, a capsule does not seem to be a strategy that is consistent with the role of this structure in other microorganisms (i.e., antiphagocytic). However, Vi protects the outer membrane from effective interactions with membrane attack complexes generated by the complement system. This is useful in protecting extracellular salmonellae.

3.1.2.3 Cell Wall

The lipopolysaccharide (LPS) in the outer membrane is an important virulence determinant. Not only is the lipid A toxic component (endotoxin), but the length of the side chain in the O repeat unit hinders the attachment of the membrane attack complex of the complement system to the outer membrane. LPS binds to lipopolysaccharide-binding protein (a serum protein), which in turn transfers it to the blood phase of CD14. The CD14-LPS complex binds to Toll-like receptor proteins on the surface of macrophage cells and results in the release of proinflammatory cytokines.

3.1.2.4 Effector (toxin) Proteins

Several of the genes that are responsible for *Salmonella* virulence are located in clusters, called pathogenicity islands, including a cluster of genes encoding virulence determinant(s), an integrase protein, a specific insertion site, and mobility. There are at least five *Salmonella* pathogenicity islands (SPIs). While SPI-1 is found in the species of *Salmonella*, SPI-2 (and presumably SPI-3 through SPI-5) is found only in *S. enterica*. All five SPIs contain genes encoding the necessary proteins for the Type III secretion system (though the genes and their products are different for each island), and may or may not contain the effector proteins (those proteins that interact with the host "target" cell). The Type III secretion system consists of an assemblage of proteins (more than 20) that form a hollow tube-like structure through effector proteins "injected" into host "target" cells.

The effector proteins associated with SPI-1 include Ssps (*Salmonella* secreted

proteins, encoded by a number of *ssp* genes located within SPI-1), and Sops (*Salmonella* outer protein, encoded by a number of *sop* genes, located outside of SPI-l). Ssps and Sops are related to uptake of salmonellae, the target cell(s) membrane of salmonellae is induced to "ruffles" that follow the rearrangement of the actin cytoskeleton following the activation of the small GTP-binding proteins, CDC42 and Rac. Additionally, Ssps also results in the death of activated macrophages via interacting with caspase-1.

The effector proteins associated with SPI-2 include Sses (secretion system effectors, encoded by a number of *sse* genes located within SPI-2), Ssas (secretion system apparatus, encoded by a number of *ssa* genes, located within SPI-2). Both Sses and Ssas are induced by low pH (stomach, phagosome), and interfering with macrophage function.

The effector proteins associated with SPI-3 include Mgts (magnesium transport system, encoded by a number of *mgt* genes located within SPI-3). These genes are induced by magnesium ions in low concentration (as occurs within macrophages), and the encoded proteins seem to be important for survival inside of macrophages.

It is not clearly defined whether the effector proteins associated with SPI-4 and SPI-5 are associated with intracellular survival.

3.1.2.5 Enterotoxin

Members of the genus *Salmonella* secrete an enterotoxin, Stn (*Salmonella* enterotoxin) associated with water and electrolyte secretion by host target cells. Stn differs from cholera toxin and LT of *E. coli* by being a peptide rather than being composed of subunits. The role (if any) of Stn in the production of diarrhea is unclear.

3.1.2.6 Iron Acquisition

Salmonellae produce siderophores (enterobactin) when growing in iron-limiting conditions.

3.1.2.7 Stress Proteins

Stress proteins are defined as proteins when the microorganism is placed under conditions of stress (e.g., heat, cold, low pH, high pH). RNA polymerase containing RpoS preferentially transcribes genes which are responsible for acid tolerance (survival at pH 5) and regulates genes found on Spv plasmids. RNA polymerase containing RpoE is involved with survival within phagocytic cells.

3.1.2.8 Virulence Plasmids

Salmonellae possess various plasmids that have different sizes, some have been associated with virulence. The most notable is a family of large (50-100kb) plasmids. Termed "*Salmonella* virulence plasmids" (Spv plasmids) that are found within those

species of salmonellae with potential to produce disseminated disease. Some of the genes (*spv* genes) carried by these plasmids are necessary for intracellular growth and regulated in part by RNA polymerase containing the stationary phase sigma factor, RpoS. Other genes on these plasmids are responsible for serum resistance and may be related to adherence and invasion of the cellular target.

3.1.2.9 Miscellaneous Products

The transcriptional regulator, SlyA (for salmolysin), is in part responsible for survival of salmonellae within macrophages, perhaps affording protection from the toxic products generated by oxygen-dependent pathways. The products of the phoP/phoQ operon are responsible for resistance of salmonellae to defensins found in the lysosomal granules of phagocytic cells (by directing the remodeling of the salmonella outer membrane). The product of the *shdA* (for shedding) gene governs fecal shedding of salmonellae in an infected host. This gene is restricted to serotypes of *S. enterica* subsp. *enterica*. Arc (for aerobic regulation control), is a two-component global regulator system, and related to intracellular survival.

3.2 Ecology

3.2.1 Reservoir

The reservoir for members of the genus *Salmonella* is the gastrointestinal tract of warm- and cold-blooded animals. Sources of infection include contaminated soil, vegetation, water, and components of animal feeds (such as bone, meat, and fish meal), particularly those containing milk-, meat-, or egg-derived constituents, and the feces of infected individuals. Lizards and snakes (usually asymptomatic) are commonly infected, sometimes with several serotypes. *Salmonella enterica* subsp. *enterica* are almost exclusively found in warm-blooded mammals and birds (evidence suggests that the possession of the *shdA* gene product is responsible).

3.2.2 Transmission

Infection occurs following the ingestion of salmonellae. The result of the interaction between host and *Salmonella* depends upon the condition of the colonization resistance to the host (a measure of the "barrier" produced by the normal flora), the infectious dose, and the particular species of *Salmonella*. Disease may or may not occur following ingestion. If it occurs, it may do so immediately, or later. In the later instance, the initial interaction may lead to the colonization (without disease) of the host, but with a change in the intestinal environment, brought on, for example, by stress or antibiotics (activities that affect the normal flora), and disease may follow.

3.2.3 Pathogenesis

3.2.3.1 Mechanisms

The most common clinical manifestation of salmonellosis is diarrhea. Some cases (defined by host factors, the strain and dose of *Salmonella*) occurs septicemia. Host factors include age, immune status, concurrent disease, and "health" of the normal flora (colonization resistance).

Stationary phase salmonellae appear best suited to initiate disease, because under these conditions, RNA polymerase containing the alternative sigma factor, RpoS, initiates transcription of the genes which are responsible for adding tolerance and subsequent survival through the stomach. Also, RNA polymerase containing RpoS is a positive regulator for the genes found on the Spv plasmids.

The target cells are the M cells atop the lymphoid nodules and the epithelial cells of the distal small intestine and the upper large bowel. If the target cell is "vacant" relative to the numbers of salmonellae (a reflection of the colonization resistance), it may result in disease. Vacancy of the target cell depends upon the status of the normal flora. If the flora is disrupted by stress, antibiotics, then the infectious dose does not have to be as high for salmonellae approach to the target cell. It appears that the M cell is the preferred target, and it is this cell that is affected first. Adhesion is the first step in the disease process, mediated by one or more of the adhesins Agf, Pet, Lpf, or by others yet to be proved. Following adhesion, salmonellae are internalized following the induction of membrane ruffles in the target cells triggered by Ssps and Sops subsequent to their "injection" by the Type III secretion system. The target cell is irreversibly damaged via this interaction, undergoing apoptosis. Salmonellae are now found within the target cells, the lymph nodule, and submucosal tissue. An inflammatory response is initiated by release of various chemokines from affected host cells, as well as release of proinflammatory cytokines following host interaction with cell wall. LPS-activities that result in an influx of polymorphonuclear neutrophil leukocytes (PMNs) and macrophages. The influx of PMNs may be reflected in a transient peripheral neutropenia. PMNs are highly efficient in phagocytosing and destroying salmonellae. If the immune status of the host and the characteristics of the salmonellae are such, the infectious process is arrested at this stage. Diarrhea is considered to result from prostaglandin synthesis by the recruited PMNs (and perhaps by the affected host cells), as well as activation of various inositol-signaling pathways within affected host cells. The net result is the secretion of chloride ions and water. The role of Stn in the process of diarrhea is unclear.

If the infecting strain of *Salmonella* has properties that allow dissemination (possession of SPI-2, 3, 4, and 5-associated gene products that allow growth within macrophages; Spv plasmid encoding ability to grow intracellularly and serum resistance; phoQ/phoP system allowing resistance to defensins; SlyA allowing resistance to oxygen-dependent

by-products; *areA*), septicemia may result. The likelihood of this occurring is increased if immune status of the host is diminished. Salmonellae disseminate and multiply within phagocytic cells (macrophages mainly) and phagosomes. Not only are the invasive strains better able to withstand the action of lysosomal contents, some "sort" to phagosomes that do not fuse with lysosomes. The presenting signs are usually, but not always, septicemia and shock. The form of disease produced by strains can escape destruction by the host and multiply within macrophages of the liver and spleen, as well as intravascularly. During the dissemination process, salmonellae are occasionally outside of the intracellular environment and therefore at risk from the formation of complement membrane attack complexes on their surfaces. This occurrence is discouraged by at least two mechanisms: a product of the Spv plasmid and the length of the O repeat unit of the LPS (there is a direct correlation between O repeat length and virulence).

Invasive salmonellae are capable of secreting a siderophore, enterobactin that removes iron from the iron-binding proteins of the host, although it is doubtful whether this is needed within the cells of the host.

Multiplication of the organism results in endotoxemia, which accounts for most signs and the course of illness.

3.2.3.2 Pathology

If the infectious process is limited to the intestinal tract, the lesions will consist of a hemorrhagic inflammation of the distal small intestine and large bowel. There may be superficial necrosis. For the septicemic form, there are inflammatory changes in liver, spleen, and intestinal tissue. There may be hemorrhages on pericardium, peritoneal surfaces, and adrenal cortices.

3.2.4 Disease Patterns

3.2.4.1 Swine

Salmonellosis in swine can present as an acute, fulminating septicemia or as a chronic debilitating intestinal disease. The form depends upon the strain of *Salmonella*, the dose, and the colonization resistance of the infected animal. The disease is the most common in stressed pigs. Such conditions occur often in feeder pigs, an age group in which salmonellosis commonly occurs. *S. typhimurium* and *S. enterica* are the predominant serotypes.

3.2.4.2 Dogs and Cats

Salmonellosis is rare in dogs and cats, although carrier reportedly high in clinically normal dogs (upwards of 35%). They are usually associated with a common source, such as contaminated dog food or "treats" (e.g., dried pig's ears). *Salmonella* should be

high on the microbiological differential list for cats with signs of septicemia.

3.2.5 Epidemiology

Salmonella are ubiquitous geographically and zoologically. Some serotypes are relatively host-specific (*S. dublin-cattle*; *S. enterica* serotype Typhisuis-swine; *S. enterica* serotype Pullorum-fowl), however others, notably *S. typhimurium*, *S. anatum*, and *S. newport*, affect a wide host range, the feral birds and rodents play an important role in interspecific dissemination of infection. Long periods of asymptomatic and convalescent shedding ensure widespread, unchecked distribution of the organisms.

Clinical outbreaks are correlated with depressed immune states, as in newborn animals (calves, foals) and stressed adults, parturient cows, equine, and swine with systemic viral diseases. All animals are at increased risk of developing disease if their normal flora is disrupted (stress, antibiotics). These circumstances lead to animals susceptible to exogenous exposure or activation of silent infections.

Humans seem to be susceptible to all *Salmonella* serotypes, the most important sources are animals and their by-products. Poultry and poultry products (eggs) are a primary source of *Salmonella* in humans. *Salmonella enterica* serotype Enteritidis (e.g., phage type 4) is especially adapted for egg transmission. When one person ingests salmonellae from the environment, whether he develop disease depends upon the dose of organisms, the serotype of *Salmonella*, and the colonization resistance of the infected individual. *Salmonella typhimurium* is most common, usually producing gastroenteritis. Some serotypes have greater invasion potential, for example, *S. choleraesuis* (from swine), and *S. dublin* (infected milk). Though *S. typhimurium* DT104 (the definitive type designation specifies a particular phage type), which is acquired from cattle, appears more prone to systemic disease, though this has been difficult to prove experimentally, Asymptomatic reptiles have become an important source of *Salmonella* to humans.

3.3 Immunologic Aspects

Protection depends upon specific and nonspecific immunological factors and microbiological defense mechanisms. Disease may be prevented by an intact colonization resistance at the level of the target cell. Whether infection can be prevented is not known.

Antibodies are specific for surface structures of *Salmonella*, possibly adhesins, prevent adherence to target cells. The newborn is protected passively by ingesting specific sIgA or IgG_1 (bovine). The adult animals are protected by exudation of specific immunoglobulins (IgM and IgG) at the site of invasion or by the production of secretory immunoglobulins.

Another, more novel approach has been applied to feed normal animal microorganisms that out-compete salmonellae for niches along the gastrointestinal tract. This

phenomenon called competitive exclusion, may be quite useful because, theoretically, salmonellae of any serotype would be excluded as long as they shared the same niche as the competing strain.

Antibodies in the circulation promote the phagocytosis of the organism as opsonins. Destruction of the salmonellae that have been phagocytosed follows the immunological activation of the macrophages by specifically stimulated lymphocytes (T cells); though activated macrophages are damaged or killed by Ssps. NK cells lyse *Salmonella*-infected cells.

Acquired immunity revolves around activation of macrophages, which takes place as follows. After initial interaction between salmonella and macrophage, IL-12 is released by the affected macrophage. IL-12 activates the TH1 subset of T helper cells. This subset secretes cytokines, interferon gamma, which activates macrophages. Activated macrophages are efficient killers of intracellular salmonellae.

Artificial immunization against salmonellae is difficult. Bacterins have limited success. Apparently, they do not stimulate cellular immunity, even though abundant antibody is produced. Antibodies that are produced locally or passed in colostrum or milk interfere with adsorption to the target cell and protect against disease in this location. Macrophages can be activated and antibody production stimulated in response to modified live vaccines. If given orally, these vaccines stimulate local secretory immunity and cell-mediated activation of phagocytic cells. Aromatic-dependent mutants of *Salmonella* show promise as effective modified live vaccines, especially for calves. *aroA* mutants of *Salmonella* can not multiply within the host since vertebrate tissue does not contain the needed precursors for aromatic acid synthesis.

3.4 Laboratory Diagnosis

In the cases of intestinal infection, fecal samples are collected; in systemic disease, a blood sample is collected for standard blood culture. Spleen and bone marrow are cultured for the salmonellae if postmortem diagnosis of systemic salmonellosis is required.

Fresh fecal samples are placed onto one or more selective media, including MacConkey agar, XLD agar, Hektoen enteric medium, and brilliant green agar. For enrichment, selenite F, tetrathionate, or Gram negative broth (GN) is recommended.

Salmonellae appear as lactose-nonfermenting colonies on lactose-containing media (lactose-fermenting strains of *Salmonella* have been reported, but these are rarely encountered). Since most serotypes of salmonellae produce H_2S, colonies on non-containing media (e.g., XLD agar), they will have a black center. Suspicious colonies can be tested directly with polyvalent anti-*Salmonella* antiserum or inoculated into differential media and then tested with antisera.

To cultivate salmonellae from tissue, blood agar can be used.

Definitive identification includes determination of somatic and flagellar antigens and possibly bacteriophage type.

Various *Salmonella*-specific DNA probes and primers for the polymerase chain reaction have been developed for identification in samples containing food, feces, water and other microorganisms.

A multiplex polymerase chain reaction (PCR) assay by primers designed to detect the common diarrhea-associated microorganisms of Swine (*Brachyspira hyodysenteriae*, *Lawsonia intracellularis*, and *Salmonella*) has been reported.

3.5 Treatment, Control and Prevention

The primary treatment for the enteric form of salmonellosis is nursing care. The use of antimicrobial agents is controversial. Some studies show that antibiotics do not alter the course of the disease. In addition, there is evidence that antibiotics promote the carrier state and select for resistant strains. Proponents of antibiotic usage recommend a member of the fluoroquinolones (e.g., enrofloxacin or ciprofloxacin).

Treatment of the systemic form of salmonellosis includes nursing care and appropriate antimicrobial therapy. This kind of antimicrobial drug includes ampicillin, enrofloxacin, trimethoprim-sulfonamides, and chloramphenicol/florfenicol. Treatment options may be compromised due to acquisition of R plasmids or integrons encoding resistance to multiple antibiotics. A serious global epidemic of *S. typhimurium* DT104 (the definitive type designation specifies a particular phage type), a type of salmonella that affects humans and other animals worldwide, contains a "cluster" of antibiotic resistance-encoding genes within its chromosome. This "cluster" called "*Salmonella* genomic island 1" (SGI l), contains the genes encoding resistance to ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides, and tetracyclines bounded by two integrons. SGI l has moved into *S. enterica* serotype Albany (fish in Southeast Asia) and *S. enterica* serotype Paratyphi B (tropical fish in Singapore).

Salmonellosis is controlled through strict protocols designed to curtail the spread to susceptible animals of any contagious agent found in feces. Artificial immunization with modified live products has shown promise (e.g., *aroA* mutants). Attempts have been made to treat and prevent the endotoxemia produced by the systemic form of the disease by administering serum containing antibodies to the core LPS. Likewise, the administration of J5, a rough variant of *E. coli*, has been shown to stimulate the production of antibody to the core LPS. Both methods seem to prevent and control the signs of disease produced by systemic salmonellosis.

Review Questions

1. Please briefly describe the basic characteristics of Enterobacteriaceae.
2. What are the common characteristics of *Escherichia* and *Salmonella*?
3. Try to describe the pathogenesis of pathogenic *E. coli*.
4. Try to describe the characteristics of the virulence factors in *Salmonella*.
5. Try to describe the basic processes of isolation and identification for *Escherichia* and *Salmonella*.
6. Try to describe the antigen structure of *Escherichia* and *Salmonella* and their presentation.
7. Try to describe the classification and their basis of *Escherichia* and *Salmonella*.
8. Try to describe the public health implications of *Escherichia* and *Salmonella*.

Chapter 9 Pasteurellaceae

Synopsis

Members of the Pasteurellaceae are Gram negative and are dyed in two poles of cell. Many members are highly pathogenic to humans and animals. *Pasteurella multocida* can cause acute or chronic infection in animals, and the key points for diagnosis of pasteurellosis include bipolar staining, no hemolysis, pathogenicity to mice. Enzyme system of *Haemophilus* is incomplete. Growth factors in blood especially X factor or and V factor are needed for growth of *Haemophilus*. *Haemophilus* can grow better in the chocolate medium, and there is a satellite phenomenon when *Haemophilus* grow with *Staphylococcus aureus*. Many species of *Haemophilus* are opportunistic or secondary infections agent for humans and animals. *Haemophilus* can be detected by many methods, e.g., isolation and culture, biochemical identification and PCR. Diseases caused by *Pasteurella multocida* and *Haemophilus* can be treatment with sensitive drugs and be prevented with effective vaccines.

Genus *Pasteurella* is Gram negative, nonmotile, non-sporulating, facultatively anaerobic coccobacillary to rod-shaped bacteria which are parasitic and often pathogens in many species of mammals, birds and reptiles. It was named to honor Louis Pasteur in 1887. Genetic studies have shown that *Pasteurella*, together with *Haemophilus* and *Actinobacillus*, constitute a family termed Pasteurellaceae.

Pasteurellaceae consists of a large and diverse family of Gram negative bacteria with members ranging from important pathogens such as *Haemophilus influenzae* to commensals of the animal and human mucosa. The information about the biology of these organisms has mushroomed in recent years, driven by the development of novel genetic and molecular methodologies. Since 1995, the family has been expanded from three genera to the current thirteen via the new genetic-based classification and identification technologies. Many members of the Pasteurellaceae make excellent natural models for the study of bacterial pathogenesis and host-pathogen-interactions, thus giving valuable insights into related human diseases. Research in this area is at a very exciting stage.

1 *Pasteurella multocida*

1.1 General Characteristic

Pasteurella multocida, as a kind of pathogenic bacterium, can cause hemorrhagic septicemia to many kinds of domestic animal. Epidemics of hemorrhagic septicemia mainly occur in cattle and buffalo; water buffalo are considered to be particularly susceptible. Infections infrequently occur in pigs, sheep, and goats. Cases have also been reported in bison, camels, elephants, horses, donkeys, deer, and yaks. Cattle, water buffalo, and bison appear to be the reservoirs of infection. Now *Pasteurella multocida* is divided into three subspecies: *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica* and *P. multocida* subsp. *gallicida*.

Owing to the economic relevance of *P.* multocida-mediated infections, work in several fields concerning this bacterial species, such as molecular characterization of pathogenic factors and construction of mutants to be used as vaccines, is increasing. However, few genetic tools for *P. multocida* genetic manipulation are available.

1.1.1 Morphology and Staining

Cells measure 0.2-2.0μm. Bipolarity (Fig. 9-1), that is, the staining of only the tips of cells, is demonstrable with polychrome stains (e.g., Wright's stain). The capsules of *P. multocida*, *P. haemolytica*, and *P. trehalosi* are the basis for type specificity.

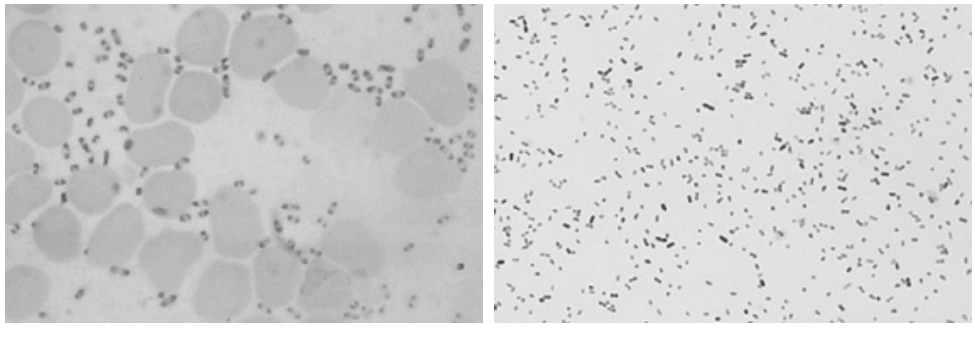


Fig. 9-1 *P. multocida* in a bovine blood smear (left) and in a culture (right) (×1,000) (Markey *et al.*, 2013)

1.1.2 Growth Characteristics

Pasteurella grow best in the presence of serum or blood. After overnight incubation, colonies are about 1mm in diameter, clear, and smooth or mucoid. *Pasteurella haemolytica* and *P. trehalosi* produce hemolysis on ruminant blood agar.

1.1.3 Serotype

Pasteurella multocida is a pathogenic Gram negative bacterium that has been classified into three subspecies, five capsular serogroups and 16 serotypes. *P. multocida* serogroup A isolates are bovine nasopharyngeal commensals, bovine pathogens and common isolates from bovine respiratory disease (BRD), both enzootic calf pneumonia of young dairy calves and shipping fever of weaned, stressed beef cattle. *P. multocida* A: 3 is the most common serotype isolated from BRD, and these isolates have limited heterogeneity based on outer membrane protein (OMP) profiles and ribotyping.

1.2 Ecology

Development of *P. multocida*-induced pneumonia is associated with environmental and stress factors such as shipping, co-mingling, and overcrowding as well as concurrent or predisposing viral or bacterial infections. Lung lesions consist of an acute to subacute bronchopneumonia that may or may not have an associated pleuritis. Numerous virulence or potential virulence factors have been described for bovine respiratory isolates including adherence and colonization factors, iron-regulated and acquisition proteins, extracellular enzymes such as neuraminidase, lipopolysaccharide, polysaccharide capsule and a variety of OMPs. Immunity of cattle against respiratory pasteurellosis is poorly understood; however, high serum antibodies to OMPs seem to be important for enhancing resistance to the bacterium. Currently available *P. multocida* vaccines for use in cattle are predominately traditional bacterins and a live streptomycin-dependent mutant. The field efficacy of these vaccines is not well proved in the literature.

Pasteurella multocida of serogroups A and D are mainly responsible for disease in poultry and pigs and to a lesser extent in cattle. Fowl cholera in chickens is caused by various serotypes of *P. multocida* serogroup A and characterized by acute septicemia and fibrinous pneumonia or chronic fibrinopurulent inflammation of various tissues.

Current biologicals are live *P. multocida* vaccines and bacterins. Potency tests for avian *P. multocida* biologicals are a bacterial colony count for vaccines and vaccination and challenge of birds for bacterins. Somatic antigens, particularly lipopolysaccharide (LPS), appear to be of primary importance in immunity. In cattle, *P. multocida* serogroup A is associated mainly with bronchopneumonia (enzootic pneumonia) in young calves; however, it is occasionally isolated from fibrinous pleuropneumonia of feedlot cattle (shipping fever). Biologicals currently available are modified-live vaccines and bacterins. The potency test for vaccines is bacterial colony counts. The test for bacterin potency is vaccination and change of mice. Important immunogens have not been well characterized for *P. multocida* infection in cattle. In swine, *P. multocida* infection is sometimes associated with

pneumonia; however, its major importance is in atrophic rhinitis. A protein toxin (dermonecrotic toxin), produced by toxigenic strains of *P. multocida* types A and D, and concurrent infection with *Bordetella* bronchiseptica appears to be the major factors in development of atrophic rhinitis.

Currently available biologicals are bacterins and inactivated toxins (toxoids). The toxin appears to be the major immunogen for preventing atrophic rhinitis. There are, however, no standardized requirements for potency testing of *P. multocida* type D toxoid. Various serotypes of *P. haemolytica* biotype A are responsible for severe fibrinous pleuropneumonia in cattle and sheep, occasionally septicemia in lambs, and mastitis in ewes. Several serotypes of *P. haemolytica* biotype T are isolated from acute septicemia in lambs. The currently available *P. haemolytica* biologicals are modified-live vaccines, bacterins, bacterial surface extracts, and culture supernates that contain an exotoxin (leukotoxin).

1.3 Immunity

The role of LPS in immunity was studied via monoclonal antibodies (MAbs) and active immunisation experiments. A panel of six MAbs produced against *Pasteurella multocida* serotype B: 2 only reacted with the LPS of serotypes B: 2 and B: 5. The MAbs could opsonise *P. multocida* for phagocytosis by mouse macrophages, but were not bactericidal in the presence of complement. They conferred only partial passive protection in mice. Similar results showing only partial protection were obtained when purified LPS was used to actively immunise mice prior to challenge, suggesting that LPS plays a partial role in immunity to infection.

The *aroA* gene from *P. multocida* serotypes A: 1 and A: 3 was cloned and inactivated by inserting a kanamycin resistance gene. The mutated gene was reintroduced into the chromosome by allelic exchange. The resultant of *aroA* mutants were highly attenuated in a mouse model system, with a dramatically decrease in ID_{50}. Virulence could be restored by complementation with a functional *aroA* gene. Mice immunised with two doses of the live mutants were protected against lethal challenge of the homologous parental strain, but not against the heterologous strain. *P. multocida* A: 1 and A: 3 express unique proteins if they grow in iron-restricted medium. Moreover, the outer membrane (OM) fractions of these cells contain novel proteins of 75kDa, 85kDa and 94kDa molecular mass. Mice were immunised with OM fractions prepared from serotype A: 3 grown in iron-restricted (OM Fe^{-}) or iron-replete (OM Fe^{+}) media. When low challenge doses were used, both immunogens protected mice against serotype A: 3, but only the OM Fe^{-} fraction protected mice against heterologous challenge with serotype A: 1. When higher challenge doses were used, only partial protection was observed.

1.4 Diagnosis

1.4.1 Direct Examination

Exudates, tissue impressions, sediments of transtracheal aspirates, and blood smears can be stained with a polychrome stain (e.g., Giemsa, Wright's, Wayson's) and examined for bipolar organisms. Their presence is suggestive but not unique to *Pasteurella*. On Gram stain, *Pasteurella* do not look distinctive.

1.4.2 Isolation and Identification

Pasteurellae that grow culture overnight on bovine or sheep blood agar are identified via differential tests. Serotyping is done in a reference laboratory.

1.5 Treatment and Control

Pasteurellae respond to timely antimicrobial therapy. Strains from carnivores and humans are generally susceptible to almost all antimicrobials. Most pasteurellae show moderate resistance to aminoglycosides, which is probably not significant clinically. In these species, the requirements of cost and with drawal before slaughter are additional considerations. Consequently, sulfonamides, penicillin G, ceftiofur, tilmicosin, florfenicol, and tetracyclines are preferred. Whether they are appropriate should depend on the result of susceptibility tests, Sulfonamides, penicillin G, quinolones, and tetracycline is used to treat fowl cholera.

Sulfonamides, tetracyclines and penicillin are also suitable for mass medication via feed or water in swine and poultry, therapeutically or prophylactically. Management practices directed at reducing stress are important in preventing pasteurellosis in livestock. Immunization has been dependable only in bovine hemorrhagic, septicemia and atrophic rhinitis control.

2 *Haemophilus*

2.1 General Characteristic

This genus is Gram negative, pleomorphic bacteria that are facultative anaerobes and nonmotile and non-spore-forming. *Haemophilus influenzae* is the first of the species which is isolated and considered. It was originally recovered during the influenza pandemic of 1889 and for a time was considered to be the causative agent of influenza; therefore it was named the influenza *Bacillus*. However, when this fallacy became apparent, the organism was renamed, still reflecting the historical association with

influenza.

Haemophilus parasuis, a resident of the normal nasopharynx of swine, can cause septicemic disease or secondary respiratory infections. *Haemophilus paragallinarum* results in infectious coryza in chicken. "*Haemophilus somnus*" causes the infections of septicemic, respiratory and genital in cattle. It is semblable between "*Histophilus ovis*" and "*Haemophilus agni*", which produce the similar conditions in sheep.

2.1.1 Morphology and Staining

Haemophilus spp. are less than a micrometer wide and 1-3μm long (Fig. 9-2), but sometimes form longer filaments. Some species (*H. influenza*, *H. paragallinarum*) are encapsulated. Pili have been described in *H. influenzae.*

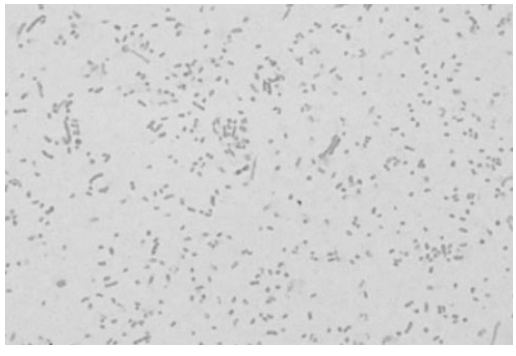


Fig. 9-2 *Haemophilus* species with microscopic appearance (Rosenberg *et al.*, 2013)

2.1.2 Growth Characteristics

Haemophilus spp., in adequate media, produces turbidity in broth and colonies 1mm in diameter on agar within 24-48h. Growth factors may be supplied as hemin and NAD about 1.0μg/ml of each. A medium naturally is chocolate agar, a blood agar supplemented with blood when the melted agar is at 75-80℃ rather than 50℃ (when making regular blood agar). This procedure releases NAD from cells and inactivates enzymes destructive to NAD.

Alternatively, a "feeder" bacterium (e.g., *Staphylococcus*) may be inoculated across plates where *Haemophilus* has been streaked. Otherwise growth occurs only near the feeder streak on inadequate media, the phenomenon is named satellitism (Fig. 9-3). It may be duplicated via commercially prepared X and V factor, impregnated filter papers placed on the inoculated area for the X and V factor requirements of *Haemophilus* spp.

2.1.3 Resistance

Haemophilus spp. are readily killed by heat, and die rapidly in culture and storage unless freeze dried or stored at –70℃. At cool temperatures, *H. paragallinarum* survive

Fig. 9-3 Satellitism, typical of *Haemophilus* species (Markey *et al.*, 2013)

in exudate for several days. Strains of *H. influenzae* are increasingly resistant to kinds of antibiotics. Thus, an extended-spectrum cephalosporin is generally recommended for empiric treatment of serious disease.

2.1.4 Variability and Serotypes

Serotypes may differ in pathogenicity and geographic prevalence, and determine the specificity of bacterin. There are three serotypes (A-C or Ⅰ-Ⅲ) of *H. paragallinarum* and at least seven of *H. parasuis.*

Haemophilus species are distinguished by a number of criteria. Strains of *H. influenzae* can be divided into encapsulated and nonencapsulated forms. Encapsulated strains express one of six distinct capsular polysaccharides that are designated serotypes. Nonencapsulated strains are referred to as non-typable.

2.2 Ecology

2.2.1 Pathogenicity

The antiphagocytic capsules and heat-labile cytotoxic factors of *Haemophilus paragallinarum* are suspected virulence factors. The lesions of *Haemophilus* infections also suggest endotoxin involvement. *Haemophilus somnus* adheres to epithelium and endothelium, is toxic to endothelial cells, is resistant to serum and phagocytic killing, and binds immunoglobulins such as staphylococcal protein A.

All infections have suppurative components. Infection of lungs body cavities, and joints tends to be serofibrinous to purulent. Bacterial colonization of the meningeal vessels produces a thrombotic vasculitis that results in encephalitis and meningitis. Hemorrhagic necrotizing processes are caused by *H. somnus.* Fowl coryza is marked by catarrhal inflammation with heterophil exudates.

2.2.2 Epidemiology

In swine, *H. parasuis* can cause bronchopneumonia that is secondary to virus infections (e.g., swine influenza). Other bacteria (e.g., *Pasteurella* spp. and *Mycoplasma* spp.) may also participate.

In young weaned pigs, *H. parasuis* also causes polyserositis, an acute inflammation that affects pleura/peritoneum, mediastinum, pericardium, joints, and meninges. The stress of weaning, transport, and management are predisposing causes. The disease outbreaks sporadically within days on account of the stressing event. Morbidity and mortality are often low because of widespread acquired resistance, but may be high in previously uninfected herds (e.g., specific pathogen-free herds). Disease manifestations include fever and general malaise, respiratory and abdominal distress, lameness, and paralytic or convulsive symptoms. Recovery begins in 1-2 weeks. The similar syndromes are due to *Mycoplasma hyorhinis*.

In chickens, infectious coryza (caused by *H. paragallinarum*) is an acute contagious upper respiratory infection. It affects chickens in different ages practically. The symptoms include nasal discharge, swelling of sinuses, facial edema, and conjunctivitis, with air sac and lung involvement. In the uncomplicated infection, mortality is low. Loss of productivity is the most damaging. Superimposed infections with mycoplasmas and helminth parasites exacerbate and prolong outbreaks. Other species, only Japanese quail are highly susceptible.

In cattle, *H. somnus* causes a septicemia resulting in thrombotic meningoencephalitis (infectious thromboembolic meningoencephalitis, TEME) and infarcts in brain and cerebellum. The pre-encephalitic stage is marked by high fever. With central nervous system (CNS) involvement, motor and behavioral abnormalities.

Haemophilus somnus occurs in pneumonic processes, usually with other agents, e.g., *Pasteurella* spp. Isolations have been made from normal and inflamed female genitalia and aborted fetuses. It is common in the genital tract of cow.

In sheep, *Haemophilus*, like organisms (*H. somnus*, *Histophilus ovis* and *H. agni*) cause respiratory and mammary infections, epididymitis of immature rams, and septicemias occasionally.

In dogs, *H. haemoglobinophilus*, a commensal of the canine lower genital tract, sometimes causes cystitis and neonatal infections. Its role in balanoposthitis and vaginitis, where it is frequently found, is unclear.

Haemophilus influenzae is also a human-specific pathogen that inhabits the upper respiratory tract and is spread by exposure to airborne droplets or contact with respiratory secretions. Non-typable strains can be isolated from the nasopharynx of up to 80% of normal children and adults at any time, usually in association with asymptomatic colonization. Overall, these organisms are the primary cause of

exacerbations of chronic bronchitis, and the secondary common etiology of acute otitis media and sinusitis. Occasionally, non-type *H. influenzae* causes invasive disease such as meningitis, septicemia, endocarditis, epiglottitis, or septic arthritis. Invasive disease frequently occurs in neonates and in patients with underlying immunodeficiency, especially when humoral immunity occurs abnormalities.

Encapsulated strains of *H. influenzae* are present in the nasopharynx of only 2-5% of children and an even smaller percentage of adults. Historically, The strains of *H. influenzae* type b were primary cause of childhood bacterial meningitis and a majority of other bacteremic diseases in children. However, in recent years the incidence of disease caused by *H. influenzae* type b has plummeted in developed countries, reflecting the routine use of *H. influenzae* conjugate vaccines. These vaccines provide effective protection against disease caused by *H. influenzae* type b but fail to protect against non-type b strains.

Haemophilus aphrophilus, *H. haemolyticus*, *H. parahaemolyticus*, *H. parainfluenzae* and *H. segnis* are members of the normal flora in the human oral cavity and oropharynx with low pathogenic potential. Among these species, *H. parainfluenzae* is the most common pathogen that has been reported in association with a variety of diseases.

2.3 Diagnosis

Recovery of the organism from infected tissues or fluids is usually needed to establish a diagnosis. Observation of Gram negative rods in these specimens prior to culture may suggest *Haemophilus* infection. Isolation of such an organism is followed by demonstration of a growth factor requirement. Organisms requiring X factor cannot convert delta-aminolevulinic acid to urobilinogen and porphyrin. The porphyrin test determines this role of X factor. Definitive assignment to a species usually requires additional tests. Fowl coryza can be diagnosed by agglutination, agar gel, immunodiffusion, and hemagglutination-inhibition tests.

2.4 Treatment and control

The hemophili of most animals are susceptible to penicillin G, ceftiofur, and tetracyclines. Calves with shipping fever involved in *H. somnus* respond to tilmicosin. For fowl coryza therapy, erythromycin or sulfonamides can be added in feed or water. *Haemophilus somnus* is susceptible to penicillin G and tetracycline. For the septicemic-meningoencephalitic form, timeliness and maintenance of treatment are critical.

Infectious coryza of fowl is controlled by elimination of the carriers and immunization of individuals at risk. Bacterins are of value in prevention of TEME but are less effective in other infections of *H. somnus*. When breeding stock must be

preserved, flock additions are vaccinated at 16 weeks, four weeks before joining the infected flock.

Review Questions

1. What are the genera in *Pasteurellaceae* which have important pathogenic effects on animals?
2. Try to describe the dyeing properties of *Pasteurella multocida*.
3. Try to describe the virulence factor and its pathogenicity of *Pasteurella*.
4. How to do the microbiology diagnosis of pasteurellosis?
5. Try to describe the changes about members of *Haemophilus*.
6. Try to describe the morphological and structural characteristics of *Haemophilus*.
7. Try to describe the culture characteristics of *Haemophilus*.
8. How to do the microbiology diagnosis of haemophilosis?

Chapter 10 Gram Negative Aerobe

Synopsis

Many members of Gram negative aerobic *Bacillus* are important pathogeny of humans and animals. Brucellosis caused by *Brucella* is a major worldwide disease, chronic and progressive debility, and characterized by the inflammation in the reproductive organs and joint. *Brucella* is a small *Bacillus* that has no buds or flagellum, and is a kind of intracellular bacteria. while erythritol can promote the growth of *Brucella*. The types of *Brucella* colony include smooth-, mucous- and coarse-form colonies. *Brucella melitensis*, *Brucella abortus* and *Brucella suis* are important pathogenic species of *Brucella*. The separation and culture of *Brucella* should bet at biosafety level 3 laboratory, and the detection of *Brucella* in samples should be at biosafety level 2 laboratory. Serology, allergic reaction and PCR are suit for the diagnosis and quarantine of *Brucella*. Antibiotics are not recommended for the treatment of brucellosis in animals. While vaccination has a significant effect to brucellosis. Animal groups are purified by the deletion of positive animals with strict detection of animal antibodies.

1 Overview

Gram negative aerobic bacteria are a large group of aerobic bacteria, which show up as pink (negative) when use the Gram-staining method to dipole. This is because the cell walls of Gram negative bacteria are low in peptidoglycan and thus have low contact with violet stain and high affinity for the pink dye safranine.

2 *Brucella*

Brucellosis is an infectious bacterial disease which caused by members of the genus *Brucella*. It is a global disease and affects a number of animal species. *Brucella* are obligate parasites, requiring an animal host for maintenance. Infections tend to attack the reticuloendothelial system and genital tract for abortions in females and epididymitis and orchitis in males showed the most common clinical manifestations.

Chronic infections are common. Although DNA-DNA hybridization studies indicate all the *Brucella* belong to a single genospecies, traditional species designations are used in this group. Different *Brucella* species exhibit hosts preferences and vary in severe disease. Dye and phage susceptibility along with biochemical cultural, and serologic characteristics are used to distinguish different species. The six species of *Brucella* are *B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis*, and *B. suis*.

Many species of *Brucella* are tolerable to cause disease in humans. Infections are chronic and debilitating. Signs of brucellosis in humans are relatively nonspecific and individuals. Brucellosis sometimes are labeled by hypochondriacs because of the vague presenting clinical signs.

2.1 Morphology and Staining

Brucella is Gram negative coccobacilli, (0.6-1.5) μm× (0.5-0.7) μm in size. Cells are fairly uniform and can easily be mistaken for cocci. They are typically arranged singly but also occured in pairs and clusters. No capsules or flagella, and spores are produced (Fig. 10-1); however, an external envelope has been demonstrated by electron microscope around *B. abortus*, *B. melitensis* and *B. suis*. *Brucella* stain red with Macchiavello and modified Ziehl-Neelsen stains (Fig. 10-2).

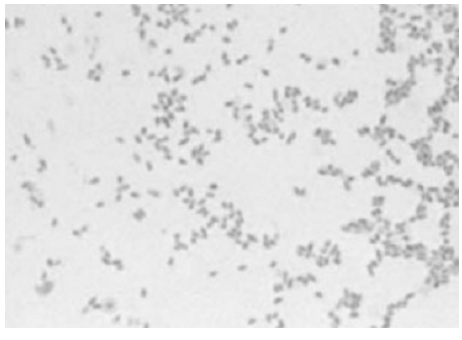


Fig. 10-1 Culture smears of *Brucella* (Rosenberg *et al.*, 2013)

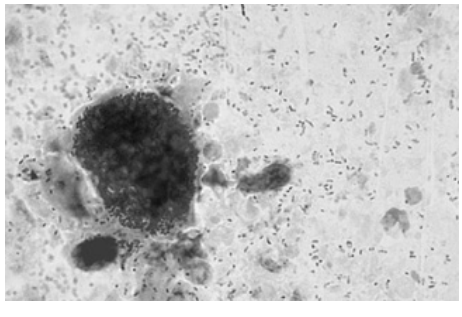


Fig. 10-2 *Brucella abortus* in clumps (×1,000) (Markey *et al.*, 2013)

2.2 Growth Characteristics

On initial isolation, colonies are not apparent until 3 to 5 days' incubation. More and more colonies are detected by 10-14 days, but in some cases incubation for up to 21 days is required. Growth is best in an aerobic environment at 37℃, but it occurs between 20℃ and 40℃. The most suitable pH is 6.6-7.4. *Brucella ovis* and some biovars of *B. abortus* require an increased concentration of CO_2. Enriched media with 5% serum are required by *B. abortus* biovar 2 and *B. ovis*.

Brucella colonies have when examined with a typical bluish color indirectly transmitted light. Colonies have smooth or non-smooth morphologies that are responsible for the presence or absence, respectively, of the polysaccharide side chain in the lipopolysaccharide. These morphologic changes are the result of spontaneous mutation and are influenced by categorical growth factors. Smooth colonies are white, convex with a whole edge and have a creamy consistency. Non-smooth colonies have intermediate, rough, or mucoid formation. Rough colonies are dull yellow, opaque, and friable. They are difficult to suspend in the solution and agglutinate spontaneously. The mucoid colonies are analogous to the rough colonies except for having a glutinous texture.

2.3 Resistance

Brucella can survive in freezing and thawing, under proper environmental conditions, they can survive for up to 4 months in milk, urine, water and damp soil. Most disinfectants active against other Gram negative bacteria kill *Brucella*. Pasteurization very effectively kills *Brucella* in milk.

2.4 Diversity

Colony morphology of *Brucella* can change from rough to smooth forms. *B. abortus*, *B. melitensis*, *B. suis*, and *B. neotomae* are typically isolated in the smooth form but can develop rough forms on subsequent laboratory passage. *Brucella ovis* is invariably in a rough form. Isolates of *B. canis* have a mucoid appearance. Generally, smooth strains of *Brucella* are more virulent than rough strains.

Variation in CO_2 requirement, H_2S production, urease production, susceptibility to varying concentration of certain dyes (thionin and basic fuchsin), and susceptibility to naturally or mutagen-derived bacteriophages lead to the diversity of species and biovars within species. Different species of *Brucella* vary in host preference and degree of virulence within and among animal species.

2.5 Pathogenesis

2.5.1 Mechanisms

Following exposure, *Brucella* pierces intact mucosal surfaces. In the alimentary tract, the epithelium covering the ileal Peyer's patches are a favored site for entry. After penetrating mucosal defence, organisms may be swallowed up by phagocytic cells. Specific receptors on macrophages appear to mediate attachment and uptake of *Brucella*. Various mechanisms are gone into service by *Brucella* to allow for survival inside phagocytic cells. They are capable of surviving and multiplying inside macrophages by inhibiting phagolysosome fusion.

Adenine from *Brucella* suspensions have been shown to restrain phagolysosome fusion in neutrophils. Intracellular survival in macrophages and neutrophils lesser extent is intensified by suppressing the myeloperoxidase-H_2O-halide system. The superoxide dismutase and catalase production may play a part in defense against oxidative killing. The stress proteins have been demonstrated in *Brucella* and it could be a factor in intracellular survival in the host. These proteins are taken for playing a part in protecting organisms from hydrolytic enzymes, oxygen radicals and myeloperoxidase killing systems in the macrophage phagolysosome.

The lipopolysaccharide of *Brucella* is directly connected with virulence and it is thought to play a part in enhancing intracellular survival. It is convinced that the variations in virulence, and in some ways, it is related to the greater ability of some species to avoid hosting defenses.

Following entry into the extracellular host, *Brucella* organisms, environment or phagocytic cells, localize to regional lymph nodes. On the one hand, they proliferate and infect other cells. On the other hand, they are likely to be killed and the infection is terminated. Some cattle seem to be innately resistant to infection. This resistance is involved in the macrophages' ability to contain the organisms. From the regional lymph nodes, *Brucella* disseminate hematogenously and localize in the reticuloendothelial system and reproductive tract.

There is preferential localization to the reproductive pregnant animals. Unknown factors in the gravid uterus, collectively mentioned to as allantoic fluid factors, stimulate the growth of *Brucella*. Erythritol is deemed to be one factor. Experimental infection studies at the cellular level have demonstrated that *Brucella* localizes into the cisternae of rough endoplasmic reticulum of trophoblasts of the placenta. Infection subsequently reaches to the fetus. The exact mechanism of abortion is unclear; however, likely possibilities are that abortion results from: ①interference with fetal circulation owing to the existing placentitis; ②the direct effect of endotoxin, and/or; ③fetal stress arising from the inflammatory response in fetal tissue.

Although less is aware of the factors referred to localization of *Brucella* in the reproductive tract of males, the presence of growth stimulating compounds may be a factor. The prolonged bacteremia observed with the *Brucella* species may account for the greater likelihood for extragenital manifestations to exist in those species.

2.5.2 Pathology

There are grossly visible lesions in the placenta relevant to *Brucella abortions*. Intercotyledonary thickening with yellow gelatinous fluid is present. The cotyledons are frequently necrotic, gray, yellow, and carpeted with a thick brown exudate. The degree of necrosis varies with *B. melitensis* infections in goats being most severe. The aborted fetus is frequently edematous. Abomasal contents may be turbid and have a lemon-yellow color.

The most common histologic findings in the fetus are bronchitis and bronchopneumonia with a predominately mononuclear cell infiltrate. Generally speaking, *Brucella* induces a granulomatous-type inflammatory reaction.

In males, palpable enlargement of the epididymis, especially related to the tail portion, is common. Epididymal lesions are distinguished by hyperplasia and hydropic degeneration of tubular epithelium. Extravasation of sperm results in the formation of a spermatic granuloma. In bulls with orchitis the scrotum is swollen largely due to phlegmonosis of the tunica and fibrinopurulent effusion in the tunica vaginalis. The testicular parenchyma becomes necrotic and it is sometimes replaced by pus.

Pathology of the accessory sex organs not only prostatitis in dogs but also fibrinopurulent seminal vesiculitis in bulls. Extragenital tract pathology includes lymphocytic endophthalmitis in dogs (*B. canis*), purulent or fibrinopurulent synovitis in swine (*B. suis*), osteomyelitis in dogs and swine (*B. canis* and *B. suis*), necrotizing and purulent bursitis in horses (*B. abortus*), and hygroma development in cattle (*B. abortus*).

2.6 Diagnosis

2.6.1 Specimens

Great care should be employed when working with infected tissues and cultures in the laboratory. All *Brucella* cultures should be handled following biosafety level 3 practices and all laboratory procedures should be performed in a manner that prevents aerosolization.

Appropriate samples for diagnosis of brucellosis depend on the animal species affected, the species of *Brucella* involved, and the clinical presentation. Abscess material, semen, and vaginal fluids associated with recent abortions are used in recovering organisms antemortem. Milk samples from cattle and goats are useful for in

ante, mortem isolation attempts and for immunodiagnostic evaluation. In dogs, because of the prolonged bacteremia that occurs, blood cultures are useful for isolation of *B. canis*. Serum is used for serologic evaluation.

Samples collected at necropsy not only should include spleen, liver, udder, and multiple lymph nodes, but also should include the supramammary, retropharyngeal, internal iliac, lumbar, and mesenteric lymph nodes. The supramammary lymph node is superior to other lymph nodes for isolating *Brucella* from dairy cattle. Abomasal fluid and the lungs of the aborted fetus and the placenta are the preferred specimens in the case of abortion. In males the epididymis, testicle, and accessory sex organs are examined.

Gram stains of fetal stomach contents from an aborted fetus and the placenta reveal large numbers of Gram negative coccobacilli. Modified Ziehl-Neelsen and Macchiavello stains are also useful for demonstrating *Brucella*. Organisms can be detected in semen but also are usually in low numbers. *Brucella* is difficult to detect by forthright examination in other samples, particularly from chronically infected animals.

2.6.2 Isolation

Tissues are cultured directly on solid media. Milk cultures are performed by the centrifuging milk at 5,900-7,700×*g* for 15min or by allowing gravity cream separation to occur overnight. Both the cream layer and the sediment, if the centrifugation technique is used, they should be plated on solid media. Commonly used media include serum dextrose, tryptose, and *Brucella* agars. If contamination is capable to be a problem, isolation attempts should be made using media containing actidione (30mg/L), bacitracin (7,500U/L), and polymyxin B (1,800U/L). Selective media are useful for both with and without the incorporation of ethyl violet 1∶800,000). Cultures should be incubated at 37℃ in 10% CO_2, for a minimum of 10 days and up to 21 days in the highly suspicious cases.

Animal inoculation is the most sensitive way for detection of *Brucella* and is sometimes necessary when very low numbers of organisms are present. Guinea pigs are the most sensitive laboratory animals for the purpose. Two guinea pigs are inoculated and at 3 and 6 weeks post-inoculation an animal is sacrificed. Serum is examined for antibodies and tissues are cultured for organisms.

2.6.3 Identification

Preliminary identification of *Brucella* species demands demonstrating colonies of Gram negative coccobacilli that are catalase positive, and oxidase positive and non-hemolytic (except for *B. ovis* and some strains of *B. abortus*). Lots of species, except *B. ovis*, are strongly urease positive. Glucose and lactose are not fermented by any of the species. Agglutination in un-adsorbed anti-smooth *Brucella* serum assists in preliminary identification of smooth strains.

Definitive identification is always performed by a *Brucella* reference laboratory. A fluorescent antibody test is used to do the rapid identification. Urease production, oxidation of metabolic substrates, agglutination in monospecific antisera CO_2 requirement, H_2S production and growth in the presence of varying concentration of thionin and basic fuchsin. Phage typing is used to determine species and biovars within species.

Brucella abortus strain 19 can be differentiated from field strains of *B. abortus* by its short of demand for CO_2, for growth and inhibition by 5mg/ml penicillin or 1mg/ml of erythritol. Strain RB51 can be differentiated from field strains and strain 19 by demonstrating its resistance to rifampin (200μg/ml), staining with crystal violet, and agglutination with acriflavin. Polymerase chain reaction (PCR) have been described for differentiation of *Brucella* species.

2.6.4 Immunodiagnosis

Antibody detection is usually used for diagnosing brucellosis and in controlling programs. Samples tested include milk, blood and occasionally semen. Many of immunodiagnostic tests have been developed for cattle. These tests detect types of antibodies, detect different classes and vary in their sensitivity and specificity. Individual blood samples can be tested by plate agglutination, tube agglutination, card tests and rose bengal plate. Other tests including the buffered plate agglutination assay, rivanol agglutination, complement fixation, and enzyme-linked immunosorbent assay (ELISA).

Generally, highly sensitive but less specific tests are used for screening purposes and are followed by more specific tests for confirmation purposes. A similar pathway to that used in cattle is employed when testing goats and sheep for *B. melitensis*. Sera are screened with a test such as the rose bengal test. Its results confirmed with a more specific test.

Milk is screened with the *Brucella* milk ring test, which identifies specific antibodies in milk. The test is performed on bulk tank milk samples as a meaning of screening dairy herds. Stained *Brucella* antigen is added to milk. If antibodies are presented, agglutinated antigen is buoyed to the top by the rising cream and a purple ring develops at the top of the tube.

Serologic tests are commonly used to make sure infected swine herds and monitor herd status. These tests are less accurate when testing individual pigs. Because some infected swine do not have detectable antibody titers. Herds can be screened by the brucellosis card test. For example, rivanol agglutination and 2-mercaptoethanol agglutination are used for confirmation. Rams are tested for antibodies to *B. ovis* using either ELISA or a complement fixation test.

For canine brucellosis, screening is performed with a rapid slide agglutination test

(RSAT). The RSAT is sensitive but not very specific, hence positive results should be confirmed with additional tests that are more specific. An agar gel immunodiffusion test using cytoplasmic antigen is more specific but not as sensitive to the RSAT and is used as a confirmatory test.

2.6.5 Nonculture Detection Methods

A number of noncultural methods, including PCR, immunoperoxidase staining, DNA probes, and coagglutination, have been described for detection of *Brucella* in tissues and fluids.

2.7 Treatment

As a general rule, treating infected livestock is not attempted because its treatment failure rate is high, its cost is expensive, and it has many potential problems related to keeping infected animals in the face of ongoing eradication programs. People always use tetracycline and dihydrostreptomycin to treat *B. ovis* infections in rams with variable results. Once obvious antibiotic treatment, epididymal lesion will present, not be beneficial because the epididymal lesion is the result of extravasation of sperm. The presence of abscesses and fibrosis in tissues of the accessory sex organs is difficult for penetration with antibiotics into these areas.

Treatment of dogs with brucellosis needs a prolonged course of antibiotic therapy. People commonly use the combination of dihydrostreptomycin and tetracycline or minocycline for 2-4 weeks. Quinolones may also be useful; but only limited information about their effectiveness is available. Treatment failures are also common. Treatment should also consist of neutering affected animals. Treatment should not be taken in canine breeding colonies. In this case, infected dogs should be killed.

2.8 Control and Prevention

Approaches on control and prevention of brucellosis depend on the animal species that have been involved, *Brucella* species, management practices, and the availability and efficacy of vaccines. Ways usually used to control brucellosis include: ①immunization alone, ②removal and test of infected animals in conjunction with an immunization program, and ③removal and test of infected animals without immunization.

2.8.1 Control by Immunization Alone

Immunization reduces the number of abortions and potential for exposure. Immunization will not result in eradication of the infection in a herd by itself. Immunization alone should be considered as a way that can only control the level of disease.

2.8.2 Immunization Followed by Test and Slaughter

Control of bovine brucellosis usually uses a combination of vaccination of females and a test and slaughter program. Cattle are inoculated when they are young and tested by immunodiagnostic tests when they reach sexual maturity and vaccination titers have disappeared. Vaccination with strain 19 is probably 70% effective on an individual basis but more effective when evaluated on a herd basis. In experimental challenge, vaccination with strain RB51 provided protection similar to vaccination with strain 19. Every animal identified as infected is culled from the herd and slaughtered. Routine testing is done by the milk ring test in dairy cattle or blood tests from beef cattle at slaughter. A similar program is followed with *B. melitensis* infections in sheep and goats.

2.8.3 Test and Slaughter without Immunization

Immunization control programs are not suitable for swine brucellosis. The most triumphant method of control is vaccination other not depopulation. For example, removing only adult animals and retaining weanlings are less successful. Removal of only the serological reactors will not control infection in the flock. Confinement operations and closed herds make establishing and maintaining a swine herd free of brucellosis readily achievable. In some cases, depopulation is experienced on *B. melitensis* infections in sheep and goats.

2.8.4 Control of *Brucella ovis*

Removing infected rams and preventing new infections in rams are the main keys to control *B. ovis* transmission in a herd. Practices that allow for introduction of infected rams into a flock, such as loaning of rams, should be avoided. Yearling rams should be kept separately from adult rams. All rams should be palpated for epididymal lesions at least twice a year before breeding season and rams with perceived lesions culled. Serologic tests (ELISA, CF) are used to identify infected rams without lesions.

Serologic testing should be carried out a minimum of two times before the rams are turned in to breed ewes. Vaccination can be adopted but its workpiece ratio is restricted and it interposes with serologic interpretation. No effort is made to control infections in ewes. Ewes, although acting a part in transmission of infection at breeding, are only short-lived infected and naturally passes the infection before the next breeding season.

2.8.5 Control of *Brucella canis*

Efforts to hold back canine brucellosis contain serologic testing of dogs by the breeding. Males are also assessed by palpation for epididymal and testicular lesions. In breeding colonies with brucellosis infected animals identified by serologic tests are removed. Repeat serologic testing is carried out to identify formerly unperceived infected animals. Until at least three passive test results are achieved, a kennel should

not be regarded as free of brucellosis. Kennel areas should be carefully disinfected with quaternary ammonium compounds or iodophors.

Review Questions

1. What are Gram negative aerobic *Bacillus* which have the important pathogenic effects?
2. Try to describe the classification of *Brucella*.
3. Try to describe the pathogenicity of *Brucella*.
4. Try to describe the form and dyeing properties of *Brucella*.
5. Try to describe the culture properties of *Brucella*.
6. How to do microbiological diagnosis of brucellosis?
7. Describe the antigenic properties of *Brucella*.
8. How to prevent brucellosis?

Chapter 11 Gram Positive Asporogenous Rod

Synopsis

Listeria and *Erysipelothrix* is typical representative of Gram positive bacteria without spores. *Listeria monocytogenes* is a kind of intracellular parasitic bacteria, and has pathogenicity to humans and animals, has several virulence factors including hemolysin LLO. *Listeria monocytogenes* can be detected with cold enrichment method. Inactivated vaccine with virulent strain was proved to have certain effect. At early infection stage, sensitive antibiotics are available for treatments. *Erysipelothrix rhusiopathiae* is a representative species of *Erysipelothrix*, and widely distributed in nature. Erysipeloid in human is caused by the infection of *Erysipelothrix rhusiopathiae. Erysipelothrix rhusiopathiae* has pathogenicity for a variety of animal, and is one of the main important pathogenic bacteria of pigs. *Erysipelothrix rhusiopathiae* can grow in the gelatin which present brush. Attenuated vaccine or inactivated vaccine of *Erysipelothrix rhusiopathiae* have good immune effect. High-immunized serum against *Erysipelothrix rhusiopathiae* can be used for emergency treatment, while penicillin also is better for treatment.

This part includes the Lactobacillaceae with one genus *Lactobacillus* and three other genera of uncertain affiliation namely *Listeria*, *Erysipelothrix* and *Caryophanon*. Organisms in the Lactobacillaceae are straight or curved rods occurring singly or in chains and generally are non-motile. These are anaerobic or facultatively anaerobic, highly fermentative with complex nutritional requirements.

1 *Listeria*

Listeriosis is a sporadic disease in humans and many species of animals and birds, and is of zoonotic importance. Recognized species of *Listeria* include *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. rocourtiae*, *L. seeligeri* and *L. welshimeri. L. monocytogenes* and *L. ivanovii* are important pathogens in veterinary medicine.

Listeria ivanovii has two subspecies, namely *Listeria ivanovii* subsp. *ivanovii* and *Listeria ivanovii* subsp. *londoniensis*.

Ruminants are the most frequently affected domestic animals. Principal forms of listeriosis include septicemia, meningoencephalitis, and abortion. In sheep and cattle, abortion is the usual manifestation of *L. ivanovii* infections. Listeriosis occurs worldwide, especially in temperate climates.

1.1 Morphology and Staining

Listeria is Gram positive, non-acid-fast, non-spore-forming, facultatively intracellular, acapsular rod-shaped bacteria, which measure (0.5-2) μm × (0.4-0.5) μm (Fig. 11-1).

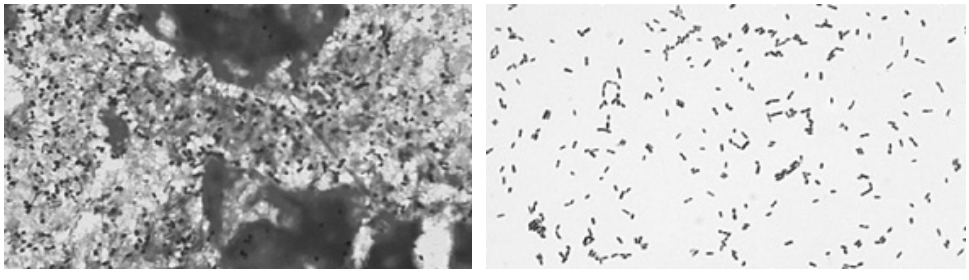


Fig. 11-1 *L. monocytogenes* from a placenta (left) and a culture (right) (×1,000) (Markey *et al.*, 2013)

1.2 Structure and Composition

Listeria has a typical Gram positive cell wall. Meso diaminopimelic acid is the major diamino acid. O antigen is determined by cell wall polysaccharides. Peritrichous flagella and motility are present at 22℃, but the motility is poor at 37℃.

1.3 Growth Characteristics

Listeria is facultative anaerobes that grow best under reduced oxygen and increased carbon dioxide concentration. Growth occurs at 4-45℃, with an optimum at 30-37℃. Simple laboratory media support growth, preferably at an alkaline or neutral pH. On sheep blood agar, most strains of *L. monocytogenes* produce a narrow zone of hemolysis. Colonies are usually 1-2mm in diameter and appear blue-green in obliquely transmitted light on solid media such as tryptose agar. Colonies of *L. ivanovii* typically produce a larger and more intense zone of hemolysis (Fig. 11-2). Many commercial Listeria selective media are available. Most contain inhibitory compounds for non-Listerial organisms. The compounds include cycloheximide, colistin, acriflavine, amphotericin B, and cefotetan.

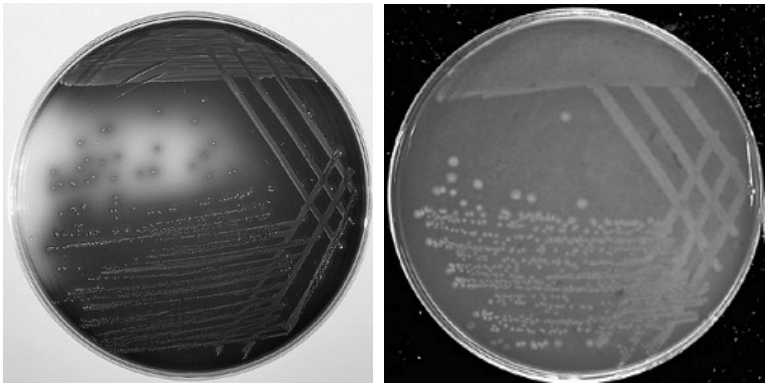


Fig. 11-2 *L. monocytogenes* on *Listeria* selective agar (left) and sheep blood agar (right) (Markey *et al.*, 2013)

Listeria can tolerate 0.04% potassium tellurite, 0.025% thallium acetate, 3.75% potassium thiocyanate, 10% NaCl, and 40% bile in media. Most strains grow over a pH range of 5.5-9.6. It has greater heat tolerance than other non-spore-forming bacteria. However, short-time high-temperature pasteurization is effective for killing *Listeria*.

1.4 Variability

There are 13 recognized serovars in the *L. monocytogenes* based on somatic (O) and flagellar (H) antigens. Most clinical isolates belong to serovars 1/2a, 1/2b, and 4b; and most food strains belong to serovar 1/2c. Although there is no correlation between serovars and species, strains of serovar 5 are *L. ivanovii*. No relationship between serovars and host specificity has been recognized. Various nucleic acid-based methods have been used to further discriminate between *Listeria* strains for epidemiologic analysis and strain tracking purposes. Whole genome sequencing has recently identified numerous genes found in virulent species but absent in avirulent species.

Both smooth and rough colonial variants can be observed. In rough colonies, filaments 20μm or more in length may be observed. L-forms develop on media containing penicillin and have been isolated from clinical cases in humans.

1.5 Pathogenesis and Immunity

1.5.1 Mechanisms

Exposure to *Listeria* occurs via the oral or less commonly the nasal route. Most *Listeria* pathogens are destroyed by gastric acids. Use of antacids and H_2 blockers can increase survival rate and are considered as risk factors for developing listeriosis. Intestinal translocation appears to be a passive process which involves both intestinal epithelial

cells and M cells overlying Peyer's patches. Internalin A and B, two surface proteins, interact with host cell receptors to mediate entry. Internalin A interacts with cell adhesion molecule E-cadherin, and internalin B interacts with hepatocyte growth factor receptor Met. After passage through the intestinal barrier, *Listeria* can be observed in phagocytic cells within the lamina propria. Further dissemination occurs via the bloodstream. *Listeria* can be internalized by phagocytic cells or by non-phagocytic cells through induced phagocytosis. Various bacterial ligands have been identified for adherence and include internalin family, ActA and p60. Nonphagocytic cells can internalize *Listeria* through a zipper-type mechanism. After internalization, it escapes from the phagosome, becomes associated with actin filaments in the cytoplasm, and propels itself to the host cell's plasma membrane via polar assembly of actin filaments (ActA). In this way, it can pass to neighboring cells in plasma membrane protrusions and so to avoid host defense mechanisms.

The other way entrying into host has been proposed for CNS (central nervous system) infections through damaged oral, nasal, or ocular mucosal surfaces via the neural sheath of peripheral nerve endings, particularly the trigeminal nerve. It is postulated that centripetal migration along cranial nerves leads to infection of the CNS. Organisms have been demonstrated in the myelinated axons of the trigeminal nerve and cytoplasm of medullary neurons. The lack of visceral involvement supports a route other than hematogenous, although a primary hematogenous route cannot be discounted.

1.5.2 Pathology

The brain stem is the most commonly involved area in the encephalitic form. The cerebrospinal fluid may be cloudy and the meningeal vessels congested. Areas of softening in the medulla are occasionally observed. Histologically, multifocal perivascular cuffing predominated by lymphocytes and histiocytes is commonly observed. Focal necrosis and microglial and neutrophilic infiltrates are seen in parenchymal tissue. Resulting microabscesses are characterized by liquefaction of the neuropil. Lesions distributed throughout the brain stem or more frequently presented unilaterally may further support the neural-migration to the brain. The medulla and pons are most commonly involved. In septicemic forms, multiple foci of necrosis in the liver and, less frequently, the spleen may be noted. In the aborted fetus of ruminants, gross lesions are minimal. Autolysis is frequent because the dead fetus had been retained for a period before being expelled.

1.5.3 Immunity

The majority cases of listeriosis in human are associated with immunosuppressed individuals, the elderly, the neonates, and pregnant women. In animals, neonates and pregnant animals likewise are predisposed. However, in some cases a predisposing immunosuppressive factor is not apparent.

As a facultatively intracellular parasite, *Listeria* is primarily contained by cell-mediated responses. Humoral factors may play some limited role in host defense. No immunizing preparations have met with significant success. Killed preparations have been ineffective, while live attenuated vaccines afforded some protection in sheep. The sporadic nature of listeriosis has not warranted vaccination as a primary medium for disease prevention.

1.6 Diagnosis

1.6.1 Specimens

Laboratory diagnosis is based upon isolation of the organism. Spinal fluid, blood, brain tissue, spleen, liver, abomasal fluid, and/or meconium are collected, depending on symptoms, lesions, and tissues available.

1.6.2 Direct Examination

A direct smear of infected tissue may reveal numerous Gram positive rods in septicemias and abortions. However, fewer numbers of organisms are typically observed in the encephalitic form. Negative results are inconclusive. Immunohistochemical staining with specific antisera is useful in diagnosing encephalitic cases.

1.6.3 Isolation

Samples are plated on sheep blood agar and incubated at 35℃ in 10% CO_2. Isolation of *L. monocytogenes* from brain tissue may be enhanced by pour plate methods. After the first attempts, remaining tissue is stored at 4℃ for "cold enrichment". Such tissue is subcultured weekly for up to 12 weeks. Cold enrichment is not necessary for isolation from Listerial abortions or septicemias. For contaminated samples, enrichment and the use of selective media (lithium chloride, phenylethanol-moxalactam medium, Oxford medium, or PALCAM *Listeria* selective medium) are advisable. Modified University of Vermont broth, MOPS-buffered *Listeria* enrichment broth, Fraser broth, and modified Oxford agar are essential components for USDA recommended isolation methods from food products. Various DNA-based and antigen-capture methods for detection of *Listeria* have been described, especially in food products.

1.6.4 Identification

Typical colonies consisting of Gram positive, regular rods are suggestive. *Listeria* is catalase-positive, motile at 25℃, and hydrolyses esculin. *L. monocytogenes* is CAMP-positive when cross-streaked with a beta-toxin-producing *Staphylococcus aureus* on 5% washed sheep blood agar. A similar phenomenon is observed when *L. ivanovii* is cross-streaked with *Rhodococcus equi*. A weak CAMP-like reaction is sometimes observed between *L. monocytogenes* and *R. equi*. In semisolid motility

media incubated at room temperature, a characteristic umbrella pattern of motility develops 3 mm to 4 mm below the surface, due to the microaerophilic nature of *Listeria*. An end-over-end tumbling type of motility with intermittent periods of quiescence is seen in hanging drop preparations. Acid is produced from glucose and L-rhamnose but not D-mannitol or D-xylose by *L. monocytogenes*. *L. ivanovii* differs by fermenting D-xylose but not L-rhamnose. Fluorescent antibody staining or agglutination with specific antiserum is helpful. Mouse inoculation causes death within 5 days with necrotic foci present in the liver. This procedure differentiates *L. monocytogenes* from non-pathogenic species of *Listeria*. However, it is rarely necessary for definitive identification.

1.6.5 Immunodiagnosis

Serology has not been useful for diagnosis due to the prevalence of positive titers in apparently normal animals and cross-reactions with *S. aureus*, *Enterococcus faecalis*, and *Arcanobacterium pyogenes*.

1.7 Treatment and Prevention

L. monocytogenes is susceptible *in vitro* to penicillin, ampicillin, chloramphenicol, erythromycin, enrofloxacin, lincomycin, nosiheptide, rifampin, salinomycin, tetracycline, vancomycin, and virginiamycin. Recent studies report that some isolates are resistant to tetracyclines, fluoroquinolones and penicillin. Minimum inhibitory concentrations are on the rise. Chlortetracycline and penicillin may be effective in timely treatment of cattle with meningoencephalitis. Treatment of sheep has been less successful. Control measures include reduction or elimination of feeding of silage, particularly poor-quality silage. All forms of stress should be minimized. Infected animals should be isolated and infected material should be disposed properly. Vaccination has not been proven to be highly successful and may not be warranted due to the sporadic nature of the disease.

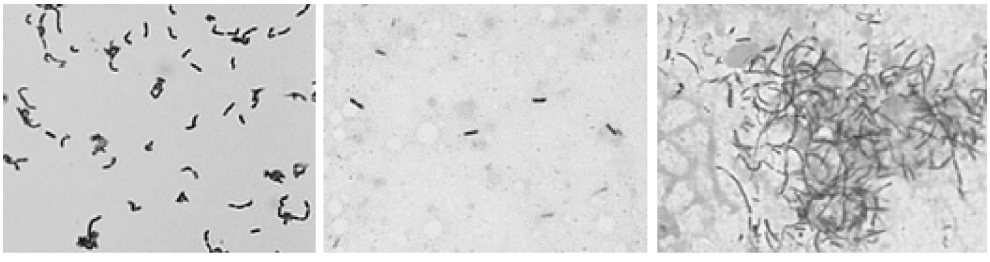
2 *Erysipelothrix*

Erysipelothrix rhusiopathiae is the type species of the genus and is also primary important. It causes erysipelas, an important disease of swine and poultry and a sporadic disease of sheep and lambs. Clinical presentations include septicemia, arthritis, vegetative endocarditis, and generalized skin lesions. The organism is commonly isolated from alimentary and lymphoid tissues of healthy animals as well as the exterior slime layer on fish. *E. rhusiopathiae* can survive for long periods without replication in soil and marine environments. In humans *E. rhusiopathiae* causes erysipeloid, an occupational disease of fish handlers, butchers, and veterinarians

and generally results in a self-limiting infection of the hands. A second species, *E. tonsillarum*, has been described for some strains previously designated as serotypes of *E. rhusiopathiae*. *Erysipelothrix tonsillarum* is biochemically and morphologically similar to *E. rhusiopathiae*, but is genetically distinct by DNA-DNA homology. *Erysipelothrix tonsillarum* is only occasionally involved in clinical disease and is non-pathogenic for swine. An additional species, *E. inopinata*, and minor *Erysipelothrix* groups have been described.

2.1 Morphology and Staining

Erysipelothrix is a Gram positive, non-motile, non-acid-fast, non-spore-forming *Bacillus*, which measures (0.2-0.4) μm × (0.8-2.5) μm in size. On subculture, rough colonies may develop and produce filamentous forms 60μm or more in length (Fig. 11-3).



from a culture　　from the liver of pig　　from the heart valves of pig

Fig. 11-3　*Erysipelothrix rhusiopathiae* in a culture (×1,000) (Markey *et al.*, 2013)

2.2 Structure and Composition

Erysipelothrix exhibits a cell wall typical of Gram positive organisms. It contains murein of the B1 delta type. The diamino acid of cell wall peptidoglycan is lysine. The DNA base composition is 35-40mol% G+C. A polysaccharide capsule has been described for *E. rhusiopathiae* and related to virulence. A protective protein, SpaA, shares similarities in the C-terminal region with choline-binding proteins of *Streptococcus pneumoniae*. To date, four different spa types have been identified in *Erysipelothrix* spp. (spaA, spaB1, spaB2, and spaC). Other surface proteins include a 16kDa hemolysin and 64-66kDa antigen. It has been suggested that antibody to the latter protein has protective activity.

2.3 Growth Characteristics

E. rhusiopathiae grows readily on most standard media and grow best on media supplemented with serum and glucose. *Erysipelothrix* is a facultative anaerobe

preferring an environment containing 5-10% CO_2. Optimal growth occurs in 24-48h at 30-37℃ and at a pH of 7.2-7.6. However, it is capable of growing over a temperature range of 5-42℃ and a pH range of 6.7-9.2.

2.4 Resistance

E. rhusiopathiae is resistant to drying and withstands salting, pickling, and smoking. It survives for up to 6 months in swine feces and fish slime at cool temperatures. It is killed by moist heat (55℃) in 15min, but grows in the presence of potassium tellurite (0.05%), crystal violet (0.001%), phenol (0.2%), and sodium azide (0.1%).

E. rhusiopathiae is susceptible to penicillin, cephalosporin, clindamycin, and fluoroquinolones, but is resistant to novobiocin, sulfonamides, and aminogly-cosides. Resistance to erythromycin, oleandomycin, oxytetracycline, and dihydrostreptomycin has been observed. Resistance is apparently not plasmid-mediated.

2.5 Variability

Common heat-labile antigens account for cross-reactions between strains. Heat-stable, somatic antigens account for the existence of at least 25 serotypes. No relationship between host species and serotype has been recognized. Serotypes 3, 7, 10, 14, 20, 22, and 23 exhibited a higher degree of DNA-DNA homology with the type strain of *E. tonsillarum* than with the type strain of *E. rhusiopathiae*. Cultures dissociate on passage from convex, circular, smooth colonies with entire edges to rough colonies with undulate edges. L-forms have been reported.

2.6 Pathogenesis

2.6.1 Mechanisms

Strains of *E. rhusiopathiae* vary in virulence for reasons not fully understood. Virulent strains produce high levels of neuraminidase in the logarithmic growth phase, which is considered an important virulence factor in acute septicemic infections. Neuraminidase cleaves sialic acid present on cell surfaces, leading to vascular damage and hyaline thrombus formation. Neuraminidase also plays a role in bacterial attachment and invasion into cells. Antibodies to neuraminidase are protective against experimental infections in mice. The presence of capsules has been described and appears to play a role in resistance to phagocytosis by polymorphonuclear leukocytes. Survival inside professional phagocytes is important for pathogenicity. In the presence of normal serum, acapsular mutants do not survive inside phagocytes, whereas encapsulated organisms survive and multiply. In the presence of immune sera, opsonized bacteria are

readily eliminated by macrophages and polymorphonuclear leukocytes. Cutaneous lesions may be seen in animals during the acute septicemia phase or following recovery. These are due to swelling of the endothelium leading to vasculitis, thrombosis, diapedesis, and fibrin deposition. Skin lesions may also appear with a non-systemic infection due to partial immunity of the host and attack from low-virulence strains.

Localization of *E. rhusiopathiae* in joints of swine leads to fibrinous exudation and pannus formation. Subsequent proliferative changes are primarily due to an immunological response from the formation of immune complexes, activation of complement, and presence of neutrophils, which damages the auricular cartilage and synovial tissues. Joint deterioration becomes chronic and progresses for years. Although *E. rhusiopathiae* can no longer be cultured from joint fluid, the organism or bacterial antigens may persist. Valvular endocarditis is less common than joint involvement and is believed to be initiated by bacterial emboli and vascular inflammation, resulting in chronic changes and damage to the heart valves. Vegetative lesions can lead to valvular insufficiency and congestive heart failure or release emboli that may cause sudden death.

2.6.2 Pathology

Pigs dying from acute erysipelas infections exhibit hemorrhages of the gastric serosa, skeletal and cardiac muscles, and renal cortex. Congestion of lungs, liver, spleen, skin, and urinary bladder is frequent. Vascular damage with microthrombi is observed microscopically. A mononuclear infiltrate predominates in most cases. Raised, pink to purple cutaneous lesions result from vasculitis, thrombosis, and ischemia. In joints, acute synovitis often proceeds to more chronic articular changes. Synovial membranes become hyperplastic and villous and are infiltrated with mononuclear cells. Spreading of granulation tissue over articular surfaces and erosion of articular cartilage may occur. Ankylosis of the joint may be the ultimate outcome. Localization to intervertebral disks leads to a destructive discospondylitis. In valvular endocarditis, the mitral valve is most commonly involved with development of large, valvular vegetations due to fibrin deposition and connective tissue proliferation. Emboli may produce infarcts in the spleen, kidney, and other internal organs.

In turkeys, the pathology associated with erysipelas infections is generally marked by congestion and intramuscular and subpleural hemorrhages, particularly affecting breast and leg muscles. Hemorrhages are also found in the mucosa of the gizzard and small intestine as well as serosal surfaces of the heart. The liver and spleen are often swollen and the abdominal fat is petechiated. A swollen, cyanotic snood and diffuse reddening of the skin are frequent observed.

In lambs with polyarthritis, affected joints are swollen and thickened with granulation tissue present on the inner surface of the joint capsule. A clear cloudy fluid is present

with variable numbers of infiltrating polymorphonuclear leukocytes.

2.6.3 Immune Mechanisms in Pathogenesis

Persistence of antigen in the joint tissue is thought to act as a chronic stimulus for immune reaction and development of arthritis. In addition, an autoimmune process secondary to the erysipelas infection may be responsible for some of the chronic joint changes.

2.6.4 Mechanisms of Resistance and Recovery

Cell-mediated and humoral responses occur, directed at neuraminidase, protective surface protein, and other cell wall components. Serum opsonins apparently play a decisive role. Phagocytosis is carried out primarily by mononuclear phagocytes.

2.7 Artificial Immunization

Attenuated vaccines and bacterins have been used for vaccination in swine and turkeys. The duration of immunity varies from 6 to 12 months for these vaccines and efficacy is variable. While effective against the acute infections, neither type appears to be highly protective against chronic erysipelas. Attenuated vaccines are given orally, parenterally, or in some countries by aerosol. Whole-cell bacterins and soluble antigen are given subcutaneously or intramuscularly. Most commercial vaccines are prepared from serotype 2. Cross-protection to acute disease caused by serotypes 1 and 2 has been demonstrated. However, certain strains have been refractory to vaccine-induced immunity. Formalin-inactivated, aluminum hydroxide-absorbed bacterins appear to be effective in turkeys. Recent studies have focused on the surface protection antigen (spa) as potential vaccine candidates, but to date no commercial products are available.

2.8 Diagnosis

2.8.1 Specimens

Specimens are collected from appropriate sites according to signs. Blood cultures from several infected animals are useful in diagnosing septicemia. Necropsy specimens include liver, spleen, kidney, heart and synovial tissue. Recovery of the organisms from skin lesions is also possible. In the more chronic forms, cultures from joints or heart valves are less successful.

2.8.2 Direct Examination

Specimens are examined by Gram stain for the presence of Gram positive rods. A negative result does not preclude infection.

2.8.3 Culture

Culture methods for the isolation of *E. rhusiopathiae* have traditionally involved the use of selective and enrichment media. One commonly used enrichment broth is *Erysipelothrix* selective broth (ESB), which contains horse serum, kanamycin, neomycin, and vancomycin. Other enrichment broths described include Bohm's medium containing sodium azide, kanamycin, phenol, and water blue and Shimoji's enrichment broth containing tryptic soy broth, Tween-80, *tris*-aminomethane, crystal violet, and sodium azide. In addition, selective agar media including sodium azide crystal violet, nalidixic acid medium, and a modified blood azide agar have been described. These media take advantage of the organism's resistance to various antimicrobials and chemicals. ESB is perhaps the most commonly used enrichment broth in conjunction with selective media. One study found that ESB combined with selective agar medium (colistin nalidixic acid or sodium azide crystal violet) markedly increased detection of *E. rhusiopathiae* from tissues versus direct culture. Samples are plated on blood agar and incubated at 37℃ in 10% CO_2. Colonies often appear non-hemolytic and pinpoint after 24h incubation. At 48h, a greenish hemolysis may be apparent. *E. rhusiopathiae* is catalase and oxidase negative and non-motile. Inoculation of triple sugar iron agar slants will show an acid reaction and H_2S production along the stab line. A "pipe cleaner" type of growth occurs in gelatin stab cultures of rough colonies held at room temperature for 3-5 days. *E. rhusiopathiae* does not hydrolyze esculin or urea, reducing nitrates, or producing indole. Fermentative activity is weak. Fermentable carbohydrates include glucose, lactose, levulose and dextrin. *Erysipelothrix tonsillarum* usually ferments saccharose while *E. rhusiopathiae* does not.

Selective media containing various amino glyco, sides and vancomycin may be used to isolating *Erysipelothrix* from contaminated tissue. Other selective media contain sodium azide (0.1%) and crystal violet (0.001%). Serology is of little value in diagnosing erysipelas infections.

2.9 Treatment and Prevention

Treatment with penicillin for at least 5 days is effective against the acute forms of erysipelas in swine and usually results in dramatic improvement with 24-36h. Other antimicrobials that *E. rhusiopathiae* appears highly sensitive include ampicillin, ceftiofur, clindamycin, enrofloxacin, erythromycin, tiamulin, tilmicosin, and tylosin. Intermediate sensitivity is seen with chlortetracycline, florfenicol, gentamicin, oxytetracycline, and trimethoprim. Resistance to apramycin, neomycin, sulfadimethoxine, sulfachlorpyridazine, and sulfathiazole appears very high. Antiserum (equine origin) is sometimes used in conjunction with antibiotic therapy. Treatment of chronic forms is less successful.

Good sanitation and nutrition are beneficial in preventing outbreaks. Infected carcasses should be disposed of in a proper manner and replacement animals isolated for at least 30 days before introduction into the herd. A number of single or combinatorial *E. rhusiopathiae* products are available. Vaccination is recommended in areas with previous history of erysipelas.

In turkeys, penicillin is the drug of choice. Subcutaneous injection of penicillin and vaccination with erysipelas bacterin are recommended, if practicable, penicillin in the drinking water for 4-5 days has been effective in controlling some outbreaks. Injectable erythromycin is a recommended alternative treatment.

Good management practices including preventing fighting among farms, ensuring proper insemination practices of hens, rotating ranges away from contaminated areas, and using vaccination in areas with a history of erysipelas are useful prevention and control measures.

Review Questions

1. What are Gram positive *Bacillus* without spores which have pathogenicity?
2. Please briefly describe the morphological and dyeing properties of *Listeria monocytogenes*.
3. Please briefly talk about the culture properties of *Listeria monocytogenes*.
4. Please briefly describe the pathogenesis of *Listeria monocytogenes*.
5. Please briefly describe the process of the microbiological diagnosis of *Listeria monocytogenes*.
6. What are the members of *Listeria* and *Erysipelothrix*?
7. Please briefly describe the morphological and dyeing properties of *Erysipelothrix rhusiopathiae*.
8. Please briefly describe the pathogenesis of *Erysipelothrix rhusiopathiae*.

Chapter 12 Gram Positive, Spore-forming Bacilli

Synopsis

There are more Gram positive bacteria with spores which have big body. *Bacillus* and *Clostridium* have great significance to humans and animals. *Bacillus anthracis*, a representative species of *Bacillus*, is an important pathogenic agent for humans and animals. Capsule and toxin are the main virulence factors of *Bacillus anthracis*. Because spores of *Bacillus anthracis* have strong resistance, the anatomy of died animals caused by *Bacillus anthracis* is forbidden. However, sensitive antibiotics can be used in the treatment of the infection of *Bacillus anthracis*, and vaccines or antiserum can effectively control the infection of *Bacillus anthracis*. Most pathogenic members of *Clostridium* are anaerobic and can cause disease to humans and animals through toxins such as neurotoxins, enterotoxins, and tissue toxins. *Clostridium perfringens* is a typical invasive clostridium, which can produce a variety of toxins and causes diseases and food poisoning in humans and animals. The detection of toxin is mainly used in the diagnosis of the disease caused by *Clostridium perfringens*. *Clostridium tetanus* is a typical non-invasive clostridium, which has a spore like a rattle-drum and cause neurotoxic symptoms characterized by rigidity and spasm in humans and animals. Neurotoxin of *Clostridium tetanus* as its main virulence factors can cause animal disease with very high mortality rate.

1 *Bacillus anthracis*

Members of the genus *Bacillus* are Gram positive, spore-forming, facultatively anaerobic rods that typically inhabit soil and water. They are ubiquitous in nature, and are commonly isolated from a wide variety of surfaces, soils, and animal byproducts. Average numbers of *Bacillus* spp. present in soil are between 10^6 and 10^7 per gram of soil. During periods of nutrient deprivation, a *Bacillus* cell undergoes a process known as sporulation by which it forms a dense, resistant endospore. Endospores are resistant

to heat, desiccation, ultraviolet and ionizing irradiation, disinfectants, and a variety of other environmental stresses. They can remain viable in soil and water for decades, awaiting nutrients, or in the case of pathogenic members of *Bacillus*, entry of spores into their respective hosts.

Three species are considered pathogenic. *Bacillus anthracis* is a zoonotic pathogen and the causative agent of anthrax. *Bacillus cereus* is a causal agent of food poisoning, and *Bacillus thuringiensis* is a lepidopteran insect pathogen. Genomic studies, including DNA-DNA hybridization, 16S and 23S rRNA sequence comparisons, multilocus sequence typing, multilocus enzyme electrophoresis, and amplified fragment length polymorphism analysis, have revealed a high degree of relatedness among these organisms, so it suggests that *B. anthracis*, *B. cereus* and *B. thuringiensis* may be viewed as a single species. Their virulence and host range are determined by their virulence gene-encoding plasmid content. For example, virulence of *B. anthracis* requires the presence of the pXO1 and pXO2 plasmids, required for production of the anthrax toxins and capsule, respectively.

1.1 Descriptive Features

1.1.1 Morphology and Staining

Bacillus anthracis are Gram positive, nonmotile, roughly rectangular rods with square ends (about 1μm by 3-5μm) (Fig. 12-1). Chains of rods are common. Chain formation is thought to contribute to the bacterium's ability to resist opsono-phagocytic killing. Spores are produced within the cells during conditions of nutrient deprivation and cause no swelling. A capsule is formed *in vivo* (Fig. 12-1), or in bicarbonate-supplemented culture media and an elevated CO_2 atmosphere *in vitro*.

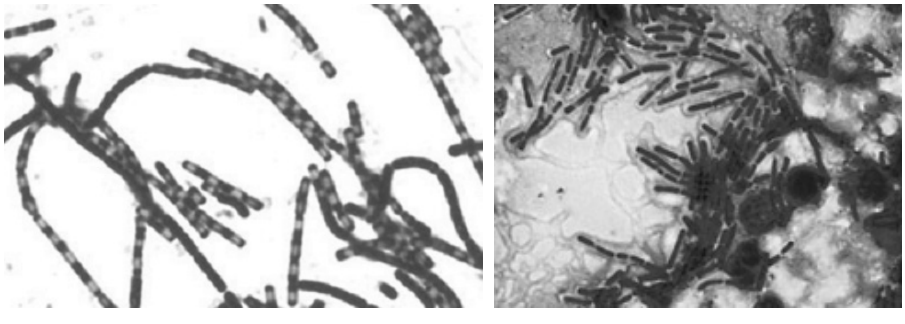


Fig. 12-1 *Bacillus anthracis* (×1,000): spore type in a culture (left) (Scott McVey *et al.*, 2013) and capsule type in a tissue (Markey *et al.*, 2013)

1.1.2 Cellular Composition

Bacillus anthracis has a Gram positive cell wall structure. Covering the cell wall is a

protein paracrystalline structure referred to as the S-layer. Although the S-layer has not been shown to be essential for virulence, S-layer deficient mutant strains were reported to be more sensitive to binding of the C3 component of the complement. Spores are the infectious form of *B. anthracis*. Spores are immunogenic and the addition of inactivated spores has been shown to increase the degree of immunity against highly virulent strains of *B. anthracis* in animal models of infection. The spores consist of multiple layers. The core is the inert bacterial cell. It is surrounded by the cortex, a layer of modified peptidoglycan. This is surrounded by the inner and outer coat layers, protein layers that confer most of the resistance properties on the spore. The outermost layer of the spore is the exosporium layer that is separated from the coat by the interspace layer. The exosporium consists of a paracrystalline basal layer and its outer surface covered by filaments of the hair-like nap composed of the BclA collagen-like glycoprotein. Because the exosporium is the outermost structure of the spore, it is probable that it plays a major role in interactions with the environment and with the host immune system. The *B. anthracis* exosporium was reported to play a role in limiting access to inducers of cytokine responses *in vitro* in macrophages. The uptake of spores by macrophages has been shown to involve interactions between BclA and the integrin Mac-1 (CR3). Spores lacking the BclA glycoprotein do not specifically target these cells, but bind more generally to epithelial cells. Spores lacking BclA have been shown to bind to extracellular matrix components such as laminin and fibronectin. The spore-surface BclA fibers may act to promote uptake by professional phagocytes and to inhibit non-specific interactions between *B. anthracis* spores with non-professional phagocytic cells in the early stages of infection.

1.1.3 Cellular Products of Medical Interest

1.1.3.1 Capsule

The vegetative form of *B. anthracis* produces a capsule composed of a polymer of poly-γ-D-glutamic acid. The genes encoding this structure are on a plasmid called pXO2. The expression of the genes encoding the capsule is under control of two regulatory gene products, AtxA and AcpA (for anthrax toxin activator and anthrax capsule activator, respectively) that respond to environmental cues. The capsule enables the vegetative form to avoid phagocytosis. The Sterne vaccine strain is attenuated for virulence because it lacks the capsule encoding pXO2 plasmid.

1.1.3.2 Toxins

A number of toxins are made by *B. anthracis.* The most important of these in the production of disease are the lethal toxin and the edema toxin.

(1) Lethal Toxin

The genes encoding the lethal toxin (LeTx for lethal toxin) are found on a plasmid

termed pXO1. LeTx is a binary toxin composed of protective antigen (PA) and lethal factor (LF). PA is responsible for binding of LeTx to target cells, while LF is responsible for its toxic activity. PA and LF associate only after PA has bound to its receptor and then proteolytically processed by endopeptidases of the furin family. Processed PA forms a homoheptameric ring on the target cell surface. The PA can then serve as the binding component for the LF protein or the edema toxin. The toxin is endocytosed and when the reduction of pH in the endosome occurs, conformational changes ensue and the PA forms a transmembrane channel to deliver LF into the cytosol. LF is a zinc metalloprotease that inactivates mitogen-activated protein kinase kinases (MAPK kinases) by cleaving off their N-terminal sequences resulting in the disruption of signaling pathways within the affected cell, and leads to inhibition of cell proliferation and ultimately to apoptosis of affected cells. Macrophages from numerous species have been shown to react to LeTx exposure by undergoing apoptosis. LeTx derives its name for its lethal effects in animal models of infection including guinea pigs, rats, and mice. LeTx induces vascular collapse (pleural edema and rapid shock) and yet exposure to purified LeTx often results in no observable histopathology. Activity of the toxin on heart tissue is thought to lead to the pulmonary edema. In certain mouse strains, LeTx exposure results in rapid lysis of macrophages and production of inflammatory cytokines. However, other strains do not show this property. Macrophage lysis is not a common feature in other animal models. LeTx exposure does result in apoptosis in a variety of cell types and a cell-death independent loss of barrier function in epithelial and endothelial cells *in vitro*. Production of LeTx is under control of the regulatory gene product, AtxA (for anthrax toxin activator), which responds to as yet unknown environmental cues AtxA, is also encoded on pXO1. LeTx production by cells cultured *in vitro* is upregulated by the presence of bicarbonate in the medium and an elevated CO_2 atmosphere in an AtxA-dependent manner, suggesting this may be the cue recognized by AtxA. Lethal toxin is produced at levels five times greater than that of edema toxin.

(2) Edema Toxin

The genes encoding the edema toxin (EdTx for edema toxin) are found on a plasmid termed pXO1. EdTx is a binary toxin composed of protective antigen (PA) and edema factor (EF). PA delivers EF to the target cells cytoplasm by the same mechanism as lethal toxin. EF is a calmodulin dependent adenylyl cyclase that increases levels of cAMP within the affected cell. This disrupts cell signaling pathways, resulting in cell type-specific physiologic changes. In animal models of infection, purified EdTx produces hemorrhagic lesions in multiple organs accompanied by hypotension and bradycardia. EdTx has not been shown to be cytotoxic for multiple cell types. EdTx has been reported to suppress thrombin-induced platelet aggregation and clotting function. Production of EdTx is also under AtxA control.

(3) Miscellaneous Toxins

Several homologues of proteins shown to be important in virulence of other microorganisms have been discovered in the DNA sequence of the genome of *B. anthracis.* These include genes encoding proteins important in survival within phagolysosomes and on mucosal surfaces (InhA and MprF), and escape from phagolysosomes, phagocytic cells (anthrolysins), and iron acquiring products (Dip).

1) InhA and MprF

Immune inhibitor A (InhA) is a metalloprotease similar to that produced by *B. cereus*. It contributes to pathogenicity through several mechanisms, including the cleavage of antibacterial proteins, escape of bacteria from macrophages, control of blood coagulation, and degradation of matrix-associated proteins. InhA recently has been postulated to play a role in increasing blood-brain barrier permeability and contributing to cerebral hemorrhages. The genome of *B. anthracis* contains the genes encoding the homologues of InhA (for immune inhibit or protein) in *Bacillus cereus*, and MprF (for multiple peptide resistance factor) in *Staphylococcus*, which have been shown to confer resistance to defensins (found within the phagolysosome, and in secretions bathing mucosal surfaces) by lysinylation of phospholipids in the bacterial cell membrane.

2) Anthrolysins

The genome of *B. anthracis* contains genes encoding anthrolysins (also referred to as anthralysins), which are homologues of several phospholipase C and cholesterol-binding cytolysins (anthrolysin O) in other pathogenic bacteria. The phospholipases are pore-forming cytolysins, and anthrolysin O, also a poreforming toxin, binds to cholesterol contain in grafts in the eukaryotic cell membranes. Although each is dispensable for virulence, loss of all four results in attenuation. It is hypothesized that these are important in escape of the organism from the phagolysosome following germination of spores in macrophages. Anthrolysin O is poorly expressed by *B. anthracis* (which is why *B. anthracis* is non-hemolytic). The reason is that its regulator, PlcR, is inactive owing to a truncation of its coding sequence. Recombinant anthrolysin O is lethal to human monocytes, neutrophils, macrophages, and lymphocytes.

3) Dlp

The genome of *B. anthracis* contains genes encoding Dlp (for Dps like protein) which is an iron-binding protein homologue of that found in *E. coli* called Dps (for DNA protecting protein under starved conditions).

4) Regulation of the cellular products of medical interest

There are two proteins, AtxA and AcpA (for anthrax toxin activator and anthrax capsule activator, respectively) that are produced in response to environmental cues. What these cues ate *in vivo* is unknown. Under the appropriate conditions, however,

AtxA increases the production of LeTx, EdTx, and proteins involved in the escape from phagolysosomes and macrophages. AcpA production is amplified under increased amounts of CO_2 (5% or greater). AtxA acts synergistically with AcpA in this regard. The genes encoding AtxA and AcpA are on the plasmids, pXO1 and pXO2, respectively. The *B. anthracis* transcriptional regulator CodY activates toxin gene expression by post-translationally regulating the accumulation of the AtxA virulence gene regulator. CodY is also required for heme utilization as an iron source.

1.1.4 Growth Characteristics

Bacillus anthracis is a facultative anaerobe and grows on common media between 15℃ and 40℃. Colonies reach a diameter of 2mm or greater in 24h at 37℃. Colonies grown in air have a dull surface and wavy margin formed by strands of bacterial chains (medusa-head). Cells are non-encapsulated unless grown in greater than 5% carbon dioxide on serum agar containing 0.7% bicarbonate bicarbonate, in which case the colonies are mucoid. No hemolytic zone is produced.

Sporulation occurs under conditions of nutrient deprivation *in vitro*. The process is oxygen requiring, and does not occur while inside a living animal host. Organisms in infected tissue or fluids exposed to air sporulate after several hours.

1.1.5 Resistance

Although generally antibiotic sensitive, all sequenced strains of *B. anthracis* possess a latent β-lactamase gene, which is transcriptionally silent. Strains have been identified in which this gene is activated and inducible penicillin resistance is observed.

Vegetative cells in unopened carcasses may survive for up to 1-2 weeks, but spores can persist for decades in a stable, dry environment. Spores are killed by autoclaving (121℃, 15min) and dry heat (150℃, 60min), but not by boiling (100℃) for less than 10min. They are not highly susceptible to phenolic, alcoholic, and quaternary ammonium disinfectants. Aldehydes, oxidizing and chlorinating disinfectants, beta-propiolactone, and ethylene oxide are more useful. Spores are efficiently inactivated by exposure to 10% bleach. Heat fixation of smears does not kill spores.

1.1.6 Variability

The peracute nature of the infectious process and spore-based life cycle mean that *B. anthracis* undergoes a limited number of divisions in infected hosts and do not replicate while dormant in the soil. Thus, these organisms have undergone far fewer replication cycles than other forms of pathogenic bacteria. This results in much less mutational alteration of their genomic sequences and very limited strain-to-strain variation. The genome is more homogeneous than is the case with other pathogens.

1.2 Ecology

1.2.1 Reservoir

The soil is the source of anthrax infection for herbivores. Other species, including humans, are exposed via infected animals and animal products.

1.2.2 Transmission

The spore is the infectious form. Infection usually takes place by ingestion of contaminated feed or water. Exposure via wound infection and arthropod bites can occur.

Human infections occur following exposure to spores on infected hides or other animal products, from soil, or exposure to infected animal blood or tissue.

1.3 Pathogenesis

1.3.1 Mechanisms

Spores are acquired from the environment (e.g., soil, animal products). They are phagocytosed by macrophages or dendritic cells (polymorphonuclear neutrophil leucocytes do not appear to play a role in the disease process). The spores germinate within the phagolysosome compartment. Vegetative bacteria escape from the phagolysosome and later from the phagocytic cell. During the intracellular replication process, the phagocytic cells traffic to the regional lymph nodes. Release of the bacteria permits access of the organisms to the bloodstream.

1.3.2 Pathology

In tissue, spores germinate and the vegetative form proliferates, producing gelatinous edema. Inflammatory reactions are minimal. Infection disseminates to reticuloendothelial sites. When these are saturated, a terminal bacteremia occurs, with enormous numbers of organisms in circulation. There are no consistent pathognomonic lesions and considerable similarities are seen to other infectious and toxic causes of acute death. Postmortem findings are widespread hemorrhages; a black, engorged, friable spleen; tarry, non-clotting blood; and absence of rigor mortis. Bleeding at body orifices is common.

Experimental transmission of *B. anthracis* has revealed that cattle are resistant to spores introduced parenterally, but sensitive to spores introduced by the oral route. The LD_{50} is likely to be $< 10^7$ spores. With sheep, the LD_{50} is between 50 and 250 spores by the subcutaneous inoculation route. Swine are resistant to lethal infections by *B. anthracis* spores. Other species including dogs, rabbits and chickens are highly resistant to anthrax infection as well.

1.3.3 Disease Pattern

1.3.3.1 Ruminants

The process described above is typical for the most susceptible species–cattle and sheep. The course, following an incubation period of 1-5 days, ranges from a few hours to 2 days. Some animals die without overt clinical signs. Others develop high fever, agalactia, and they may abort. There is congestion of mucous membranes, hematuria, hemorrhagic diarrhea, and often-regional edema. These forms are regularly fatal. Occasional animals show just localized edema or an ulcerative skin lesion and recover.

1.3.3.2 Horses

Horses develop colic and diarrhea; edema also occurs, particularly of dependent parts and at the point of infection (e.g., the intestine or the throat) where it may cause death by asphyxiation. Alternatively, the course may be septicemic, as in ruminants.

1.3.3.3 Swine

In swine, localization in pharyngeal tissues is typical. An ulcerative lesion at the portal of entry is associated with regional lymphadenitis. Obstructive edema may cause death. Ulcerative hemorrhagic enteritis and mesenteric lymphadenitis sometimes occur.

1.3.3.4 Humans

Three distinct disease forms arise depending on the route of spore entry into the patient. Exposure through skin wounds or abrasions results in cutaneous anthrax that constitutes 95% of naturally occurring human infections. The hallmark lesion is the malignant pustule, a local ulcerative inflammatory lesion covered by a black scab (eschar). Possible complications are subcutaneous edema and septicemia. The case fatality rate for cutaneous anthrax is 10-20%. Inhalation of spores leads to pulmonary anthrax or "wool-sorter's disease" which is highly fatal if it is untreated, and 50% to >90% lethal even with antibiotic therapy. The poor prognosis is due to the initial non-descript influenza-like symptoms that delays correct diagnosis and the rapid time-course of the infection. Radiographic evidence of mediastinal widening is characteristic. Pulmonary edema, hemorrhagic pneumonia, and meningitis have been described in affected patients. Pulmonary anthrax is the form of human disease of concern from a bioterrorism standpoint.

1.3.4 Epidemiology

A soil rich in calcium and nitrate, with a pH range of 5.0-8.0, favors sporulation and bacterial proliferation at temperatures above 15.5℃, especially after flooding. The geography and seasonality of outbreaks reflect such circumstances. In cattle, sheep,

and possibly horses, outbreaks begin with a few cases contracted from the soil. After excretions and postmortem discharges seed the area, secondary cases occur. Floods and industrial effluents from rendering works, tanneries, carpet mills, brush factories, or wherever else carcasses are salvaged may contaminate areas. Bone meal, an animal feed supplement, is a common vehicle in non-endemic areas. Carnivores (mink) are usually exposed via infected meat.

Human exposures are contracted in occupations dealing with animals and animal-derived material such as imported hides, wool and bone. Anthrax occurring under industrial conditions is often the lethal airborne version. Non-industrial exposures have recently been associated with products such as decorative drums. Cutaneous anthrax infections are the usual form of non-industrial exposure.

1.4 Immunologic Aspects

Hyperimmune sera can prevent and alleviate disease. Antibacterial and antitoxic factors are thought to be involved. In most species, immunity is directed against the protective antigen. Capsular polypeptide fails to stimulate protective antibody.

Immunization of livestock has utilized mostly modified live spore vaccines. Currently, these are derived from avirulent (non-capsulated) mutants. The most widely used is the Sterne vaccine (a strain of *B. anthracis* that lacks the pXO2 plasmid). A cell-free vaccine consisting of concentrated culture filtrate has been used on humans exposed to industrial anthrax, researchers working with *B. anthracis*, or victims of suspected bioterrorism events involving *B. anthracis*. Five intramuscular injections over an 18-month schedule are recommended. Recombinant PA-based vaccines show promise.

1.5 Laboratory Diagnosis

1.5.1 Sample Collection

During sample collection, precautions against contamination of the environment are important. Blood may be aspirated from a superficial vessel. Aqueous humor has the added advantage of remoteness from sources of early postmortem contamination. For direct examination, bloody discharges from orifices are sampled. If the carcass has been opened, spleen material may be collected. *B. anthracis* is categorized as a select agent and once identified is subject to strict regulations for possession or transport. Only registered facilities and individuals can possess or work with select agents.

1.5.2 Direct Examination

Blood and organ smears are stained by Gram stain and a capsule stain such as McFadyean's methylene blue. Chains of encapsulated, Gram positive, non-spore-forming rods

suggest *B. anthracis*. Contaminant *Bacillus* species are usually not encapsulated and lack the clipped, squared-off appearance of anthrax bacilli. Fluorescent antibody helps in the differentiation.

1.5.3 Isolation and Identification

Bacillus anthracis grows on all common laboratory media. No hemolytic zones are evident around the colonies, although this feature is true of certain other *Bacillus* contaminants, and the "string of pearls" test (the characteristic cell rounding that occurs when *B. anthracis* contacts penicillin producing a chain of spherical cells). Definitive identification is by sensitivity to the γ-bacteriophage.

Experimental animals (mice, guinea pigs) are injected subcutaneously with suspect material. Death from anthrax occurs after 24h. Lesions include hemorrhages, gelatinous exudate near the inoculation site, and an engorged spleen. The encapsulated agent is demonstrable in blood and tissue.

1.5.4 Immunodiagnosis

B. anthracis antigens can be demonstrated in extracts of contaminated products by a precipitation test using antiserum prepared in rabbits by subcutaneous immunization with the Sterne strain (Ascoli test). The test lacks high specificity, in that the thermostable antigens of *B. anthracis* are shared by other *Bacillus* spp., and is dependent on the probability that only *B. anthracis* would proliferate throughout the animal and deposit sufficient antigen to give a positive reaction. Nowadays, it appears to be only used in Eastern Europe.

1.5.5 Molecular Techniques

Various gene sequences have been targeted in the design of primers that are used to amplify segments of DNA by using the polymerase chain reaction. Examples include portions of the gene encoding the 16S ribosomal RNA and parts of pOX1 and pOX2.

1.6 Treatment, Prevention, and Control

B. anthracis does not normally exhibit antibiotic resistance. It is susceptible to penicillins, chloramphenicol, streptomycin, tetracycline, fluoroquinolones, and erythromycin. Treatment should continue for at least 5 days. In some areas, antiserum is given simultaneously. Antiserum is not available in the United States. In acute anthrax, antimicrobial treatment is often unsuccessful.

Populations at risk are vaccinated annually. When an outbreak or a case of anthrax has occurred, animal health authorities are notified to supervise control measures. Carcass disposal involves incineration (preferred) or deep burial (>6.5ft) under a layer of quicklime (anhydrous calcium oxide). Surviving sick animals are isolated and

treated. Susceptible livestocks are vaccinated. The premises are quarantined for 3 weeks subsequent to the last established case. Milk from infected animals is discarded under appropriate precautions. Barns and fences are disinfected with lye (10% sodium hydroxide). Boiling for 30min will kill spores on utensils. Surface soil is cleared of spores by treatment with 3% peracetic acid solution at the rate of 8 liters (2 gal) per square meter. Some other material can be gas-sterilized with ethylene oxide.

Prevention of anthrax exposure through animal products imported from endemic areas requires disinfection of such material as hair and wool by formaldehyde. Bone meal is sterilized by dry heat (150℃, 3h) or steam (115℃, 15min).

2 *Clostridium*

Members of the genus *Clostridium* are Gram positive, sporeforming, anaerobic rods characterized also by production of powerful extracellular toxins. The diseases produced by members of this genus will be discussed under three categories: enterotoxic, including the enterotoxemias and diarrheas, produced by *C. perfringens*, *C. colinum*, *C. difficile*, *C. piliforme*, *C. septicum*, *C. spiroforme*, and *C. sordellii*; histotoxic, produced by *C. perfringens*, *C. chauvoei*, *C. haemolyticum*, *C. novyi*, *C. septicum*, and *C. sordellii*; and neurotoxic, produced by *C. botulinum* and *C. tetani*. Clostridial infections are serious infections because of the powerful toxins produced by these organisms. Many have been controlled successfully by immunization almost since the dawn of bacteriology. However, other clostridial infections are emerging as ever more common and important, perhaps partly because their ability to form resistant spores gives them selective advantage in growing in the intestinal tract whenever antibiotics are administered. Much remains to be discovered.

2.1 Descriptive Feature

2.1.1 Morphology and Staining

Members of the genus *Clostridium* are Gram positive rods measuring 0.2-4μm by up to 20μm. Location and shape of endospores are consistent within a species. Their ability to form spores is crucial to persistence in the intestine and the environment, and contributes to difficulties in their control (Fig. 12-2).

2.1.2 Structure and Composition

Little of medical relevance is known of the ultrastructure and composition of clostridia. A surface-associated structure characterized by orderly paracrystalline protein arrays (S-layer) in the cell wall of *C. difficile* may contribute to the ability of this bacterium to resist antimicrobial peptides in the intestine. The role played by the S-layer proteins is

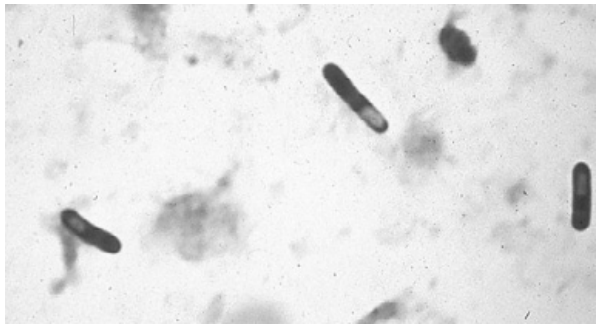


Fig. 12-2 A strain of *Clostridium* with a spore (Scott McVey *et al.*, 2013)

unknown. Some clostridia have been shown to produce pili or fimbriae (*C. difficile*), and others produce adhesive structures, presumably cell wall proteins. Considerable cellular intraspecific antigenic diversity and interspecific cross-reactivity exist but are of less interested than the antigenic properties of toxins because these latter are crucial in immunity. Those that are motile have peritrichous flagella or pilus-mediated "twitching motility". Of pathogenic species, *C. perfringens* and *C. difficile* may form capsules.

2.1.3 Growth Characteristics

Strictness of anaerobic requirements varies among clostridial species. In addition to an anaerobic environment, clostridia prefer 2-10% CO_2 in their atmosphere. For example, *C. difficile* is far more readily killed by exposure to air than is *C. perfringens*.

Most pathogenic clostridia require complex media including amino acids, carbohydrates, and vitamins. Blood or serum is beneficial. An ear-neutral pH and temperature of 37℃ are optimal.

Growth is usually visible within 1-2 days. Colonies are often irregular in shape and contour. Several clostridia swarm across moist agar media without forming colonies. Most clostridia products hemolysis when they grow on blood agar. In liquid media, clostridia often grow in air provided a reducing agent is present (cooked meat pieces and thioglycolate), though growth occurs only in the anaerobic portions of the medium.

2.1.4 Biochemical Activities

Most clostridia are metabolically highly active and masters at attacking carbohydrates, proteins, lipids, and nucleic acids. Clostridial cultures typically emit putrid odors due to produces of peptide catabolism, which is a common mode of energy production. Most clostridia attack carbohydrates, proteins, lipids or nucleic acids. Biochemical react ions and their end products furnish a basis for species identification.

2.1.5 Resistance

The vegetative form is as susceptible to environmental stresses and disinfectants as

other bacteria. Endospores impart resistance to drying, heat, irradiation, and disinfectants.

2.2 *Clostridium perfringens*

2.2.1 Descriptive Features

C. perfringens is a Gram positive, spore-forming, non-motile, encapsulated obligately anaerobic rod (Fig. 12-3) that produces a variety of toxins. Four of these toxins are used to type members of this species. There are five types, designated A through E. Although the toxinotyping scheme is now widely recognized as inadequate to describe the range of enteric diseases caused by this organism, *C. perfringens* is associated with enterotoxic and other enteric disease including diarrhea in a variety of species, as well as histotoxic infections such as wound infections (gas gangrene) and serious mastitis. Understanding of the role of *C. perfringens* in enteric disease in animals, particularly in serious hemorrhagic or necrotizing enteric disease, resembles that of understanding of *Escherichia coli* in enteric disease 50 years ago, when it was thought to be exclusively normal microflora. *C. perfringens* is as dynamic a bacterium as *E. coli*, increasingly recognized as having, like *E. coli*, the ability to adapt to cause disease in different host species. One important base of its adaptability is its possession of different conjugative plasmids that can move readily between *C. perfringens* in the intestine and that can also acquire mobile genetic elements carrying virulence determinants, and likely to change these through DNA recombination.

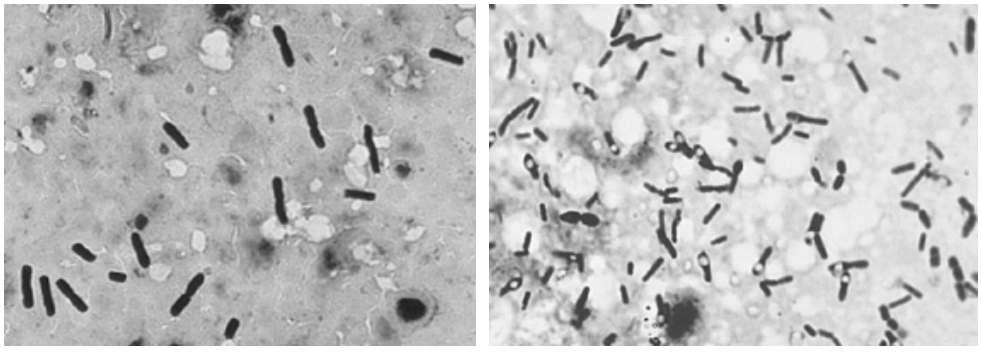


Fig. 12-3 *Clostridium perfringens*: large rods (left) and spores (right) (×1,000) (Markey *et al.*, 2013)

2.2.2 Cellular Products of Medical Interest

(1) Adhesion

C. perfringens possesses genes encoding fibronectin-binding and collagen-adhesion proteins. These proteins are thought to be involved in binding to the extracellular matrix during infection.

(2) Capsule

The role of the capsule in disease produced by *C. perfringens* is undefined, but it probably acts as a deterrent to phagocytosis. Encapsulation is an important virulence determinant in wounds (e.g., gas gangrene), but probably not in the intestinal canal (e.g., enterotoxemias, diarrheal diseases).

(3) Toxin

C. perfringens produces a variety of protein toxins and tissue-degradative enzymes consistent with its adaptation to break down tissue quickly and efficiently. It is auxotrophic for 15 amino acids, which it requires to obtain intact rather than being able to synthesize. Under optimal conditions, it divides every 10min, the fastest known multiplication rate of any bacterium. It has been aptly described as an "anaerobic flesh-eater". Most toxins are regulated by a global regulatory system ("VirR/VirS"), which is itself under the control of a "quorum sensing" system. The "major toxins" are the mouse-lethal α-, β-, ε-, and ι-toxins. The others are currently described as "minor".

Alpha toxin (Cpa or Plc for *C. perfringens* alpha toxin or phospholipase C, respectively), is produced by all *C. perfringens.* It is a mouse-lethal phospholipase C (a lecithinase) that hydrolyzes host cell membranes as part of its action in breaking down tissues to extract nutrients. Alpha toxin also displays the "hot-cold" lysis phenomenon.

Beta toxin (Cpb for *C. perfringens* beta toxin) encoded by a gene located on a conjugative plasmid, is a mouse-lethal pore-forming toxin, which damages host target cells (intestinal epithelial cells and endothelial cells). In addition, beta toxin affects nervous tissue by influencing the distribution of calcium ions across their membranes thereby disrupting normal nerve conduction. It is susceptible to the proteolytic activity of trypsin. The beta$_2$ toxin (Cpb2 for *C. perfringens* beta$_2$ toxin) is newly described, but the role this toxin plays in associated disease is not well characterized. The gene encoding this toxin is sometimes located on a plasmid. Its mechanism of action is unknown. It is produced by *C. perfringens* isolated from the intestinal contents of pigs with necrotic enteritis, horses with enterocolitis, and dogs with diarrhea (beta$_2$ toxin-producing strains are isolated less often from clinically normal animals).

Epsilon toxin (Etx for epsilon toxin) encoded by a gene located on a conjugative plasmid targets lipid (cholesterol and sphingolipids) rafts found in eukaryotic cell membranes, though the toxin concentrates in brain and kidney. It is a permease that acts by affecting the cellular cytoskeleton resulting in an increase in the permeability of epithelial and endothelial cells (both in the intestine but especially the microvasculature of the brain, leading to leakage of toxin into that organ). The toxin seems to target neuronal granule cells in the cerebellum and elsewhere in the brain to release the neurotransmitter glutamate. Etx is secreted as a protoxin that is activated in the intestine by proteolytic enzymes. The toxin may delay intestinal transit time in affected

animals.

Iota toxin (Itx for iota toxin) is a binary mouse-lethal toxin composed of a binding portion (Ib) that binds the toxin to target epithelial cells, and an enzymatically active portion (Ia). After the toxin binds to specific receptors on the cell surface, Ia gains entry into the cytoplasm. Although it is not clear precisely how entry occurs, it seems that a pore (composed of Ib) is formed in the cell membrane through which Ia traverses. Ia is an ADP-ribosylating toxin that ribosylates actin within the host cell, resulting in disorganization of the cellular cytoskeleton and death of the affected cell.

Kappa toxin (Col for collagenase) is a collagenase. Col is thought to aid spread of clostridial cells through the tissue.

Mu toxin (Nag for *N*-acetylgalactosaminidase) is a hyaluronidase. Nag is thought to aid spread of clostridial cells through the tissue.

Sialidase (neuraminidase or Nan) removes sialic acid residues from glycoconjugates on cell walls of eukaryotic cells resulting in disruptions of the intercellular matrix.

A number of hemolysins are produced by *C. perfringens*. Their role in disease is unknown.

Enterotoxin (Cpe for *C. perfringens* enterotoxin) is coded by a gene located in chromosomal (isolates from cases of human food-related gastrointestinal disease) or plasmid-based (isolates from dogs with diarrhea, or from human patients with non-food-related gastrointestinal disease). Enterotoxin is produced during sporulation of Cpe containing *C. perfringens* (less than 5% of type A strains in which Cpe is most commonly found). When the endospore is released, enterotoxin is also released into the surrounding milieu. Enterotoxin is a bifunctional toxin, first forming a pore in the apical portion of small intestinal epithelial cells resulting in fluid and electrolyte abnormalities, as well as providing access to tight junction proteins (specifically claudins and occludins). Interactions of Cpe with tight junction proteins result in further losses in control of fluid and electrolytes.

Perfringolysin O (Pfo), also known as theta toxin, is a cholesterol-binding cytolysin. Pfo binds to cholesterol-containing rafts in the eukaryotic cell membrane, and forms a pore, which results in the death of the cell. In addition, Pfo is responsible for the lysis of membranes enclosing *C. perfringens*-inside of phagolysosomes, resulting in their escape into the cytoplasm of the phagocytic cell.

(4) Regulation of Toxin Genes

C. perfringens globally synchronously regulates its toxin production and production of major metabolic enzymes by the two-component regulatory system, VirR/VirS. VirS is a histidine kinase that acts as a “sensor” of environmental cues resulting in autophosphorylation of one of its histidine residues. This phosphate is then serially transferred to an aspartate residue, then to another histidine before being used to phosphorylate VirR, the “regulator”. Phosphorylated VirR is a transcriptional activator

of the genes encoding the proteins mentioned above. What environmental cues are "sensed" by *C. perfringens* is unknown, but the composition of the intestinal contents appears to play an important role in the pathogenesis of the enterotoxemias.

2.2.3 Ecology

2.2.3.1 Reservoir

C. perfringens, type A, occurs in intestinal tracts of humans and other animals and in most soils. Types B, C, D and E are found mostly in the intestinal barn of animals, and their survival in soil is variable.

2.2.3.2 Transmission

Transmission is by ingestion and wound infection.

2.2.4 Pathogenesis and Disease Patterns

C. perfringens causes both serious enteric diseases, including important enterotoxemias, and histotoxic infections.

2.2.4.1 Wound Infections (Gas Gangrene)

C. perfringens, type A, alone or with other bacteria, causes anaerobic cellulitis and gas gangrene following inoculation into a normally sterile site. The membrane active toxins (alpha and perfringolysin O) accounted for the tissue destruction. Spread of the process is aided by collagenase (kappa toxin or Col), sialidase (Nan) and hyaluronidase (Mu toxin or Nag). Encapsulated *C. perfringens* resist phagocytosis. Those that are phagocytosed escape the phagolysosome by secretion of perfringolysin O. The process is a necrotizing cellulitis or myonecrosis with edema, hemorrhage, emphysema and a febrile, often fatal, toxemia. This type of *C. perfringens* infection in animals is rare, but when it occurs it is associated most often with injection sites deep in muscle (mainly horses).

2.2.4.2 Enterotoxemias

Most animal diseases due to *C. perfringens* are intestinal and involve types A, B, C, or D (rarely E).

C. perfringens type A has been implicated in enteritis, notably hemorrhagic or necrotizing forms, in numerous species, but understanding of the molecular basis of these infections (and hence of optimal microbiological diagnosis) is in some cases poor. For example, type A *C. perfringens* causes necrotic enteritis of chickens and other birds. It has only recently been recognized that strains that cause this infection are characterized by production of the NetB toxin, the gene for which is on one of three pathogenicity loci that are characteristic of chicken necrotic enteritis strains. This unexpected discovery illustrates the largely unrecognized likely ability of this bacterial

pathogen to adapt to cause distinct diseases in different animal species. Type A *C. perfringens* is important causes of necrotizing and emphysematous abomasitis in young calves, as well as sporadic hemorrhagic gastroenteritis in numerous species including dogs and foals, although the basis of these diseases is still unclear. Tissue destruction is usually attributed to the virulence determinants important in gas gangrene, but this assumption is probably incorrect. Cpb2-producing *C. perfringens* may be associated with fatal typhlocolitis in adult horses following gentamicin treatment. Extraordinarily, the *cpb2* toxin gene is usually out-of-frame and therefore unread in horse isolates, but treatment with gentamicin may cause ribosomal distortion such that the mRNA transcript is read in frame, and the toxin produced. Type A isolates are also involved in non-enterotoxemic food poisoning in humans (strains of which possess the chromosomally encoded Cpe enterotoxin as well as being more resistant to heat) and antibiotic-associated diarrhea in humans (associated with plasmid-borne carriage of the *cpe* and *cpb2* genes). There is a suspicion that type A *C. perfringens* encoding the Cpe enterotoxin may be involved in watery diarrheal illness in dogs and cats, but this is not proven. There is also a suspicion that Cpb2-positive type A strains can cause a mild diarrheal illness and growth setback in neonatal swine, but again this is unproven.

C. perfringens type B is an "old world" disease. *C. perfringens* type B causes "lamb dysentery" in newborn lambs. Occasionally foals, calves, and mature sheep and goats are affected. Beta toxin is considered the principal factor producing hemorrhagic enteritis and affecting the small intestine. Its trypsin susceptibility explains in part the predilection of the disease for the newborn, since colostrum contains antitrypsin substances. The signs are depression, anorexia, abdominal pain, and diarrhea. The course is rapid, with mortality rates approaching 100%. A chronic form occurs in older animals. The characteristic intestinal lesion is hemorrhagic enteritis. Extraintestinal lesions include congestion, edema, serosal effusions, and hemorrhages in various organs. The signs and pathology associated with this disease are due to the action of the membrane-active toxins (beta and epsilon), as well as those products that destroy the connective tissue components. Epsilon toxin, being a permease, increases intestinal permeability, ensuring its absorption into the circulation where it affects vascular endothelium, leading to fluid loss and edema, as well as damage to kidney function. Beta and epsilon toxins also affect the nervous system, and the severe depression, lack of response to corrective therapy, and high mortality may be due in part to this activity. Since epsilon toxin requires activation by proteolytic enzymes, its role in disease caused by type B strains is less important than that played by beta toxin.

C. perfringens type C causes hemorrhagic enteritis in neonatal calves, foals, piglets, and lambs worldwide. In some other species, including humans and rarely chickens, the organism can also produce necrotic enteritis. An often rapidly fatal intestinal

toxemia of older sheep is called "struck" (as in "struck dead", because the associated sudden death might suggest lightning strike). Beta toxin is the principal factor producing hemorrhagic enteritis, affecting the small intestine. Its trypsin susceptibility explains in part the predilection of the disease for the newborn, colostrum contains antitrypsin substances. The signs are depression, anorexia, abdominal pain, and diarrhea. The course is rapid, with mortality rates near 100%. The signs and pathology associated with this disease are due to the action of the membrane-active toxins beta toxin. In humans in New Guinea, type C enteritis ("pig bel") has been associated with feasting on undercooked and contaminated pork as well as on cassava; the trypsin-inhibiting properties of cassava prevent the breakdown of β-toxin produced by type C organisms in the small intestine.

C. perfringens type D produces an enterotoxemia ("overeating disease", or "pulpy kidney disease") in older lambs (<1 year), in goats of all ages, and occasionally in calves. Epsilon toxin is secreted as protoxin activated by intestinal proteases, explaining the predilection of this disease for older animals since colostrum contains antitrypsin activity. Epsilon toxin increases intestinal permeability, ensuring its absorption into the circulation where it damages vascular endothelium, leading to fluid loss and edema. When toxin levels are high, affected capillary endothelial cells in the brain are damaged, and the resultant edema greatly increases the intracranial pressure. When the amount of toxin is lower, however, as might be the case in a partially immune animal or when the amount of toxin produced in the intestinal canal is less, it damages the capillary endothelial cells in the brain so that toxin levels are increased in that organ. This results in a focal symmetrical encephalomalacia. In addition to these changes (which are toxin-dose related), epsilon toxin triggers catecholamine release, resulting in adenylyl cyclase activation, cAMP-related hyperglycemia, and glycosuria, a frequent finding in enterotoxemia. Gross lesions may be absent and death rates may be high in lambs, and postmortem autolysis is rapid because of vascular endothelial damage. Subserous and subendocardial hemorrhages and excess fluid in the body cavities are sometimes seen in lambs. Cerebral hemorrhage and degenerative lesions are common in less acute cases. Histopathology may reveal enteritis. Lambs may die without premonitory signs. But convulsions may occur in agonal stages and diarrhea in protracted cases. Cattle and older sheep show neurological manifestations. In adult goats, local necrotizing enteritis with diarrhea is common. Death rates are high in lambs. In calves and goats, non-fatal subacute and chronic cases occur.

C. perfringens type E produces a relatively uncommon form of enterotoxemia in calves, lambs and rabbits. The membrane active toxins (alpha, iota, and perfringolysin O) together with those toxins affecting connective tissue substances combine to produce this disease. Hemorrhagic enteritis and ulcerative abomasitis (gastritis) are the

pathologic lesions. Type E disease was erroneously described in rabbits since the *C. spiroforme* toxin is neutralized by ι-toxin antiserum.

2.2.4.3 Non-enterotoxemic Diarrhea

The role of *C. perfringens* in non-enterotoxemic and mild diarrhea in animals remains to be confirmed. For example, neonatal piglets may have a syndrome of weight loss, failure to thrive, and diarrhea associated with β2-toxin-producing type A *C. perfringens*. It is possible that non-enterotoxemic diarrhea occurs in other species subsequent to the interaction of the Cpe enterotoxin (with epithelial cells of the small intestine following sporulation of the microorganism in that environment). Although any type of *C. perfringens* can harbor the genes encoding Cpe, type A is the most common. This disease is one of the most commonly occurring food-related diseases in human patients. In addition to altering fluid and electrolyte flow of the epithelium, Cpe damages epithelial cells and tight junctions leading to sloughing with accompanying inflammatory changes.

2.2.5 Epidemiology

Some normal animals, especially adults, commonly carry *C. perfringens* in their intestinal tracts, probably as frequently as they carry *E. coli*. During outbreaks of diarrheal disease, pathogenic strains survive in soil long enough to infect other animals.

The determinant of enterotoxemic disease is the intestinal environment, which is influenced by diet and age. Overeating, especially on protein and energy-rich food (milk, legume forage, grain) is almost a prerequisite. In young animals, the excess feed is often passed, inadequately digested, into the intestine, where it provides a rich medium for proliferation and toxigenesis (up regulation of the VirR/VirS system) by ingested or resident bacteria. Overloading slows intestinal motility, thereby favoring retention of bacteria and absorption of their toxins. In addition, there are likely to be bacterial factors promoting intestinal colonization, but these are not well described. In broiler chickens, necrotic enteritis is predisposed by concurrent coccidiosis, as well as by wheat based diets, and diets containing trypsin inhibitors (such as unheated soybean meal).

The age predilection of these enteric diseases is due to the diet and the infantile digestive tract, which often lacks enzymes to inactivate the toxins. In particular, colostral antitrypsin activity exacerbates this aspect. *C. perfringens* spores are destroyed in the functional rumen, an organ that is rudimentary at birth. Type D proliferation, for which the ε-toxin requires trypsin activation, in older lambs appears to be favored by high-carbohydrate intake. Seasonal prevalence relates to the seasonal abundance of susceptible populations and rich forage. With warm temperatures, favoring proliferation of bacteria in the type A-associated conditions occur worldwide. Type B lamb dysentery occurs in Europe and South Africa, while type B enteritis of

sheep or goats is reported from Iran. Type C occurs worldwide, and type D is prevalent wherever sheep are raised. Type E is found in Britain, the United States, and Australia. A toxin similar to its ι-toxin is also produced by *C. difficile* and *C. spiroforme*.

2.2.6 Immunologic Aspects

Immunity is antibody-mediated and correlates with antitoxin levels. Immunizing preparations often include bacterial components as well. Active immunization is important in the control of the diseases.

2.2.7 Laboratory Diagnosis

C. perfringens is relatively aerotolerant, and is easy to isolate and to identify in the diagnostic laboratory. Spores are rarely demonstrable in exudates obtained from normally sterile sites.

Isolation follows inoculation of blood containing agar media, and incubation in an anaerobic environment. If *C. perfringens* is to be isolated from a contaminated environment (e.g., intestinal contents), the sample can first be heated to 80℃ for 15min since endospores will resist this treatment, whereas vegetative forms will not, and then the sample is placed into or onto isolation media. Diagnostic features include: ①alpha toxin-associated hemolytic activity (hot-cold lysis), ②the clotting of milk followed by gaseous disruption ("stormy fermentation"), and ③neutralization of alpha toxin activity on egg yolk agar containing specific anti-alpha toxin antibody (Nagler reaction).

In cases of enterotoxemia, stained (e.g., Gram's, Wright's and Giemsa) contents of the small intestine often contain large numbers of Gram positive rods resembling *C. perfringens.* However, this test is of limited value due to rapid postmortem bacterial overgrowth in all parts of the gut. DNA primers specific for the various genes encoding the toxins have been developed for detection in feces or cultures by using the polymerase chain reaction (multiplex PCR).

Demonstration of toxin in the contents of the small intestine is definitive and involves injecting small amounts of clarified intestinal contents into the tail vein of mice. Death after more than a few minutes post injection constitutes presumptive evidence of enterotoxemia and the toxin can be neutralized using specific antitoxins. Such a procedure, or guinea pig intradermal toxin testing, is now generally regarded as barbarism, and therefore most diagnostic laboratories rely on PCR testing. Lambs with Type D enterotoxemia usually test positively for glycosuria.

Cpe is detected immunologically in feces of affected dogs or cats by an enzyme-linked immunosorbent assay (ELISA). Although sporulation and Cpe production are co-regulated, there is disagreement regarding the usefulness of determining the presence of spores in stained smears of feces as a method of diagnosis.

2.2.8 Treatment and Control

Most cases of enterotoxemia are too acute for successful treatment. The best method of preventing enterotoxemia is active immunization of dams with two injections of bacterin-toxoid combinations prior to parturition. Commercial immunizing products usually cover type C and D. This ensures nurslings passive protection for the first weeks of life. During outbreaks, antitoxin and toxoid are sometimes given and a second dose of toxoid is administered some weeks later. Protection of lambs against type D enterotoxemia requires two vaccinations at a monthly interval. The course should be completed 2 weeks before the lambs are placed on full feed. Milking goats respond poorly to immunization with pulpy kidney (type D) vaccines, and therefore, vaccination is often repeated several times a year.

Antitoxin of appropriate type may be given to sick animals and those at risk. Protection lasts 2 to 3 weeks. Prophylactic dosages, given subcutaneously, can be doubled and given intravenously for therapy. However, antibiotics now usually replace antitoxins because of the cost and unavailability of antiserum. Active immunization of dams with two injections of bacterin-toxoid combinations prior to parturition ensures nurslings passive protection for the first weeks of life.

During outbreaks, antitoxin and toxoid are often given and a second dose of toxoid is administered some weeks later. Protection of lambs against type D enterotoxemia requires two vaccinations at monthly intervals. The course should be completed 2 weeks before the lambs are placed on full feed.

Commercial immunizing products usually cover types C and D.

Ensuring against overeating is a worthwhile preventive measure where practicable. Feeding broad-spectrum antibiotics reduces the prevalence of enterotoxemia of lambs, but creates other problems. Feeding antibiotics to poultry prevents mortality and illness in chicken due to necrotic enteritis caused by *C. perfringens*, type A. Diarrhea in dogs and cats associated with *C. perfringens*, type A producing Cpe, responds to metronidazole, macrolides (tylosin), or ampicillin.

Review Questions

1. What are Gram positive bacteria with spores, which have important pathogenic effects to humans and animals?

2. Try to describe the difference of the form and structure of *Bacillus anthracis in vivo* and *in vitro*.

3. Try to describe the virulence factors of *Bacillus anthracis* and its pathogenic effect.

4. How to conduct the microbiology diagnosis of *Bacillus anthracis*? And what are precautions in the diagnosis process of *Bacillus anthracis*?

5. Briefly describe the basic characteristics of *Clostridium*.

6. Try to describe the classification of the pathogenic *Clostridium* and the diseases caused by pathogenic *Clostridium*.

7. Try to describe the culture characteristics, toxins and diseases of *Clostridium perfringens* and *Clostridium tetanus*.

Chapter 13 *Mycobacterium*

Synopsis

Mycobacterium is a representative of Ziehl-Neelsen staining positive bacteria in Gram positive bacteria. The composition of cell wall is more special, which can be widely used as the classic immunoadjuvant. Mycobacteria have no spores, no flagella or no capsule. The growth of mycobacteria requires special nutritional conditions. According to the growth rate, mycobacteria have slow growth type and rapid growth type. Most members of *Mycobacterium* are saprophytic bacteria in the environment. Important pathogenic mycobacteria in humans and animals mainly include *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium paratuberculosis* and *Mycobacterium avium*, while *Mycobacterium tuberculosis* is as a representative. Cellular immunity and humoral immunity are separated, while infection immunity is special. Delayed allergy, IFN-gamma release test, ELISA methods can be used for the detection of mycobacteria. Sensitive drugs can be used for the treatment of mycobacterium infection. BCG vaccination can be used to prevent the infection of *Mycobacterium tuberculosis* in humans. Generally, animals can not be vaccinated BCG elated vaccines. The control and purification of tuberculosis in animals mainly rely on the monitoring and elimination.

1 Overview

Mycobacterium spp. can cause tuberculosis which is a chronic granulomatous disease. The tubercle bacilli of *M. tuberculosis*, *M. bovis* and *M. avium* are the agent of the disease in primates, other mammals, and birds, respectively. Host specificity is relative.

Of the 40-odd other *Mycobacterium* spp., some ("non-tuberculous" "atypical" "anonymous", or saprophytic) can cause tuberculosis-like infections. For example, *M. microti* can cause tuberculosis of voles; *M. leprosy* leads to human leprosy; and *M. avium* subsp. *paratuberculosis* causes Johne's disease in ruminants. Other mycobacteria produce granulomatous skin diseases or bovine mastitis, while fish and other poikilotherms can still be infected by other mycobacteria.

2 Tubercle Bacilli

2.1 Morphology and Staining

Tubercle bacilli are mainly rod-shaped, about 0.5μm wide, and are variable in length, with no spores, flagella, and capsules. Mycobacteria, though are cytochemical Gram positive, often resist staining with Gram stain. Acid fastness is their most significant staining property: they can resist discoloration with 3% HCl in ethanol once stained. *Mycobacteria* can be stained with fluorescent dyes (auramine-rhodamine) (Fig. 13-1).

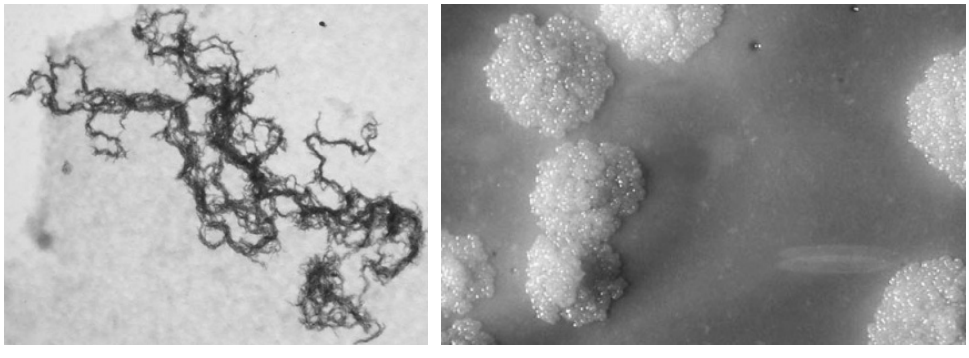


Fig. 13-1 Tubercle bacilli: showed cording (left) and colonies on L-J medium (right) (Rosenberg *et al.*, 2013)

2.2 Structure and Composition

2.2.1 Lipids

Mycobacterial cells abound in lipids, especially in their walls. Lipids are responsible for acid fastness, pathogenic, and immunologic properties. The colonial characteristics, serologic specificities, and bacteriophage susceptibilities are often determined by mycolic acid (mostly glycolipids and peptidoglycolipids). They are considered to be instrumental in ensuring bacterial survival within macrophages. The bulk of cell wall lipids are made up of subsurface layers of long-chain branched mycolic acids and their esters. Acid fastness somehow depends on these cell wall constituents. Mycolic acids are linked to the innermost peptidoglycan layer by way of arabinogalactans.

2.2.2 Wax D

"Wax D", an autolysate of *M. tuberculosis*, contains elements of all portions of the cell wall, has adjuvant activity. It can activate macrophages, stimulate particularly cell-mediated immune responses including delayed-type hypersensitivity, and induce

granuloma formation. One of its constituents, cord factor (dimycoyl trehalose), among other effects, immobilizes neutrophils, acts as an adjuvant, evokes granulomatous responses, and causes mitochondrial disruption, which leads to disturbances in cellular respiration. Its relation to the "cord" pattern of mycobacterial growth has not been proved. Sulfolipids (or sulfatides) and a phospholipid, phosphatidyl inositol mannoside (PIM), may also aid in preventing respiratory burst and phagolysosomal fusion, and may interfere with function of reactive oxygen intermediates following ingestion of tubercle bacilli by macrophages.

2.2.3 Mycobactins

Mycobactins are cell wall amines, which are involved in iron acquisition. The presence of carotenoid pigments in some mycobacteria ("chromogens" vs. "non-chromogens") and their light dependence ("photochromogens" vs. "scotochromogens") are the basis for classifying non-tuberculous mycobacteria.

2.2.4 Tuberculins

Tuberculins are bacterial peptides liberated into the culture media during bacteria growth. To some of them, or to their parent proteins, delayed-type hypersensitivity can develop during infection, making them useful diagnostic reagents. There have been no exotoxins produced.

2.3 Growth characteristics

Tubercle bacilli are strict aerobes that grow best on complex organic media such as Lowenstein-Jensen's, which contains whole eggs and potato flour among other ingredients. A dye, malachite green, inhibits contaminants. Oleic acid-albumin media, such as Middlebrook's 7H10 agar, are also used for isolation, often in combination with Lowenstein-Jensen's. Simple synthetic media containing ammonium salts, asparagine, citrate, glycerol, and minerals and vitamins are unsuitable for isolation, but support growth from large inocula.

The presence of glycerol favors growth of *M. tuberculosis* (eugonic) and *M. avium*, but not for *M. bovis* (dysgonic). Non-tuberculous mycobacteria often grow on routine laboratory media.

Generation time of tubercle bacillis more than 12h, and it may take weeks before colonies are visible. A wetting agent, tween-80, expedites growth in liquid media, and transparent oleic acid-albumin agar media permits early discernment of colonies. *M. avium* grows more rapidly than mammalian types.

Temperature from 33℃ to 39℃ is suitable for the mammalian species' growth, while avian and related mycobacteria (see below) are from 25℃ to 45℃, with the optimum being near the top of that range. Colonial growth of mammalian tubercle

bacilli is dry and crumbly. Avian forms grow in dome-shaped colonies.

2.4 Resistance

Tubercle bacilli survive with exposure to 1mol/L NaOH or HCl for 30min, a circumstance utilized in decontaminating diagnostic specimens. Mycobacteria are resistant many antimicrobial drugs, bacteriostatic dyes and disinfectants. Phenolic disinfectants are the most effective.

Tubercle bacilli can endure dry condition and survive for long periods in soi1, while they can be killed by sunlight, ultraviolet irradiation, and pasteurization.

2.5 Variability

Genetically, mammalian tubercle bacilli are variants one species, *M. tuberculosis*: human, bovine, and murine. The vaccine strain BCG (Bacille de Calmette et Guerin) is a modified *M. bovis*. Avian tubercle bacilli involve one species within the *M. intracellulare* group of saprophytic mycobacteria.

2.6 Ecology

2.6.1 Reservoir

The source of tubercle bacilli is tuberculous individuals. Humans can perpetuate *M. tuberculosis*, cattle *M. bovis* and chickens *M. avium*. The latter two infect wild mammals and birds, respectively, which may occasionally become the sources of infection for domestic animals. Most non-tuberculous mycobacteria are saprophytes, living in surface water and soil. Some of them are normal commensal bacteria of animals. The significant sources of infection are not diseased individuals.

2.6.2 Transmission

Tubercle bacilli are transmitted via the respiratory and alimentary through contaminated airborne droplet nuclei, feces, urine, milk from infected mammary glands, genital discharges, or contaminated feed and water. Percutaneous, transplacental, and transovarian (birds) infections are unusual. Intrauterine infection of calves could occur when bovine tuberculosis was common.

2.7 Pathogenesis

2.7.1 Mechanisms

The lipid components are implicated in pathogenesis. Mycolic acid and phospholipid

and sulfolipid apparently protect tubercle bacilli avoid phagocytic killing. Other lipids can produce granulomas with tuberculo-proteins, stimulate cell-mediated responses, a central feature of tuberculosis.

2.7.2 Pathology

Infection begins with the accumulation of tubercle bacilli in the lung or on pharyngeal or intestinal mucous membranes. As for previously unexposed animals, local multiplication occurs as macrophages on the scene ingest the organisms. Resistance to phagocytic killing (cell wall chemistry and shunting to endosomal compartments rather than those which fuse with lysosomes) allows continued intracellular and extracellular multiplication, an inflammatory response (elicited by mycobacterial cell wall constituents) involving a large proportion of histiocytes and monocytes which can develop around the focus of proliferating organisms. Infected host cells and bacteria reach draining lymph nodes, where proliferation and inflammatory responses continue.

After the first week, the host response will be modified essentially by cell-mediated immune reactions from a foreign body reaction to a reaction characteristic of infectious granulomas. Infected macrophages secrete IL-12, a cytokine which is responsible for stimulating $CD4^+$ TH1 lymphocytes to produce gamma interferon (INF), granulocyte monocyte colony stimulating factor (GM-CSF), and migration inhibition factor (MIF) can attract and activate macrophages. Then, the activated macrophages acquire the capacity to kill mycobacteria. Their efficiency depends on the adequacy of the immune response and the virulence of bacteria. Stimulated $CD4^+$ TH1 as well as gamma-delta T lymphocytes lyse mycobacteria-containing macrophages. Recognition of infected macrophages is probably connected to increased expression of "stress" proteins by the infected macrophages.

Epithelioid cells are found among the macrophages. They have oblong vesicular nuclei and pale, poorly delineated cytoplasm; they often become the predominant cells. A third, rare cell type is the Langhans giant cell, which may be a cellular fusion product. It has a large amount of pale cytoplasm and, peripherally, many vesicular nuclei. Both cells are probably macrophage derivatives. They do not seem to be effective phagocytes. Their bacterial content presumably came from their macrophage precursors.

Caseation necrosis develops at the center of the lesion. A function of the allergic state, it may proceed to calcification or liquefaction. The periphery of the lesion is unaltered macrophages mixed with lymphocytes. Fibrocytes appear, and a fibrous layer eventually invests the lesion, which is called a tubercle. Tubercles may enlarge, coalesce, and eventually occupy sizable portions of organs. Such tubercles consist mostly of caseous material.

The process described is typical of human and ruminant tuberculosis caused by

mammalian tubercle bacilli.

It is chronic; the lesion is called productive or proliferative. Occasionally, an acute exudative process takes place, marked by predominantly neutrophilic responses and fluid effusion. It is thought to be favored by factors such as a large infecting dose, focally delivered; high virulence of the infecting strain; constitutional predisposition of the host; loose tissue architecture as in the lung, serous membranes, or meninges; and a high degree of tuberculous hypersensitivity. One such acute process is tuberculous pneumonia, which may cause extensive necrosis and be rapidly fatal (galloping consumption), resolve almost completely or subside into the chronic pattern.

The course of the disease can be influenced by cell-mediated responses in several ways. Primary infection is disseminated via lymphatics to lymph nodes and beyond these through the bloodstream, seeding many reticuloendothelial tissues. Cell-mediated immunity and macrophage activation eliminate these foci, except where they have developed furthest–that is, the point of primary exposure and the adjacent lymph node. Here primary lesions (Ghon or Ranke complexes) persist. Sometimes, especially with the alimentary tract exposure, they are "incomplete", that is, only the lymph node lesion is discernible.

However, once cell-mediated reactivity is established, subsequent reinfection follows a different course: antigen-specific T lymphocytes and activated macrophages promptly converge on the site, contain the infection, and prevent lymphatic spread. Antigen-specific T lymphocyte responses also mediate allergic cytotoxic reactions and cause extensive tissue destruction, which is characteristic of progressive tuberculosis, while the immune response can limit the lymphatic dissemination, tissue damage facilitates bacterial spread by contiguous extension, or erosion of bronchi, blood vessels, or viscera, introducing infection to new areas. Wherever microorganisms lodge, the allergic (as well as the immune) reaction will be repeated with cumulative consequences. Hematogenous dissemination may cause miliary tuberculosis, that is to say that multifocal tubercle formation is throughout an organ. Reinfection tuberculosis is most often endogenous and can result from reactivation of previously dormant foci.

2.7.3 Epidemiology

With the eradication of cattle tuberculosis in industrial countries, the traditional reservoir in domestic mammals has disappeared. Game farms, animal parks, and zoos remain foci of *M. bovis* in technically advanced countries. Sporadic cases of canine tuberculosis often prove to be *M. tuberculosis* infections which are traceable to human contacts, "reverse zoonoses", which are also found in nonhuman primates in laboratory colonies and zoos.

In commercial poultry establishments, rapid population turnover (<1 year) eliminating transgenerational transmission has eradicated *M. avium*. While it remains a problem in

barnyard flocks, particularly since the agent can survive in soil for several years.

Tuberculosis is a typical disease of captivity and domestication. When wild and captive infected populations were compared, clinical improvement and lack of spread in free-living animals contrasted with deterioration and high communicability in confined groups. Tuberculosis in wild populations seems to be relatively rare. Nevertheless, *M. bovis*, probably originated from cattle.

Immature individuals often can develop more severe lesions than older ones. Breed susceptibilities are different. Zebu cattle can endure more than European breeds, while fox terriers and Irish setters are less resistant than Dachshunds and Dobermans. The higher prevalence in dairy than beef cattle may reflect closer confinement, longer life span, and greater productivity stress among dairy cows. Exemption from pregnancy and lactation may explain the lower disease prevalence in bulls than cows, although in dogs the reverse sex ratio is observed.

2.8 Immunologic Aspects

2.8.1 Immune Mechanisms of Disease

The key role of cell-mediated immune responses in the pathogenesis of tuberculosis has been discussed. With their absence, the disease may progress as a disseminating inflammatory disease (as it does in athymic mice) without the development of typical lesions.

2.8.2 Recovery and Resistance

The cell-mediated responses determine the acquired resistance. Under natural conditions, the resistance develops along with hypersensitivity, both of which are demonstrated in the Koch phenomenon: an already tuberculous guinea pig suffers a rapid, destructive, but limited reaction at the site of re-exposure to tubercle bacilli, while a virgin animal can develop persistent, progressive, disseminating, and eventually fatal disease when injected at the same anatomical site. Allergic reactivity and protective responses are separable. Immunity can persist in desensitized animals and can be absent in sensitized subjects. Antibody to tubercle bacilli does not protect against natural infection.

2.8.3 Artificial Immunization

Vaccination of humans with BCG (live attenuated *M. bovis*) produces temporary immunity and hypersensitivity. The benefits of vaccination are best where exposure is most intense and negligible where prevalence is low. Vaccination in humans is focused on infants and tuberculin-negative individuals anticipating exposure.

BCG has been used in calves. This practice is inappropriate in countries which attempt to eradicate tuberculosis as it interferes the interpretation of the tuberculin test.

M. microti, the vole *Bacillus*, can stimulate immunity to bovine and human tuberculosis. Its virulence is too variable to permit its use as a vaccine. Among subcellular experimental immunogen, a ribosomal preparation is of interest because it produces protection without tuberculin allergy.

2.9 Laboratory Diagnosis

Tracheobronchial and gastric lavage are included in samples; lymph node, thoracic, abdominal, and other aspirates; urine, feces, and biopsy specimens, at necropsy, material 15 obtained from lesions.

2.9.1 Direct Examination

Fluids are concentrated by centrifugation in tightly capped containers. Samples for microscope only are digested and disinfected with hypochlorite (bleach, clorox). Smears of sediment or tissue can be stained with an acid-fast stain, auramine-rhodamine where fluorescence microscope is available. Histologic sections are stained with hematoxylin-eosin and acid-fast stains. Positive results would be confirmed.

2.9.2 Culture

Digestion and selective decontamination are advisable, especially specimens which are likely to contain a mixture of microorganisms. Identification of mycobacteria could be done almost exclusively *in vitro*. Sometimes, animal inoculations are used, and DNA probes, specifically for the main groups, are commercially available.

2.9.3 Immunodiagnosis

2.9.3.1 Tuberculin Test

Cell-mediated hypersensitivity acquired through infection, can be demonstrated systemically by fever, ophthalmically by conjunctivitis, or dermally by local swelling, when tuberculin or its purified protein derivative (PPD) is given by the subcutaneous, conjunctival, or the intradermal route, respectively. In cattle, tuberculin, the equivalent of a 0.2 to 0.3mg/dose of bovine PPD, 15 injected intradermally in the caudal, vulvar or anal skin or the neck region in some situations. In positive cases, a swelling($\geqslant$5mm) can develop within 72h. While tuberculin cannot cause the allergic state, it may desensitize animals for weeks or months.

The reacting animal to be slaughtered and necropsied is necessary for a positive test which can imply past or present infection. Where tuberculosis is rare, lesions are not often found in reactors (NVL, for non-visible lesion reactors). Such apparently false-positive reactions are explained by allergies to non-tuberculous, related agents such as other *Mycobacteria*, or *Nocardia*. Simultaneous use of avian tuberculin, which detects

hypersensitivity to several non-tuberculous mycobacteria, often can help to decide, via comparative size assessment of the two reactions, whether sensitivity is primarily due to mammalian or a heterologous tuberculin. Other explanations for NVL are early stages of infection, remote location of lesions, or microscopic sizes of lesions.

False-negatives occur in animals which are quite recently infected and in advanced cases which anergy attributes to antigen excess or immunosuppression, Nonspecific factors, such as malnutrition, stress, and impending or recent parturition, are alternative causes of anergy. While rules governing the use of tuberculin tests in eradication programs vary from country to country.

Tuberculins of appropriate specificity are used on swine and poultry. Ears are injected in swine, the wattles in poultry. The reliability of tuberculin tests on horses, sheep, goats, dogs, and cats is not established.

2.9.3.2 Serology

Serologic tests are not useful in diagnosis of mammalian tuberculosis. As a first step in poultry tuberculosis eradication, a whole-blood agglutination test is available. It is sensitive but lacks specificity.

2.10 Treatment and Control

Generally, streptomycin, isoniazid (INH), ethambutol, and rifampin are first-line drugs for tuberculosis therapy. Second-line drugs are pyrazinamide, para-aminosalicylic acid, kanamycin, cycloserine, capreomycin, and ethionamide. Because resistance often develops under a single-drug regimen, a combination is commonly used the most favored one in human medicine being INH-ethambutol-rifampin. Treatment is 9 months with rifampin included 18 to 24 months without it.

As the public health hazards inherent in the retention of tuberculous animals, anti-tuberculous chemotherapy of animals is discouraged. Prophylactic treatment with INH may be considered for pets recently exposed to tuberculosis. There have reports that some experimental successes with INH for prophylaxis and treatment of calves. In countries with eradication programs, treatment is generally discouraged or illegal.

Identification and elimination of infected animals controlled the bovine tuberculosis. This approach has resulted in near-eradication of the infection in many countries. Continued surveillance is necessary to prevent a resurgence. In poultry, tuberculosis in backyard flocks is perpetuated by retention of birds and persistence of soil contamination.

Review Questions

1. Try to describe the dyeing properties of mycobacteria and its relationship to the

cell wall.

2. What are the mycobacteria who have pathogenic effects on animals?
3. Try to describe the pathogenesis of *Mycobacterium tuberculosis*.
4. Try to describe the immunity of *Mycobacterium tuberculosis*.
5. How to quickly check tuberculosis?
6. How to control tuberculosis?
7. Briefly describe the culture characteristics of *Mycobacterium tuberculosis*.
8. What are the virulence factors of *Mycobacterium tuberculosis*?

Chapter 14 *Spirochaeta*, *Mycoplasma*, *Rickettsia* and *Chlamydia*

Synopsis

Spirochaeta, *Mycoplasma*, *Rickettsia* and *Chlamydia* are the Gram negative prokaryotic microorganism, but they are different from bacteria in morphology, structure or culture. The structure of *Spirochaeta* is similar to bacteria, but *Spirochaeta* with a spiral shape can move with endoflagella, and can be viewed with a dark field microscope. *Spirochaeta* are widely distributed in aquatic environments, and only a few members of *Spirochaeta* can cause diseases in humans and animals. The representative spirochaetae which have pathogenicity include *Borrelia burgdorferi*, *Brachyspira hyodysenteriae*, *Treponema paraluiscuniculi*, *Treponema pallidum*, and some members of *Leptospira*. *Mycoplasma* is lack of cell wall, and present more forms. *Mycoplasma* can pass through the bacterial filter. The nutrition demand for the growth of *Mycoplasma* is higher. The typical colony of *Mycoplasma* is frying egg shaped. *Mycoplasma* are widely distributed in nature, and often contaminate cell culture and biological products. The detection of *Mycoplasma* can take through the physiological and biochemical characteristics, serology and molecular biology methods. *Mycoplasma*, which is pathogenic to animals, mainly include *Mycoplasma hyopneumoniae*, *Mycoplasma gallisepticum*, members of *Mycoplasma mycoides* cluster, and members of *Mycoplasma haemotrophic* cluster. *Spirochaeta* and *Mycoplasma* can grow and multiply in cell-free media. *Rickettsia* present more forms. The size of rickettsia is between bacteria and viruses, but *Rickettsia* can not pass through the bacterial filter. *Rickettsia* is not sensitive to sulphonamide which can promote the growth of *Rickettsia*. *Rickettsia* is spread through the arthropods. The important *Rickettsia* in veterinary medicine mainly are members of *Rickettsia*, *Anaplasma*, *Ehrlichia* and *Neorichettsia*, many of which are pathogenic to humans and animals. The enzyme system of *Rickettsia* is not completely. So, *Rickettsia* is obligatory intracellular parasitism. *Chlamydia* has a unique developmental cycle, which can form the inclusion body. *Chlamydia* can be filtered. The infection of *Chlamydia* lead to more types of inflammatory, and

affect the reproductive system. *Chlamydia* which are pathogenic to humans and animals are mainly *Chlamydophila psittaci*, *Chlamydophila felis*, *Chlamydophila pecorum*, *Chlamydophila pneumoniae* and *Chlamydia trachomatis*, etc. *Chlamydia* has some enzyme systems, but they can not synthesize compounds with high-energy bond. So, *Chlamydia* is obligatory intracellular parasitism with the energy from production material in host cells.

1 *Spirochaeta*

The genus *Spirochaeta* is a group of free-living, saccharolytic non-pathogenic, obligate or facultative anaerobic helical shaped bacteria. Isolated strains can have been obtained from a variety of marine waters and freshwaters. Extremophilic species include anaerobic, thermophilic which are isolated from hot springs in New Zealand, and the moderately thermophilic *Spirochaeta caldaria* from cyanobacterial mats of hot springs in Utah and Oregon, while in Shiashkotan Island in Russia, the extremely thermophilic *Spirochaeta thermophila* from marine areas. It has been found that alkaliphilic spirochetes such as *S. alkalica*, *S. africana* and *S. asiatica* isolated from the alkaline Lake Magadi in Kenya and from sulfide-saturated mud sediments of Lake Khatyn in Siberia.

Besides, culture-independent studies confirmed that the presence of the *Spirochaeta* species in anaerobic bioreactors, and the digestive tract of termites.

Many members of the genus *Spirochaeta* inhabit extreme environments with respect to temperature, salinity and pressure. These microorganisms may hence produce enzymes which have potential biotechnological applications.

1.1 Descriptive Features

1.1.1 Cell Structure

The spirochetes present a large, heterogeneous group of spiral, motile bacteria (Fig. 14-1). They have unique morphology and cellular structure of *Spirochaeta* spp. (and most other *Spirochetes*) among prokaryotes. The cells have a helical shape and consist of an outer membrane, a protoplasmic cylinder, and axial filaments (ultrastructurally similar to bacterial flagella). The outer membrane or the outer sheath surrounds all of the structures including the axial filaments and the protoplasmic cylinder. The cell body of the organism is the protoplasmic cylinder. It is coiled, and the cytoplasm, the nuclear region and the peptidoglycan-cytoplasmic membrane complex are the composing part. Axial filaments can be called the periplasmic flagella and are also known as periplasmic fibrils, axial fibrils and endoflagella. There are normally two periplasmic

flagella in the cell with each periplasmic flagellum running most of the length of the cell, so there is region where the two flagella overlap and make an arrangement of "1-2-1", at first, there is one flagella then two flagella at the region where they overlap and then one again when only the second flagellum is present. Regions where these flagella attach can be found at the ends of the cell. An illustration of a spirocheata cell showing the outer sheath, the protoplasmic cylinder and the periplasmic flagella. It can be found that the periplasmic fibrils are between the outer membrane and the protoplasmic cylinder (Fig. 14-2). As mentioned earlier, all species of *Spirochaeta* have two periplasmic flagella except one, *Spirochaeta plicatilis*. This species is a huge member of *Spirochaeta* and has 18 to 20 periplasmic flagella inserted near each end of the protoplasmic cylinder. Also, the periplasmic flagella of the spirochete are endocellular and permanently wound around the cell body. This provides these bacteria a unique system for motility unlike other flagellated bacteria as their flagella are extracellular and are in direct contact with the environment. The axial filaments reside under the outer membrane and are responsible for the unique mobility behavior of *Spirochetes*, a trait which has been linked to the pathogenicity of certain genera.

Fig. 14-1 *Leptospira* (left) and *Borrelia* (right) (Markey *et al.*, 2013)

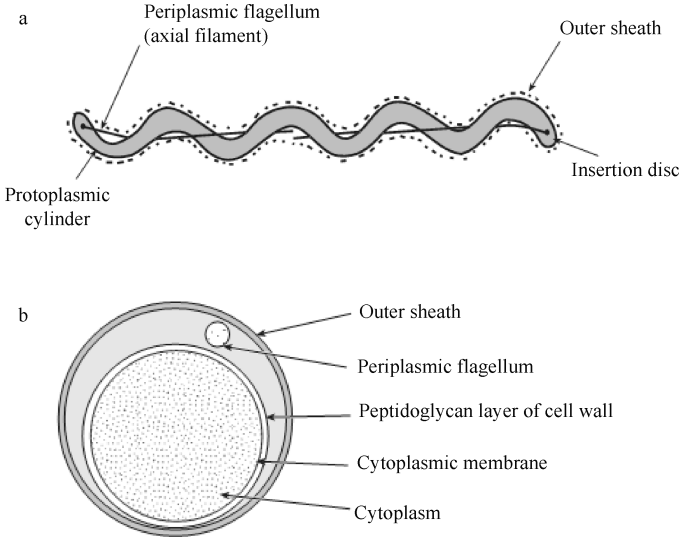


Fig. 14-2 Cell and structure of a spirochaeta (Markey *et al.*, 2013)

a. A spirochaeta cell; b. Cross section of a spirochaeta cell

The typical shape is helical for members of the *Spirochetes phylum*. While, a coccoid shaped *Spirochaeta* spp. was recently isolated from a termite gut.

1.1.2 Genome

At present, there has no a fully closed genome sequence of a member of *Spirochaeta*. However, the genome sizes of three species of *Spirochaeta* are known, *Spirochaeta asiatica* 2.1Mb, *Spirochaeta alkalica* 2.7Mb and *Spirochaeta africana* 2.5Mb. It was confirmed that DNA of spirochaeta species has G+C content range of 51- 65mol% through HPLC (high performance liquid chromatography).

1.1.3 Metabolism

The *Spirochaeta* includes a group of saccharolytic bacteria. In many aquatic environments, the saccharolytic bacteria play the role of decomposing the (poly) carbohydrates, and the major end products are acetate, ethanol, CO_2, and H_2. Besides, *Spirochaeta isovalerica* can ferment a number of amino-acids with the addition of carbohydrates. All isolated *Spirochaeta* species produce pyruvate via the Embden-Meyerhof pathway for glucose catabolization.

1.1.4 Ecology and Applications

Spirochaeta is quite common in a variety of aquatic environments, such as rivers, lakes, marshes, sediments and muds of ponds, and oceans. In these environments, *Spirochaeta* can compete with other microorganisms for available nutrients by presenting various types of mobility behaviors. The helical shape of the *Spirochetes* cell as well as the polar positioning of the axial filaments leads to the Spirochetes' unique mobility. They are also able to move onto surfaces which are similar to gliding bacteria. And they even could move through the environment of high viscosities, which is too high to move for most flagellated bacteria.

As they are saccharolytic, so only the carbohydrate can be used as their substrates. They are commonly found in areas with high activity of plant decomposition. These species can ferment sugars which are produced as the by-products by other organisms for the plant decomposition. When the cellobiose is available, the facultative and obligate anaerobes species of *Spirochaeta* will grow abundantly and richly. Cellobiose is a product when other organisms degrade cellulose. Free living species, such as *S. aurantia*, are able to exhibit strong chemotactic response to low cellobiose concentrations and they also have systems which could facilitate them to enhance the chemotactic response to low concentration of substrate in the environment. This character provides them an advantage when competing with other cellobiose-utilizing organisms. Another advantage of some species is that they have developed strategies to survive with extremely low concentrations or none in environments. *Spirochaeta isovalerica* does not ferment with amino acids. Instead, it can catabolize small amounts

of L-isoleucine, L-leucine and L-valine by thus forming isovalerate, 2-methylbutyrate and isobutyrate. This strategy also can make ATP which is as the energy source for the bacteria. Metabolism of endogenous RNA and intracellular polyglucose storage granules are other strategies employed by these spirochetes to produce ATP.

It is discovered that there are symbiotic relationships between species of *Spirochaeta plicatilis* and *Beggiatoa*. The spirochete has been found lurking among the trichomes of *Beggiatoa*. They are found to move towards the trichomes through chemotaxis of the metabolites which are produced by the *Beggiatoa*, and the metabolites are the substrates of the spirochete. It has been also known that one of its species, *Spirochaeta caldaria* is a thermophilic spirochete taken from a freshwater hot spring and may form symbiotic relationships with the *Clostridium thermocellum*. A study has shown that cellulose degradation is prompted in *Clostridium thermocellum* when the spirochete is present.

As some species of *Spirochaeta* can convert cellulose to ethanol or hydrogen gas, which can be possibly be used in the direct bioconversion of cellulose-containing wastes to fuel. Since some species are extremophilic and can survive in high temperatures as well as high salt, their enzymes can be extracted for application.

1.1.5 Classification

Spirochaeta belongs to Bacteria, Spirochaetae, Spirochaetes, Spirochaetales, Spirochaetaceae.

1.2 *Brachyspira* (*Serpulina*)

Members of the genus *Brachyspira* are spiral-shaped obligately anaerobic bacteria and Gram negative, belonging to the Spirochaetaceae. *B. hyodysenteriae* can cause swine dysentery, a disease of actively in growing pigs. *Brachyspira pilosicoli* is linked with intestinal spirochetosis of the postweaning pigs, dogs, birds, and humans (especially those immunocompromised ones). *Brachyspira aalborgi* is the causative agent of human spirochetosis. Other brachyspiras include *B. intermedia* and *B. murdochii*, *B. innocens* with uncertain pathogenic potential, as they are found in feces of symptomatic as well as asymptomatic pigs, they may have little if any pathogenic potential. *Brachyspira canis* is often found in the intestinal content of symptomatic as well as asymptomatic dogs.

1.2.1 Descriptive Features

1.2.1.1 Morphology and Staining

B. pilosicoli and *B. hyodysenteriae* are loosely coiled spirochetes, with the length of 6-11μm and 0.25-0.35μm in width. Another quite similar brachyspira, *B. innocens* (the so-called small spirochete), is often found in the feces of pigs with symptom of

dysentery as well as normal feces. *B. innocens* is measured 5-7μm by 0.2μm tightly roiled.

The brachyspiras are commonly used Romanovsky-type stains (e.g., Wright's, Giemsa) in smears to demonstrate them rather than Gram stain. Though they are Gram negative, but this characteristic is not used to identify or detect them.

1.2.1.2 Cellular Anatomy and Composition

Cells are typical spirochetes. There are 8-12 flagella inserted at either end to make up the axial filament of *B. hyodysenteriae*; *B. innocens* has 10-13 flagella and *B. pilosicoli* 4-6.

1.2.1.3 Cellular Products of Medical Interest

(1) Cell Wall

The cell wall of brachyspiras is Gram negative. The lipopolysaccharide (LPS) in the outer membrane is an important virulence determinant. Not only the lipid, but the length of the side chain in the O-repeat unit that hinders the attachment of the membrane to the outer membrane. LPS binds the plasma protein to LPS-binding protein, which then binds CD14. The CD14-LPS complex binds a toll-like receptor on the surface of macrophage cells, causing the proinflammatory cytokines.

(2) Cytoxin

The protein, Tly (for cytoxin) is related to the strong beta-hemolysis by *B. hyodysenteriae in vitro* (the degree of hemolysis *in vitro* is often used to differentiate *S. hyodysenteriae* from *B. innocens* and *B. pilosicoli*). This protein is a virulence determinant. Mutants are less virulent without producing Tly. Tly is a pore-forming cytotoxin which can affect host target cells (goblet and colonic epithelial cells).

(3) Hemolysin

The relation of another hemolysin-Hly (for hemolysin) to virulence is unclear at present.

(4) Flagella

Flagella appear necessary for virulence, though are present both on virulent and avirulent brachyspiras. This trait is linked to movement through the intestinal mucus to gain access to target cells in the large intestine. It has also been shown that virulent strains have relation with intestinal mucus.

1.2.1.4 Growth Characteristics

Members of the genus *Brachyspira* are all obligate anaerobes. *Brachyspira hyodysenteriae* and *B. pilosicoli* are resistant to high concentrations of spectinomycin, which is useful in isolating these organisms from feces. *B. hyodysenteriae* and *B. pilosicoli* presumably

remain infective for long periods if attached on organic material at 5-25℃. They cann't withstand drying or direct sunlight.

1.2.1.5 Variability

There are at least twelve serotypes of *B. hyodysenteriae*. Fingerprinting isolates which are via restriction-length polymorphisms of whole-cell DNA, DNA encoding ribosomal RNA, DNA encoding specific genes (e.g., flagellin), and multilocus enzyme electrophoresis to demonstrate the heterogeneity of members of *B. hyodysenteriae* as well as the others (*B. innocens* and *B. pilosicoli*).

1.2.2 Ecology

1.2.2.1 Reservoir and Transmission

The gastrointestinal tract of the pig is the reservoir for *B. hyodysenteriae*, especially asymptomatic carriers (animals recovered from the disease). The *B. hyodysenteriae* has been isolated from the feces of dogs, rats, and mice living on farms where the disease exists. The transmission route is through the fecal-oral.

B. pilosicoli has been isolated from dogs, birds, and humans, and there has evidences which show that humans may acquire *R. pilosicoli* from affected dogs.

1.2.2.2 Pathogenesis

B. hyodysenteriae multiplies and produces disease in the colon (swine dysentery). It seems that *B. hyodysenteriae* alone will not cause disease. Other bacteria such as *Bacteroides vulgatus*, *B. fragilis*, *Fusobacterium necrophorum*, *Campylobacter coli*, *Clostridium* spp., and *Listeria denitrificans* are usually found in the colon of pigs, and have been involved in the supporting role. The superficial coagulation necrosis is observed with epithelial cell erosion. Edema, hyperemia, hemorrhage, and influx of polymorphonuclear neutrophil leukocytes (PMN) into the mucosa and submucosa have appeared. There has failure of colonic absorption. Inflammation, brought about by cytotoxin-mediated destruction of colonic target cells (goblet cells initially, then enterocytes), may induce a secretory diarrhea. It has not been found in DNA sequences encoding which has been known as enterotoxins in *B. hyodysenteriae*.

The signs of disease are quite typical. Affected pigs will void gray to strawberry-colored feces and become dehydrated, in the extreme, be acidotic and hyperkalemic. However, temperature generally keeps normal. Morbidity rates in susceptible pigs will be high up to 90%, with mortality of approximately 20-40% in untreated herds. The duration period of illness ranges from a few days to several weeks. Survivors may be permanently stunted and remain asymptomatic shedders. There is no easy way to detect such animals.

B. pilosicoli is linked with the intestinal spirochetosis of pigs (post-weaning period),

dogs, birds, and humans. The characteristic of this disease is a mild, persistent diarrhea and low mortality. Biopsies of affected colon show large dumps of spirochetes adhering "end on" to the intestinal epithelium.

1.2.3 Immunologic Aspects

There is little knowledge about the immunologic factors of these diseases. Pigs recovered from swine dysentery are resistant to reinfection. Bacterins have showed some effectiveness in reducing the severity of the disease in affected swine.

1.2.4 Laboratory Diagnosis

1.2.4.1 Sample Collection

Fecal samples from affected animals showing signs of the disease are used to detect *B. hyodysenteriae* and *B. pilosicoli*.

1.2.4.2 Direct Examination

Smears of fecal material are stained with a Romanovsky-type stain (e.g., Wright's, Giemsa) or carbol fuchsin. Observation of large, loosely called spirochetes in diarrheal feces is presumptive evidence for infection of *B. hyodysenteriae* (swine dysentery) or *B. pilosicoli* (intestinal spirochetosis). *B. innocens* may appear in samples from pigs with swine dysentery, but these will be smaller and have tighter coils, a distinction that is somewhat difficult to make.

1.2.4.3 Isolation/Detection

Inoculation onto blood agar plates containing spectinomycin (400μg/ml) can accomplish the isolation of *B. hyodysenteriae* and *B. pilosicoli* from fecal samples. The plates are incubated 24-48h containing 10% carbon dioxide in an anaerobic environment. Colonies of *B. hyodysenteriae* are small and strongly beta-hemolytic, but those of *B. pilosicoli* are not so strongly hemolytic. A multiplex polymerase chain reaction (PCR) assay use primers designed to detect the DNA of common diarrhea-associated microorganisms (*B. hyodysenteriae*, *Lawsoniaintracellularis*, and *Salmonella*).

1.2.4.4 Identification

The best way to differentiate *B. hyodysenteriae* from *B. innocens* is by gas chromatographic analysis of volatile fatty acids or DNA probing/analysis. However, these techniques are quite incompetent in a busy diagnostic laboratory. Therefore, observing the strength of the beta-hemolysis, fructose fermentation (*B. innocens* will be positive), and indole production (*B. hyodysenteriae* will be positive) are traits used to make the distinction. The hemolysis trait seems to be relatively stable, the other tests are somewhat variable and misidentifications. *B. pilosicoli* hydrolyzes produce hippurate, while *B. innocens* does not. *B. pilosicoli* is differentiated from *B. canis* by molecular means (sequence of

the gene encoding the 16S rRNA combined with multilocus enzyme electrophoresis).

1.2.5 Treatment, Control and Prevention

Drugs are effective in treating swine dysentery and intestinal spirochetosis in swine include organic arsenicals, tylosin, gentamicin, nitrofurazone, virginiamycin, and lincomycin. These have been used at low prophylactic levels, as drugs used routinely to prevent the disease will ultimately lose their effectiveness. Metronidazole is the recommended treatment for dogs with intestinal spirochetosis.

2 *Mycoplasma*

The study of *Mycoplasma* can increase the understanding of chronic diseases. As both have an extracellular and intracellular pathogen, a better comprehension of the *Mycoplasma's* virulence mechanisms will provide fresh understanding of how to diagnose and combat this pathogen.

2.1 Descriptive Features

2.1.1 Cell Structure

The cell morphology of *Mycoplasma* is extremely pleomorphic. There are different cell shapes include spherical, ring-shaped, pear-shaped, spiral-shaped, and filamentous forms. Sometimes, cells appear as chains of beads, and this is caused by the asynchronized genomic replication and cell division. The diameter of the spherical form is 0.3-0.8μm. Gram method can hardly stain *Mycoplasma*. It is preferred to choose Giemsa, Castaneda, dienes and new methylene blue to stain.

2.1.2 Genome Structure

It is thought that *Mycoplasma genitalium* has the smallest genome of any self-replicating organism, only 580,070bp long with just 470 open reading frames. Its overall G+C content is 32%. The lowest G+C content regions are around the origin of replication. The genome of *Mycoplasma pneumoniae* is much longer at 816kb, containing 209 open reading frames in the additional 236kb, providing it 679 open reading frames in total. *Mycoplasma hyopneumoniae* 232 is 892,758bp long with 28.6% of G+C mol%. It has 53 ORFs and is the largest family of the 34-member ABC transporter family. *Mycoplasma mobile* 163K is 777,079bp long with 517 ORFs and owns an extremely low DNA G+C contents of 24.9% and most reduced set of tRNAs of any reported organism. *Mycoplasma mycoides* subsp. *mycoides* SC str. PG1 is 1,211,703bp long with the lowest G+C content (24%) and the highest density of insertion sequences (13% of the genome size) among all sequenced bacterial genomes. This organism can

lead to contagious bovine pleuropneumonia (CBPP), a severe respiratory disease in cattle that spread very rapidly, especially in developing countries. *Mycoplasma penetrans* HF-2 is 1,358,633bp long with an average G+C content of 25.7%. This organism is known to infect people with HIV-1 infection, while it can cause even more damage to the immune system. *Mycoplasma pulmonis* is 963,879bp long with a G+C content of 26.6%. It can cause murine respiratory mycoplasmosis (MRM) and genital infections. *Mycoplasma gallisepticum* R is 996,422bp long with an overall G+C content of 31%. Chronic respiratory disease in chickens and other avian species can be caused by *Mycoplasma gallisepticum* R.

The phylogeny of mollicutes is interesting as regards the degeneration of the genome. During their evolutionary history, there have occurred multiple reductions in genome size, and the usual genetic code has been adjusted, so the overall rate of evolution uncharacteristically is high. One possible reason for the reduction in genome size is the evolution of mollicutes into strict parasites making much of their metabolic machinery obsolete. *Mycoplasma* also has unique use of the amino acid codon UGA, as they use it as an additional codon for tryptophan, while other organisms use it as a stop codon.

Researchers hope to increase understanding of the pathophysiology of the pathogen in sequencing these genomes through confirming virulence factors. It will also be helpful in the identification of protective antigens for developing a vaccine to combat the parasite. In addition, it will also be useful in forming the definition of a minimal cell, as the genomes of many mycoplasmas are stripped down to the bare bones.

2.1.3 Metabolism

Attribute to seriously degraded genome, they cannot perform many metabolic functions, such as cell wall production or synthesis of purines. As stripped down such organisms, they are considered the perfect model of the minimalist cell. It means that they are thought to contain the minimum machinery necessary for survival and are considered the model organisms for the essential functions of all living cells. The *Mycoplasma* cell has a minimum set of organelles involving a plasma membrane, ribosomes, and a highly coiled circular chromosome.

To bind to the host cells is essential for *Mycoplasma*, so they have developed special tip organelles for this purpose. There have a significant percentage of genes in their genome devoted to this important function. Most species of *Mycoplasma* are extracellular pathogens, and their tip organelle which has a high concentration of adhesins can adhere to the eukaryotic cell. The organelle contains a central rod-shaped structure and is fettered by the cell membrane. *Mycoplasma* can penetrate their host cell using their tip organelle when they act as intracellular pathogens. The lack of a rigid cell wall may also help facilitate contact between mycoplasma and their host cell, by creating the possibility of fusion between the two membranes. This would enable the exchange of membrane and cytoplasmic components.

2.1.4 Ecology

As *Mycoplasma* are strictly parasites, they cannot live freely. The host that they parasitize is a wide range of organisms including humans, plants, animals, and insects. *Mycoplasma* grows very slowly with a generation time ranging up to 9h even under perfect condition in some species. Besides, they have a very long lag phase, so it is common that it may take an entire week before colonies become visible on agar plates. Due to their degraded genome and inability to perform basic functions, *Mycoplasma* relies on their host for most of their nutrition.

Many species of *Mycoplasma* are commensal, and can live innocuously with their host as part of the natural flora of the body, so they have no detrimental effects. But when *Mycoplasma* acts as pathogens, they rarely cause chronic but mild infections if ever, kill their host. They are usually surface parasites, although several species, including the aptly named *M. penetrans*, are intracellular pathogens.

2.1.5 Pathology

Several chronic diseases, including chronic fatigue syndrome, fibromyalgia syndrome, gulf war syndrome, and rheumatoid arthritis have been found definite connection with *Mycoplasma*, while the connection remains obscure. Chronic fatigue syndrome (62.9%) and fibromyalgia syndrome (50%) were detected *Mycoplasma* spp. in a study. It also found that more than 50% of patients with rheumatoid arthritis had mycoplasmal infections and 36% of these patients had multiple infections by different species of *Mycoplasma*.

The most common symptoms linked with mycoplasmal infections include night sweats, chronic fatigue, skin rashes, intermittent fevers, increased dermal sensitivity, joint and muscle pain, swelling and reduced mobility of joints, heart palpitations, pain and arrhythmia, stomach cramps and regurgitation, loss of vision, double vision, etc. Although there is a clear connection between *Mycoplasma* infection and the above mentioned diseases, it has not been determined which the precursor is. *Mycoplasma* may cause these various diseases, or it just may be an opportunistic pathogen that colonizes a host with a weak immune system, and leads to a secondary infection.

2.1.6 Classification

Higher order taxa: Bacteria, Firmicutes, Mollicutes, Mycoplasmatales, Mycoplasmataceae, *Mycoplasma*.

2.2 *Mycoplasma hyopneumoniae*

2.2.1 Description and Significance

Mycoplasma hyopneumoniae is a small genome type of bacteria without a cell wall. It is detected throughout respiratory systems in pigs, such as the cilia of cells in lungs,

and causes coughs and more specifically, enzootic pneumonia (EP). EP is known to cause weight loss in pigs, causing millions of dollars of losses in the livestock industry.

To analyze how much the bacteria are involved in respiratory diseases in pigs and antibodies against the bacteria, it is necessary to sequence the genome of *Mycoplasma hyopneumoniae.*

Nasal swabs are used to isolate strains of *Mycoplasma hyopneumoniae* from infected pigs. The organisms are then isolated in cultures and detected using PCR. Nested PCR purifies samples to sequence and align them, and then to detect the presence of the mycoplasma. After detecting strains, it is important to analyze how much vaccination that these strains affect. It is quite difficult for the growth of *Mycoplasma hyopneumoniae* in the laboratory as the bacteria require specific nutrients.

2.2.2 Genome Structure

Mycoplasma hyopneumoniae 232 has a complete genome. It is circular and the length is 892,758 nucleotides with the G+C content of 28%. It has 727 genes. The chromosome is its replicon type.

2.2.3 Metabolism

As *Mycoplasma hyopneumoniae* lacks a cell wall, it cannot perform many metabolic processes seen in other bacteria. However, it just contains the minimum amount of organelles for live along with a small genome. Like all other mycoplasmas, *Mycoplasma hyopneumoniae* only relies on its host (the cilia on lung cell, for nutrition and energy *Mycoplasma hyopneumoniae* is known to secrete toxic by-products rather than toxins, as found in other disease-causing bacteria).

2.2.4 Ecology

Mycoplasma hyopneumoniae adheres to the cells in the lungs of pigs. Since there are a few cases of EP per herd of pigs, there have detrimental effects on the economy and the livestock industry. Other species of *Mycoplasma* including *M. mycoides* have also been recognized to cause diseases in farms and affect the agriculture industry as well.

2.2.5 Pathology

Mycoplasma hyopneumoniae is a mycoplasma microorganism and does not contain a cell wall. This microorganism uses the cilia of epithelial cells lining the lungs as its host. Once adhered, it can lead to the stop of cilia function, and eventually the death of epithelial cells, causing lesions found in pigs infected with enzootic pneumonia (EP). Symptoms of EP include reduction of growth in pigs and respiratory problems, such as coughing.

2.2.6 Application to Biotechnology

Mildly toxic by-products secreted by *Mycoplasma hyopneumoniae* can stop the cilia on epithelial cells in lungs from beating. However, antibiotics of target cell walls are ineffective against all *Mycoplasma* as they have no cell walls.

2.3 *Mycoplasma gallisepticum*

2.3.1 Description and Significance

M. gallisepticum is a bacterial pathogen that can cause chronic respiratory disease. It is detected in the poultry and other avian species' respiratory system at 37℃. The pathogen has a flask-shaped appearance, blebs at the poles of the cell and specialized tip-like organelles but without a cell wall. *M. gallisepticum* can survive from a few days to months according to the environment. *M. gallisepticum* can survive one to four days on cotton, rubber, hair and feathers. Under dry conditions, it can survive 61 days at 4℃ and 10-14 days at 20℃. The pathogenic mechanism of virulence of the bacterium is determined by the genome sequencing. A clone of the strain Rlow designated Rlowc2 (isolated from the respiratory system of chickens and the respiratory organs, eyes and brains of avian species) was used to sequence the genome of *M. gallisepticum*.

2.3.2 Genome Structure

The circular DNA genome of *M. gallisepticum* is 996,422bp long with a G+C content of 31mol%. 742 coding DNA sequences (CDSs), 91% coding density, have been determined of the 996,422bp. 469 coding DNA sequences' functions have been determined, 159 CDSs are conserved hypothetical proteins and the remaining 123 CDSs are hypothetical proteins. 33 tRNA genes were identified in the genome accompanying a polypeptide release factor prfA (similar to DNA transcription UAA and UAG stop codons). *M. gallisepticum*'s genome contains two 16S rRNA genes.

Just like *M. pneumoniae* and *M. genitalium*, *M. gallisepticum*'s genes within the OriC region are also not conserved. Genes in the OriC region include *gyrA*, *gyrB*, *dnaJ*, *dnaN*, *soj* (upstream of *dnaA*), and ABC transporters, *rpl34* and *rpnA* (downstream of *dnaA*). The origin of replication contains an increased number of A-T base pairs (characteristic of prokaryotes) found between the *dnaN* and *soj* genes.

The genome of *M. gallisepticum* contains VIhA known as pMGA lipoproteins which make up the largest family of genes. This family is noted as the vIhA family that generates an antigenic variation in chickens and avian species, therefore, it is important for the bacteria to evade the hosts' immune response. The vIhA family consists of 43 genes which make up a total 43kb of the bacterial genome.

2.3.3 Cell Structure and Metabolism

M. gallisepticum lacks a cell wall. The filaments that it contains make the bacterium to attach to the host (erythrocyte) for colonization. The granular nuclear material, cylindrical ribosomal arrays and surface blebs of the bacterium can be seen by electron microscopy as a "unit" membrane.

M. gallisepticum's cell membrane is 110Å containing intramembranous particles of 5-10nm in diameter. The membrane consists of two 30Å lines separated by a 50Å area. There are many different membrane-associated proteins in the membrane: ATP binding proteins, SecA, FtsY, and proteases. It is found that 24 ATP-binding proteins in the membrane are associated with the ABC transporter which makes up the second largest gene family of *M. gallisepticum*. This family constitutes one-third of the total 75 proteins that are predicted to be involved in biomolecule transport. The membrane proteins of SecA, SecE, SecY, YidC and a trigger factor are involved in membrane-associated protein secretion. FtsY and Ffh are involved in the signal recognition particle pathway. Several transmembrane domains of the VIhA family include GDSL motif, zinc metalloproteases, and lectin-binding motifs, which suggests that some membrane-associated proteins can bind portions of sugar for the purpose of cytoadherence and nutrient uptake.

The nuclear material of the bacterium contains the DNA which is found in a centrally located region as a fibril containing granules smaller than ribosomes. The thickness of fibril is 30Å. The anterior of the cell are made up of bleb shaped structures, measuring 800-1,300Å, not including the bounding membrane.

Dihydrolipoamide transacetylase and pyruvate dehydrogenate enzymes produced by *M. gallisepticum* are part of the multienzyme pyruvate dehydrogenase complex (PDHC). Pyruvate oxidation occurs with these enzymes, then producing acetyl-CoA and generating ATP, which is the bacteria's source of energy. The ATP binding cassette transporters is also included, and it is an important transport system for acquiring many precursors needed for the bacteria's survival.

2.3.4 Ecology and Pathology

M. gallisepticum can cause chronic respiratory disease in poultry, which is most commonly found in chicken, and other avian species. The transmission of the bacterium is through direct contact of infected species or the environment (dust, soil, drinking water, food, etc.), leading to loss in poultry and egg production.

Chronic respiratory disease is caused via cyt adherence to sialic acid residues of the tracheal lumen epithelial cells to the tip-like structures of *M. gallisepticum*'s virulent R strain. The two genes, *crmA* and *gapA* are essential to the cyt adherence of *M. gallisepticum*. The prerequisite for cytopathogenicity is attachment to the epithelial cells, which eventually causes the fusion of the cell membrane with *M. gallisepticum*. The

penetration of *M. gallisepticum* into cells only use 5min after infection and the number of intracellular mycoplasmas increases in 24h. Other bacterial and viral pathogens can infect erythrocytes after secondary infection of the tracheal epithelium through ciliostasis and deceleration. The cause of this pathogen's virulence is through the family of genes *vlhA*, a virulent factor includes the protein Lpd, from the family of genes *vlhA*, which causes this colonization and pathogenesis. Nasal discharge, decreased egg production, tracheal rales and weight loss are symptoms of this disease

3 *Rickettsia*

3.1 Description and Significance

Rickettsia are well known as pathogens. *Rickettsia conorii* causes Mediterranean spotted fever in humans and is transmitted via the contact with infected brown dog ticks. Other *Rickettsia* involve *Rickettsia prowazekii* causing typhus, *R. rickettsii* causing Rocky Mountain spotted fever, and *Rickettsia akari* causing rickettsial pox. Besides, *Rickettsia prowazekii* has similar genome to mitochondrial genomes; phylogenetically, *R. prowazekii* is the most closely related to the mitochondria so far. Tick hemolymph cells are infected with *R. rickettsii*.

3.2 Genome Structure

The genome of *Rickettsia prowazeki* is 1,111,523bp in length and contains 834 protein-coding genes. It has no genes for anaerobic glycolysis and genes for the biosynthesis and biosynthesis regulation of amino acids and nucleosides in free-living bacteria similar to mitochondrial genomes. Unlike the mitochondrial genome, the genome of *R. prowazekii* has a complete set of genes encoding for the tricarboxylic acid cycle and the respiratory-chain complex. Genomes of *Rickettsia* and mitochondria are all small, highly derived, "products of several types of reductive evolution".

3.3 Cell Structure and Metabolism

Rickettsia are obligate intracellular pathogens. They are dependent on entry, growth, and replication within the cytoplasm of a eukaryotic host cell. With the lysis of the host cell, then the rickettsial progeny is released to start a new infection cycle. Generally, the infection doesn't lead to a complete shutdown of the host machinery. Apparently, "vigorous host responses" generally can clear the rickettsial pathogens. Conversely, the host's immune responses can also lead to a persistent subclinical infection even for years after primary infection and/or antibiotic treatment. One theory that explains how rickettsia survives in host cells is that the "suppression of the antimicrobial activities of

the eukaryotic target cells, specifically monocytes/macrophages".

3.4 Ecology

Rickettsia are commonly carried by anthropods like ticks, mites, lice, or fleas. Wild rodents that have been infected with a rickettsia by this louse can provide another way for animals and humans to contract the bacteria. Different forms of *Rickettsia* and the diseases that they cause can be found all over the world. While some diseases are worldwide, others are localized to one general place or area, for example, oriental spotted fever caused by *R. japonica* is localized in Japan.

3.5 Pathology

R. prowazekii is mostly known for being the agent of epidemic, louse-borne typhus in humans. Approximately 20-30 million humans were infected during the World War Ⅰ and another few million were killed after the World War Ⅱ. Typhus is listed as one of the main epidemic diseases of human history, a truly apocalyptic pestilence that happens in the wake of wars, famine, and other human misfortune. Rocky Mountain spotted fever, which is caused by *R. rickettsii*, is the most severe rickettsial illness which is a tick borne. The American dog tick (*Dermacentor variabilis*) and the Rocky Mountain wood tick (*Dermacentor andersoni*) are the primary carrying ticks. Patients infected with *R. rickettsii* generally present nonspecific symptoms like fever, nausea, vomiting, muscle pain, lack of appetite, and severe headache with an incubation period of 5-10 days after an infected tick bite. Later symptoms include rash, abdominal pain, joint pain, and diarrhea. The most common components of clinical diagnosis usually are fever, rash, and a previous tick bite. Rocky Mountain spotted fever is treated by a tetracycline antibiotic like doxycycline. People are thought to have long lasting immunity against reinfection once they had the disease.

3.6 Classification

Rickettsia belong to higher order taxa: Bacteria, Proteobacteria, Alphaproteobacteria, Rickettsiales, Rickettsiaceae.

4 *Chlamydia*

4.1 Descriptive Features

4.1.1 Description and Significance

Chlamydia has plagued humanity as the most commonly contracted STD, caused by

Chlamydia trachomatis. Combat the pathogen is significantly increased with the sequencing of the *C. trachomatis* genome.

4.1.2 Genome Structure

With the hope that a comparison between two genomes of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* will significantly enhance the understanding of both pathogens, the sequences of *Chlamydia trachomatis* and *Chlamydophila pneumoniae* have been determined. Identification of particular genes to one or the other species could reveal mutually exclusive biological, virulence, and pathogenesis capabilities, while genes both the two have owned will help researchers to understand better the metabolic capabilities necessary for living in a human host.

The genome of *Chlamydia trachomatis* is 1,042,519bp long and 894 predicted protein-coding sequences. The genome of *Chlamydophila pneumoniae* is 1,230,230bp long with 1,073 open reading frames. There are 186 genes of the *Chlamydophila pneumoniae* genome which are not homologous to sequences of the *C. trachomatis*, and *C. trachomatis* has seventy genes that are unrepresented on the *Chlamydophila pneumoniae* genome.

4.1.3 Cell Structure and Metabolism

Chlamydia trachomatis and *Chlamydophila pneumoniae* are Gram negative (classified as such causes, they are difficult to stain, but are more closely related to Gram negative bacteria), aerobic, intracellular pathogens with the typical shape of coccoid or rod and require growing cells to remain viable. *Chlamydia* cannot synthesize ATP by itself and grow on an artificial medium. Therefore, it was thought to be a virus in the past. The unique cell wall of *Chlamydia trachomatis* is thought to be one of its virulence factors, as it inhibits phagolysosome fusion in phagocytes. The cell wall contains an outer lipopolysaccharide membrane but without peptidoglycan. Instead, it contains cysteine-rich proteins which have a similar function of peptidoglycan. This unique structure of the cell wall allows intracellular division and extracellular survival.

4.1.4 Ecology

Chlamydia has a very unique life-cycle (Fig. 14-3). It has alternates between a non-replicating, infectious elementary body, and a replicating, non-infectious reticulate body. Dispersal form of the pathogen is the elementary body, which is similar to the spore. The bacterium induces its own endocytosis upon when contacting with potential host cells. Once entering a cell, the elementary body will germinate as the result of interaction with glycogen, and then converts into its vegetative and reticulate form. The reticulate form divides every 2-3h, and the incubation period is 7-21 days in its host. After division, the pathogen is back to its elementary form and the exocytosis of cell

released it. They are extremely sensitive to temperature, and must be refrigerated at 4℃ as soon as a sample is achieved.

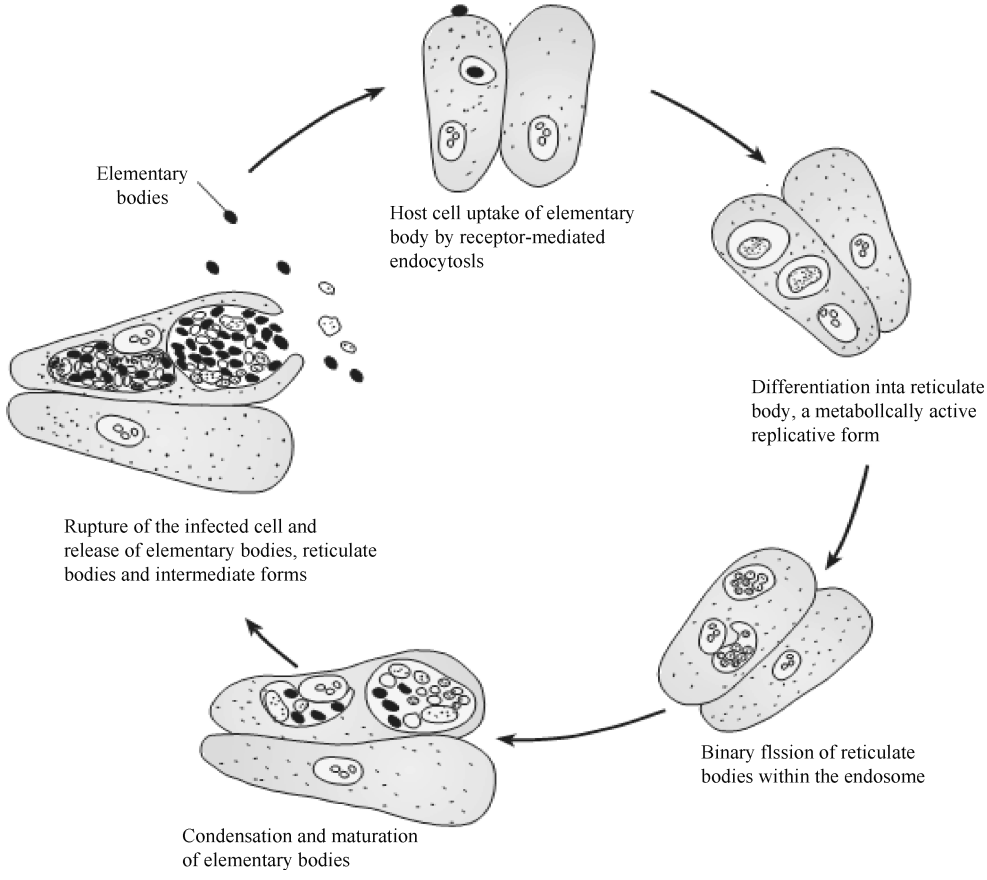


Fig. 14-3 The life cycle of chlamydiae (Markey *et al.*, 2013)

4.1.5 Pathology

Chlamydophila pneumoniae is one of the main causative agents of pneumonia, and bronchitis just as its name. Besides, it has also been associated with artherosclerosis, and multiple sclerosis. *Chlamydophila pneumoniae* is not the STD form of chlamydia and the most commonly known is *C. trachomatis*. *Chlamydophila pneumoniae* was first linked to heart disease in 1988 and *Chlamydophila pneumoniae* antibodies were identified in people with artherosclerosis. Although this connection was confirmed by subsequent studies, many studies also denied the connection. As it is an intriguing idea, more extensive and further research needs to be done. Another interesting connection is of *Chlamydophila pneumoniae* and multiple sclerosis. To date, no studies have confirmed this connection, but many patients afflicted with multiple sclerosis have

tested positive for chlamydia in their spinal fluid. *Chlamydophila pneumoniae* is not a sexually transmitted disease, and thus there is no connection between STDs and either artherosclerosis, or multiple sclerosis. While *Chlamydia* trachomatis is the more well-known of the two pathogens, as it causes the most common STD (*Chalmydia*) in the world, and has been connected with blindness and infertility. It is believed that over 50 million new cases occurred worldwide annually. The site of infection determines the symptoms of chlamydia. As it is transmitted through infected secretions, and mainly infects mucosal membranes, especially the cervix, rectum, urethra, throat and conjunctiva (eyes). Trachoma, one of the primary causes of blindness is caused by *C. trachomatis*. It can be transmitted through the eye discharge, or contact with eye-seeking flies, and then causes an inflammatory reaction of the eye, which will produce scar tissue on the conjunctiva. This compels the eye lid to turn inward, where eye lashes can rub the cornea to cause scarring.

The most dangerous thing about chlamydia is that 75% of women and 50% of men are asymptomatic when transmitted sexually, and are completely conscious that they are infected. *Chlamydia* infection is a major cause of infertility in both women and men. Women are susceptible to pelvic inflammatory disease (PID), and ectopic pregnancy. 40% of women with chlamydia will evolve PID, while 20% will become infertile and 9% will have a potentially fatal ectopic pregnancy of that 40% women. Recently, there has research shown that women infected with chlamydia are likely to contract HIV (3-5 times more). For men, infections often start from the urethra, with urethritis, and can develop in the upper genital tract, causing epididymitis and prostatitis. Besides, it can cling to sperm, which increase the risk of transmission to women. It has been indicated that chlamydia can spoil sperm health, leading to male infertility. When infected with chlamydia, a dramatic decrease in motility, and viability of sperm usually occur.

Chlamydophila psittaci has also connected with ocular adnexal lymphoma (OAL), which can affect the tissues surrounding the eye. A study showed that *Chlamydophila psittaci* DNA had appeared in 80% of the ocular adnexal lymphoma samples, in 12% of the benign lymphoid tissues, and in none of the healthy human samples. The lymphoma samples did not contain *C. trachomatis* or *Chlamydophila pneumoniae* as well. *Chlamydophila psittaci* DNA was found in the white blood cells of lymphoma patients with the percent of 43% (9 of 21), which indicates as the authors noted that *Chlamydophila psittaci* infection persisted over time in a high proportion of patients. Several of these patients received treatment of antibiotics to control *Chlamydophila psittaci*, and it leads to a remission of the lymphoma.

Recently, it has been found that *Chlamydia* contains a small needle-like projection called a type Ⅲ secretion apparatus. It serves as a conduit to make the bacteria connect to the cytoplasm of the host cell, which means that *Chlamydia* can inject

proteins directly into the cytoplasm of the cell with avoiding lysosomes. Scientists have also discovered that the chlamydia-infested vacuole divert lipids into itself rather than another compartment of the host cell.

4.1.6 Classification

Higher order taxa: Bacteria; Chlamydiae/Verrucomicrobia group; Chlamydiae; Chlamydiae (class); Chlamydiales; Chlamydiaceae; *Chlamydia*. Species: *Chlamydia muridarum*, *Chlamydia trachomatis*, *Chlamydophila pneumoniae*.

4.2 Psittacosis

In medicine (pulmonology), psittacosis (also called parrot disease, parrot fever, and ornithosis) is a zoonotic infectious disease caused by *Chlamydophila psittaci* and contracted not only from parrots (macaws, cockatiels and budgerigars), but also from pigeons, sparrows, ducks, hens, gulls and many other species of bird. The occurance of infection in psittacine birds is considered to be higher than in canaries and finches.

In certain contexts, the word "psittacosis" is considered to be that disease is carried by any species of the Psittacidae, whereas "ornithosis" is the disease carried by other birds.

In birds, *Chlamydophila psittaci* infection is referred to as avian chlamydiosis (AC). The bacteria can be shed through feces and nasal discharges of infected birds, which can reserve infectious for several months. Many strains keep actionless in birds until activated under stress. Birds are significantly suitable, highly removable vectors for the spread of chlamydial infection as they have access to the detritus of infected animals of all sorts.

4.2.1 Symptoms

Chlamydophila psittaci is often systemic and infections in birds, it can be inapparent, severe, acute or chronic with intermittent shedding. Symptoms include inflamed eyes, hard breathing, watery droppings and green urates.

4.2.2 Diagnosis

Symptoms may be as the evidence for the initial diagnosis. Actually, diagnosis is usually confirmed via an antigen and antibody test. A PCR-based test is also practicable. Although these tests can confirm psittacosis, false negatives possiblely occur and so a combination of clinical and lab tests is necessary before giving the bird a clean bill of health.

4.2.3 Epidemiology

Although it can be transmitted via feathers and eggs, the droppings of another infected bird are the prominent infection path, and is typically either inhaled or ingested.

Mucosal epithelial cells and macrophages of the respiratory tract can be infected by *Chlamydophila psittaci* strains in birds. Eventually, septicaemia develops and the bacteria settle on epithelial cells and macrophages of most organs, conjunctiva, and gastrointestinal tract. It can also be transmitted in the eggs. Severe symptoms will be triggered by stress, resulting in rapid deterioration and death. *Chlamydophila psittaci* strains have similar virulence and grow readily in cell culture. They have 16S rRNA genes that differ by <0.8%, and belong to eight known serovars. All of them are considered to be transmissible to humans. *Chlamydophila psittaci* serovar A is endemic among *Psittacine* birds and can cause sporadic zoonotic disease in humans, other mammals, and tortoises. Serovar B is endemic among pigeons, it while it has also been confirmed that Serovar B causes the abortion of a dairy herd. Serovars C and D are occupational hazards for slaughterhouse workers and people in contact with birds. Serovar E isolates (known as Cal-10, MP or MN) were isolated from a variety of avian hosts worldwide, although they were connected with the 1920s-1930s outbreaks in humans, it has not been identified the specific reservoir for serovar E. The M56 and WC serovars were isolated during outbreaks in mammals.

4.2.4 Treatment

Antibiotics, such as doxycycline or tetracycline via drops in the water, or injections are the common treatment, and many strains of *Chlamydophila psittaci* are susceptible to bacteriophage.

Review Questions

1. Term explanation: spirochaetae, mycoplasma, rickettsia, chlamydia, axial flagella, elementary body, reticulate body, inclusions.
2. What are spirochaetae, mycoplasma, rickettsia and chlamydia which has pathogenicity?
3. Describe the classification status of spirochaetae, mycoplasma, rickettsia and chlamydia.
4. Describe the basic process of microbiology diagnosis for the diseases caused by spirochaetae, mycoplasma, rickettsia and chlamydia, respectively.
5. Describe the morphology, structure and dyeing properties of spirochaetae, mycoplasma, rickettsia and chlamydia, respectively.
6. Describe the culture characteristics of spirochaetae, mycoplasma, rickettsia and chlamydia, respectively.
7. How to distinguish spirochaetae, mycoplasma, rickettsia, chlamydia from bacteria?
8. Describe the pathogenesis of spirochaetae, mycoplasma, rickettsia and chlamydia, respectively.

Ⅱ. MYCOLOGY

Chapter 15 Mycology

Synopsis

Fungi belong to eukaryotic microorganisms, which are widely distributed. Fungi have a large number of species and many kinds, and have complicated classification systems. In general, fungi can be divided into yeast, mould and basidiomycetes, and can grow with asexual reproduction and sexual reproduction. Some members of fungi can cause infection or poisoning in humans and animals. Yeasts are usually single cells. The pathogenic yeasts mainly include *Histoplasma*, *Cryptococcus* and *Candida*, etc. The structure of mould contains mycelium (hypha) and spores. The mycelium forms the colony, while spores have different morphological characteristics. In humans and animals, the infection mold mainly includes *Pneumocystis*, *Dermatophytes*, *Aphanomyces*, the poisoning mold mainly include *Penicillium*, *Fusarium*, *Claviceps*, etc. The infection and poisoning mold mainly include *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus versicolor*, etc. The toxins produced by mold are toxic and carcinogenic roles to humans and animals. Fungal diseases can be diagnosed by microbiology, molecular biology and other methods, while toxins must be detected in toxic diseases. Several methods include changing conditions to prevent the reproduction of fungi and disusing musty feed or food, are mainly used to control fungal diseases.

Mycology is also named fungi. It is reported that there are more than 50,000 species of fungi, but most of them are beneficial to human and animal. They reside in nature and are essential in breaking down and recycling organic matter. Some fungi can greatly enhance our quality of life by contributing to the production of food and spirits. Other fungi have served drugs by providing useful bioactive secondary metabolites, such as antibiotics (e.g., penicillin, streptomycin) and immunosuppressive drugs (e.g., cyclosporine, rapamycin, tacrolimus). Fungi have been exploited by geneticists and molecular biologists as model system for the investigation of all sorts of eukaryotic processes, fungi exert their greatest economic impact as phytopathogens; the agricultural industry suffers huge crop losses every year because of fungal diseases of plants. Fortunately, only a few hundred species of fungi are proven to cause human disease, and 90% of human infections by fungi can be attributed to a few dozens of species.

1 General Properties of Fungi

Fungi are eukaryotic microorganisms (domain Eucarya) that exist widely in nature. Only about 200 of the thousands of species have been identified as human pathogens, and among these known pathogenic species, fewer than a dozen are relative to more than 90% of all human fungal infections.

The basic morphological element of filamentous fungi is the hypha and a web of intertwined hyphae is known as mycelium. The basic form of a unicellular fungus is the yeast cell. Dimorphic fungi usually take the form of yeasts in the parasitic stage and the form of mycelia in the saprophytic stage. The cell walls of fungi consist of nearly 90% carbohydrate (chitin, glucans, and mannans) and fungal membranes are rich in sterol types not found in other biological membranes (e.g., ergosterol). Filamentous fungi reproduce either asexually (mitosis), by hyphae growth and tip extension, or with the help of asexual spores. Yeasts reproduce by a process of budding. Cell walls of fungi consist of nearly 90% carbohydrate (chitin, glucans, cellulose and mannans) and fungal membranes are rich in sterol types not found in other biological membranes, on the other hand, they can produce sexual spores. Fungi imperfecti or deuteromycetes are the designation for a type of fungi in which the fructification forms are either unknown or missing completely.

1.1 Definition and Taxonomy

Fungi are microorganisms in the domain eucarya. They show less differentiation than plants, but a higher degree of organization than prokaryotes bacteria (Table 15-1). The kingdom of the fungi (Mycota) comprises more than 50,000 different species, but only about 200 of which have been recognized as human pathogens, and only about a dozen of these "pathogenic" species cause 90% of all human mycoses. Many mycotic

Table 15-1 Some differences between fungi and bacteria (Kayser *et al.*, 2005)

Properties	Fungi	Bacteria
Nucleus	Eukaryotic; nuclear membrane; more than one chromosome; mitosis	Prokaryotic; no membrane; nucleoid; only one "chromosome"
Cytoplasm	Mitochondria; endoplasmic reticulum; 80S ribosomes	No mitochondria; no endoplasmic reticulum; 70S ribosomes
Cytoplasmic membrane	Sterols (ergosterol)	No sterols
Cell wall	Glucans, mannans, chitin, chitosan	Murein, teichoic acids (G^+), proteins
Metabolism	Heterotrophic; mostly aerobes; no photosynthesis	Heterotrophic; obligate aerobes and anaerobes, facultative anaerobes
Size, mean diameter	Yeast cells: 3-5-10μm; Molds: indefinable	1-5μm
Dimorphism	In some species	None

infections are relatively harmless, for instance the dermatomycoses. In recent years, however, the increasing numbers of patients with various kinds of immune defects have resulted in more life-threatening mycoses.

The taxonomy of the fungi is mainly based on their morphology. In medical mycology, fungi are classified according to practical aspects as yeasts, dermatophytes, molds, and dimorphic fungi. Molds grow in filamentous structures, yeasts as single cells and dermatophytes cause infections of the keratinized tissues (hair, skin, nail, etc.). Dimorphic fungi have both of the two forms, such as yeast cells and mycelia. Fungi are carbon heterotrophs. Saprobic or saprophytic fungi obtain carbon compounds from dead organic material whereas biotrophic fungi (parasites or symbionts) require living host organisms. Some fungi can exist in both saprophytic and biotrophic forms.

1.2 Morphology

Fungi have two basic morphological forms (Fig. 15-1).

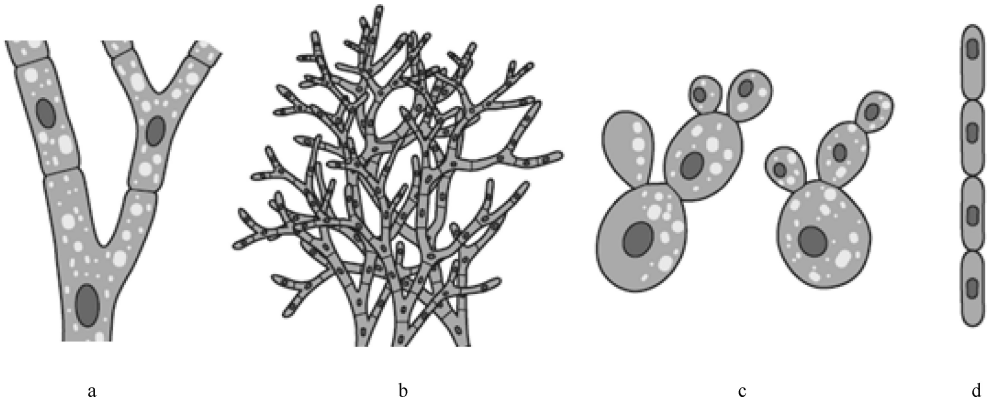


Fig. 15-1 Two basic morphological forms: hypha and yeast (Kayser *et al.*, 2005)
a. Hypha, septate, or nonseptate; b. Mycelium, web of branched hyphae;
c. Yeast form, budding (diameter of individual cell 3-5μm); d. Pseudomycelium

1.2.1 Hypha

This is the basic element of filamentous fungi with a branched tubular structure, 2-10μm in width.

1.2.2 Mycelium

This is the web or matlike structure of hyphae. Substrate mycelia (specialized for nutrition) penetrate into the nutrient substrate, whereas aerial mycelia (for asexual propagation) develop above the nutrient medium.

1.2.3 Fungal Thallus

This is the entirety of the mycelia and is also called the fungal body or colony.

1.2.4 Yeast

This is the basic element of the unicellular fungi. It is round to oval and 3-10μm in diameter. Several elongated yeast cells chained together and resembling true hyphae are called pseudohyphae.

1.2.5 Dimorphism

Some fungal species can develop either the yeast or the mycelium form according to the environmental conditions. This property was named dimorphism. Dimorphic pathogenic fungi take the form of yeast cells in the parasitic stage and appear as mycelia in the saprophytic stage.

1.3 Metabolism

All fungi are carbon heterotrophs, which mean they depend on exogenous nutrient substrates as sources of organic carbon, but with a few exceptions, fungi are obligate aerobes. Many species have the capability to maintain metabolic activity in the most basic of nutrient mediums. Known metabolic types of fungi include psychrophilic, thermophilic, halophilic, and acidophilic species. The metabolic capabilities of fungi are exploited in the food industry (e.g., in the production of wine, beer, bread, cheese, or single-cell proteins) and in the pharmaceutical industry (e.g., in the production of enzymes, antibiotic substances, citric acid, etc.). The metabolic activity of fungi can also be a damaging factor. Fungal infestation can destroy foods, textiles, wooden structures, etc. Fungi also cause many plant diseases, in particular diseases of crops.

1.4 Reproduction of Fungi

1.4.1 Asexual Reproduction

This category contains the vegetative propagation of hyphae and yeasts as well as vegetative fruits, i.e., formation of asexual spores.

Hyphae was elongated in a zone just short of the tip in which the cell wall is specifically elastic. This apical growth process can also include the formation of swellings which can develop into lateral hyphae, and they can also branch out in turn.

Yeasts reproduce by budding. This process starts from an outgrowth of the mother cell wall which can develop into a daughter cell or blastoconidium. The isthmus between the two yeast is finally cut off by the formation of septum. Some yeast reproduces in both the yeast and hypha forms.

A type of propagative form, the asexual spores, is formed in vegetative fruits. These structures show considerable resistance to exogenous pathogen and contribute fungi to spread in a natural environment. Asexual spores come in a number of morphological types: sporangiospores, conidia, blastospores, and arthrospores. These forms rarely develop during the parasitic stages in hosts, but they can be observed in cultures. The morphology of the asexual spores of fungi is one of the important identification characteristics.

1.4.2 Sexual Fructification

Sexual reproduction in fungi perfecti (Eumycetes) abides by essentially the same patterns as in the higher eukaryotes. The nuclei of two haploid partners fuse to form a diploid zygote. Then the diploid nucleus goes through meiosis to form the haploid nuclei, finally forming haploid sexual spores: ascospores, zygospores, and basidiospores. Sexual spores are rarely produced in the kinds of fungi which parasitize in human tissues.

Sexual reproduction structures are either unknown or not present in many species of pathogenic fungi, known as fungi imperfecti (Deuteromycetes).

2 Fungal Diseases

2.1 General Aspects of Fungal Disease

In all of fungal diseases, besides fungal allergies (e.g., extrinsic allergic alveolitis) and mycotoxicoses (aflatoxicosis), fungal infections are the most common fungal diseases.

Mycoses are classified clinically as follows: primary mycoses (coccidioidomycosis, histoplasmosis, blastomycoses), opportunistic mycoses (surface and deep yeast mycoses, aspergillosis, mucormycosis, phaeohyphomycosis, hyalohyphomycosis, cryptococcosis, penicilliosis, pneumocystosis), subcutaneous mycoses [sporotrichosis, chromoblastomycosis, Madura foot (mycetoma)], cutaneous mycoses (pityriasis versicolor, dermatomycosis).

Little is known about fungal pathogenicity factors. The natural resistance of the macroorganism to fungal infection is based mainly on effective phagocytosis whereas specific resistance is generally through cellular immunity. Opportunistic mycoses infect mainly this type of patients with immune deficiencies (e.g., in neutropenia). Microscopy and culturing methods are the most common laboratory diagnostic methods for fungal infections, which can detect the pathogens directly, and identify specific antibodies. Therapeutics for the treatment of mycoses include polyenes (especially amphotericin B), azoles (e.g., itraconazole, fluconazole, voriconazole), allylamines, antimetabolites (e.g., 5-fluorocytosine), and echinocandins (e.g., caspofungin). Combination of drugs is the primary method for the treatment of

fungi diseases.

2.1.1 Mycogenic Allergies

Spores of ubiquitous fungi continuously are breathed in the respiratory tract with air. These spores contain potent allergens to those who are susceptible may manifest strong hypersensitivity reactions. Based on the localization of the reaction, it can cause the following diseases, such as allergic rhinitis, bronchial asthma, or allergic alveolitis. Many of these allergic reactions are certified occupational diseases, i.e., “farmer’s lung” “woodworker’s lung” and other types of extrinsic allergic alveolitis.

2.1.2 Mycotoxicoses

Some fungi can produce mycotoxins, the well known of which are the aflatoxins produced by the *Aspergillus* species. These toxins are ingested by people with the food stuffs on which the fungi have been growing for some time. Aflatoxin B1 may contribute to primary hepatic carcinoma. This kind of disease is observed frequently in Africa and Southeast Asia.

2.1.3 Mycoses

Data on the general incidence of mycotic infections can only be approximate, because there is no requirement that they must be reported to the health authorities. It can be assumed that cutaneous mycoses are one of the most frequent infections worldwide. On the other hand, primary and opportunistic mycoses are relatively rare. Opportunistic mycoses have been increasing in recent years and decades, which reflect the fact that clinical manifestations are only observed in hosts whose immune disposition allow them to develop mycoses. The increasing numbers of patients with immune defects and a high frequency of invasive and aggressive medical therapies are the factors which can contribute to the increasing significance of mycoses. Categorization of the infections used here disregards taxonomic considerations to concentrate on practical clinical aspects.

2.1.4 Host-pathogen Interactions

The factors that determine the onset, clinical picture, severity, and outcome of a mycosis include interactions between fungal pathogenicity factors and host immune defense mechanisms. Compared with the knowledge in the field of bacteriology, it must be said that we still know little about the underlying causes and mechanisms of fungal pathogenicity. Human have the high levels of nonspecific resistance to most fungi based on mechanical, cellular, and humoral factors. Among these factors, phagocytosis by macrophages and neutrophilic granulocytes is the most importance. Close contact with fungi results in the acquisition of specific immunity, especially the

cellular immunity. However, the role of humoral immunity in specific immune defense is secondary.

2.1.5 Diagnosis

The primary concern here is identification of the pathogenic fungi.

2.1.5.1 Microscopy

Native preparation: briefly heat material under the coverslip with 10% KOH. Stained preparation: these materials include methylene blue, lactophenol blue, ink, periodic acid-Schiff (PAS), etc.

2.1.5.2 Culturing

They can be cultured on universal or selective mediums. For example, Sabouraud dextrose agar contains selective agents (e.g., chloramphenicol and cycloheximide). This medium has an acid pH of 5.6. Main identifying structures are morphological, in particular the asexual and, if present, sexual reproductive structures. Biochemical tests are used mainly to identify yeasts and are generally not as important in mycology as they are in bacteriology.

2.1.5.3 Serology

By the identification of antibodies to special fungal antigens in patient's serum, it is very difficult to explain the serological findings in fungal infections.

2.1.5.4 Antigen Detection

Specific antigens have been found in the diagnostic material in some fungal infections (e.g., cryptococcosis) by direct means using known antibodies.

2.1.5.5 Cutaneous Test

Cutaneous (allergy) tests with specific fungal antigens are effective in diagnosing a number of fungal infections.

2.1.5.6 Nucleic Acid Detection

It is helpful for rapid detection of mycotic diseases in immunocompromised patients by DNA amplification methods.

2.1.6 Therapy

At present, only a few of anti-infective agents can be useful in the treatment of fungal infections.

2.1.6.1 Polyenes

These agents can bind to membrane sterols and destroy the membrane structure.

①Amphotericin B used in systemic mycoses and fungicidal activity with frequent side effects. There is conventional galenic form and (new) various lipid forms. ②Nystatin, natamycin used in mucosal mycoses only for topical.

2.1.6.2 Azoles

These agents can disrupt ergosterol biosynthesis. Their effect is mainly fungistatic with possible gastrointestinal side effects. So, hepatic functional parameters should be monitored during therapy. ①Ketoconazole. It is one of the first azoles and no longer used because of side effects. ②Fluconazole. It can be used by oral and intravenous application on the surface or in systemic mycoses, and also in cryptococcal meningitis in AIDS patient. ③Itraconazole. It can be used by oral and intravenous application in systemic and cutaneous mycoses and also used in the treatment of aspergillosis. ④Voriconazole. It is utilized by oral and intravenous application, which has good activity against *Candida* and *Aspergillus*. But no activity against Mucorales.

2.1.6.3 Antimetabolites

5-Fluorocytosine: it can interfere with DNA synthesis (base analog), which can give by oral application in candidiasis, aspergillosis, and cryptococcosis. It is necessary to monitor the course of therapy for the development of resistance. The toxicity of amphotericin B can be reduced in combination with 5-fluorocytosine.

2.1.6.4 Allylamines

Terbinafine: it can be used by oral or topical application to treat dermatomycoses, and inhibit the biosynthesis of ergosterol.

2.1.6.5 Echinocandins

Caspofungin has been approved as a salvage therapy in refractory aspergillosis. It is also useful in oropharyngeal and esophageal candidiasis, inhibition of the biosynthesis of glucan of the cell wall.

2.1.6.6 Griseofulvin

This is an older antibiotic used in treatment of dermatomycoses. By oral application, it needs months for the treatment process.

2.2 *Candida*

Candidiasis is usually due to the parasitic yeast *Candida albicans*, which can inhabit mucous membranes of most mammals and birds. Of the more than 150 other species of *Candida* that are associated with many diverse habitats, few are associated with animal disease. Disease produced by members of the genus *Candida* usually occurs in an

immunocompromised host.

The subsequent discussion deals with *C. albicans* unless otherwise indicated.

2.2.1 Descriptive Features

2.2.1.1 Cell Morphology, Anatomy, and Composition

On routine laboratory media and mucous membranes, *C. albicans* typically grows as oval budding yeast cells (blastoconidia), 3.5μm to 6μm by 6-10μm in size. Under certain conditions of nutrition, temperature, pH, and atmosphere, yeast cells sprout germ tubes that develop into septate-branching mycelium. "Pseudohyphae" is produced by elongation of the blastoconidia and their failure to separate. *In vivo*, mycelial (a collection of hyphae) or pseudomycelial growth is associated with active proliferation and invasiveness (Fig. 15-2).

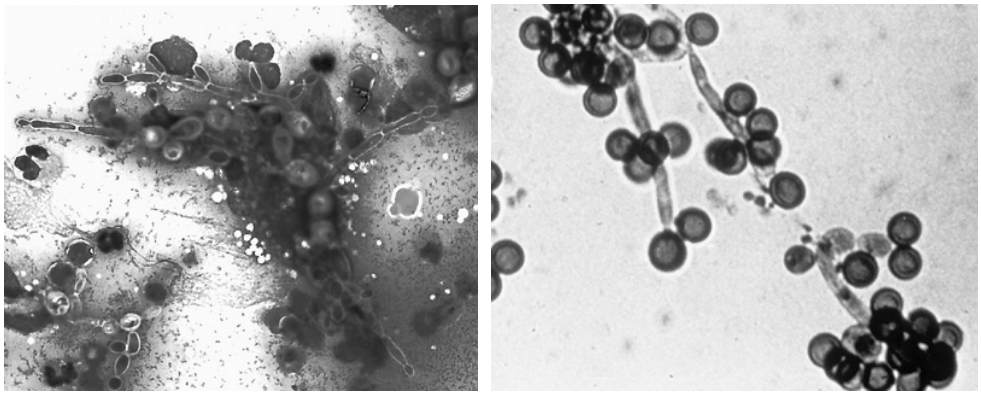


Fig. 15-2 *C. albicans* in peritoneal fluid from a dog (left) and in a culture (right)
(Scott McVey *et al.*, 2013)

The so-called chlamydospore (chlamydoconidium) is a thick-walled sphere of unknown function, which is attached by a suspensor cell to (pseudo) mycelium and essentially confined to *in vitro* growth of *C. albicans* (rarely other *Candida* spp.).

There are many glycoproteins in the cell wall; the polysaccharide portions are glucans and especially mannans, lipids and chitin can also be seen. Mannoproteins are found on the cell surface. Cellular products, such as peptidolytic enzymes, which may be the virulence factors. Two major cross reacting serogroups are found. These are termed A and B, and are identifiable with absorbed sera.

Candida can be stained with Gomori methenamine silver (GMS), periodic acid Schiff (PAS), and other fungal stains, but is usually studied in culture unstained. Polychrome stains (Wright's, Giemsa) are suitable for demonstrating it in tissue or exudate. *Candida* cells often appear Gram positive with Gram stain.

2.2.1.2 Cellular Products of Medical Interest

(1) Adhesins

Various cell wall components (chitin, lipids, and mannoproteins) have been associated with adherence to extracellular matrix proteins.

(2) Miscellaneous Products

Proteases and neuraminidases have been proven to play a role in pathogenesis. Cell wall glycoproteins have endotoxin-like activity.

2.2.1.3 Growth Characteristics

C. albicans, an obligate aerobe, can grow on ordinary media over a wide range of pH and temperature. At 25℃, creamy to pasty white colonies consisting predominantly of yeast cells can appear in 24-48h. Production of (pseudo) mycelium is influenced environmentally, but the controlling factors are disputed. Incubation temperature above 35℃, a slightly alkaline pH, and a rich, carbohydrate free fluid medium are often recommended.

Different ability to ferment or assimilate carbohydrates is the standard of species identification.

Candida can be killed by heat above 50℃, chlorine, ultraviolet light, and quaternary ammonium-type disinfectants. They withstand freezing and survive well in the inanimate environment. They are susceptible to polyene antimycotics, and usually to flucytosine and the azoles.

2.2.1.4 Reservoir

C. albicans is associated with mucocutaneous areas, particularly of the alimentary and lower genital tract, of mammals and birds. Environmental sources are important especially for other *Candida* spp.

2.2.1.5 Transmission

Most *Candida* diseases arise from an endogenous source. That is, they are caused by a commensal strain. Mastitis of bovine is infected via the teat canal by way of administered medication, during milking, by cow-to-cow spread, or from the environment.

2.2.1.6 Pathogenesis

(1) Mechanisms

Mannoprotein, chitin, and lipids are possibly the adhesions in human candidiasis; several extracellular matrix proteins have been proven to be the receptor. Germ tube formation is related with experimental pathogenicity, but the role of mycelium formation in virulence is under dispute. Proteases and neuraminidase may be virulence

factors. Cell wall glycoproteins have endotoxin-like activity.

(2) Pathology

Candidiasis affects frequently the mucous surfaces on which the agent is normally found, possibly the anterior digestive tract from mouth to stomach; it remains typically confined to areas of squamous epithelium, the genital tract, skin, and claws can be involved as well, occasional intestinal, respiratory, and septicemic infections occur.

On epithelial surfaces, candidiasis forms whitish to yellow or gray plaques, marking areas of ulceration with a varying degree of inflammation. Diphtheritic membranes may form in the gut or respiratory tract, and abscesses may form in the viscera. Granulomatous lesions are rare. Inflammatory responses are mainly neutrophilic.

2.2.1.7 Disease Patterns

(1) Birds

Avian candidiasis can affect chickens, pigeons, turkeys, and other birds. It resembles thrush of human involving the anterior digestive tract. It can be a stunting disease and cause considerable mortality in the young people.

(2) Swine

In the alimentary tract of pigs, candidiasis is regarded as ulcerative lesions that may lead to rupture.

(3) Cattle

Pneumonic, enteric, and generalized candidiasis affect calves on intensive antibiotic regimens. Candida mastitis in dairy cows is typically mild and self-limiting, ending in spontaneous recovery within about a week. Bovine abortions have been reported because of candidiasis.

2.2.1.8 Epidemiology

The common agents of candidiasis are commensal with most warm-blooded species. Disease is related to immune and hormonal inadequacies, reduced colonization resistance (a measure of the "health" of the normal flora), or intensive exposure to weakened hosts or vulnerable tissues. These conditions cause the susceptibility of infants, diabetics, subjects on antibiotic and steroid regimes, patients with indwelling catheters, and mammary glands of lactating cows.

2.2.2 Immunologic Aspects

Immunoincompetent individuals are the preferred infection targets. Polymorphonuclear neutrophil leukoeytes (PMNs) and activated macrophages form the main defense against candidiasis. The role played by opsonins (antibody or complement) is contributing to phagocytosis. Macrophages are activated by gamma interferon secreted

by TH1 cells stimulated by interleukin 12 from macrophages actively engaged in phagocytosis. In the process, there is no artificial immunization.

2.2.3 Laboratory Diagnosis

In exudate, *Candida* can be seen as yeast cells (blastoconidia) or (pseudo) hyphae. All forms are only too evident in unstained wet mounts, or in fixed smears stained with Gram's stain, Romanovsky-type stains (Wright's, Giemsa) or fungal stains, e.g., periodic acid Schiff (PAS) and Gomori methenamine silver (GMS).

C. albicans can grow well on blood or Sabouraud's agar with or without inhibitors. Other *Candida* spp. may be inhibited by cycloheximide. Yeast isolates producing (pseudo) mycelium can be considered *Candida* spp. Isolation of *Candida* spp. from mucous membranes (even in large numbers) suggests a diagnosis of candidiasis only in the presence of compatible lesions, and abundant (pseudo) hyphae forms in direct smears.

Incubation at 37℃ for ⩾2h of a lightly inoculated tube of serum will produce germ tubes if the isolate is *C. albicans*, which also produced chlamydospores on cornmeal tween-80 agar. Identification kits of yeast are commercially available.

DNA probes and tests for circulating antigen, antibody, and metabolites have had no enough trial in veterinary medicine.

2.2.4 Treatment, Control and Prevention

Correcting conditions underlying clinical candidiasis may in itself lead to recovery. In poultry, it is a traditional treatment way by drinking water dissolved copper sulfate. Nystatin can be given in feed or water. It is also used topically in mucosal and cutaneous forms of candidiasis of mammals, as are amphotericin B and miconazole. Fluconazole (preferred) or flucytosine is useful for treating dogs or cats with lower urinary tract candidiasis.

In disseminated forms, fluconazole or flucytosine are the preferred drugs. Susceptibility test is advisable. Sometimes, flucytosine-amphotericin B combined is used in humans and occasionally in animals.

2.3 *Histoplasma capsulatum* var. *capsulatum*

Histoplasma capsulatum is a dimorphic fungus existing as a mold at 25-30℃ (saprophytic phase) and as a yeast at 37℃ (parasitic phase). This fungus has three varieties: *H. capsulatum* var. *duboisii*, *H. capsulatum* var. *capsulatum*, and *H. capsulatum* var. *farciminosum*. Variety tarctmtnosum can cause epizootic lymphangitis (pseudoglanders). Varieties *capsulatum* and *duboisii* can cause histoplasmosis, a systemic fungal disease of mammals. Variety *duboisii* is found only in Africa, whereas variety capsulatum is found worldwide, and is the most common cause of histoplasmosis. In this chapter,

histoplasmosis will be discussed without regard to this varietal distinction, i.e., *H. capsulatum* var. *capsulatum*, and *H. capsulatum* var. *duboisii* will be regarded as *H. capsulatum*.

2.3.1 Descriptive Features

2.3.1.1 Morphology, Structure, and Composition

The free-living form of *H. capsulatum* consists of septate hyphae bearing spherical to pyriform microconidia 2-4μm in diameter, and "tuberculate" macroconidia, thickwalled spheroidal cells, 8μm to 14μm in diameter, studded with fingerlike projections. In animal hosts or appropriate culture, the mold becomes yeast consisting of oval, single budding cells that measure 2-3μm by 3-4μm. Asexual, ascomycetous state, *Ajellomyces capsulatus*, has been described (Fig. 15-3).

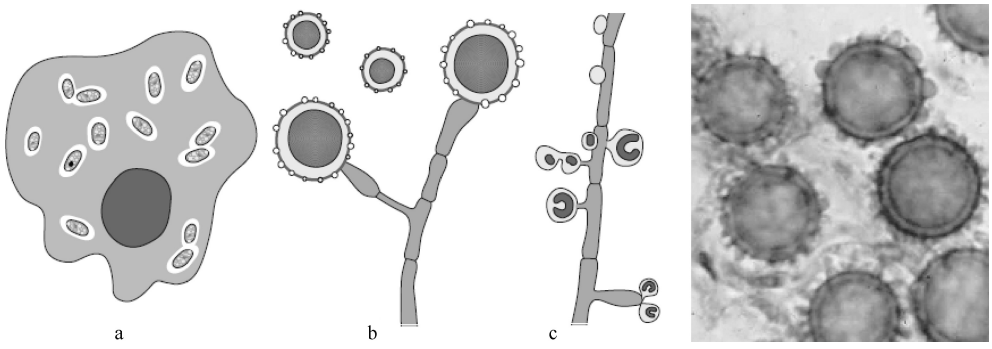


Fig. 15-3 *Histoplasma capsulatum* (left) (Kayser *et al.*, 2005), and mycelial phase (right) (Scott McVey *et al.*, 2013)
a. Yeast cells in macrophage; b. Macroconidia (7-15μm); c. Microconidia (2-5μm)

Histoplasmins, which are used in immunodiagnosis, are obtained from mycelial culture filtrates. They contain polysaccharides, with variable admixtures of glycoproteins and cellular breakdown products. Mycelial and yeast phases differ in cellular constituents. Some of which (e.g., cell wall glucan) have been related to virulence.

2.3.1.2 Cellular Products of Medical Interest

(1) Adhesins

Inhaled microconidia and yeast-phase parasitic forms are recognized by, and bind to, beta-2 integrins on the surface of neutrophils and macrophages. It is unknown what fungal structure is involved. Likewise, an unknown adhesin is related to the adherence of *H. capsulatum* to fibronectin receptors on dendritic cells. Adherence in this fashion to macrophages, neutrophils, or dendritic cells makes the fungus enter the cell without causing an effective oxidative burst and the generation of reactive oxygen and nitrogen

intermediates.

(2) Miscellaneous Products

Histoplasma capsulatum can produce a number of products which may play an important role in histoplasmosis.

1) Calcium-binding Protein (Cbp)

Yeast (parasitic) phase of *H. capsulatum* produces a calcium-binding protein (Cbp) that permits the yeast phase to grow in low-calcium environments (the phagolysosome) by efficiently chelating calcium and delivering it to the fungus. Moreover, since Cbp chelates available calcium within the phagolysosome, which impedes effectiveness of several calcium requiring lysosomal enzymes. Normal acidification of the phagolysosome is also a calcium-dependent event. *H. capsulatum* mutants which are unable to produce Cbp are avirulent.

2) H Antigen

In the previous, host immune responses to H antigen were used as a diagnostic tool in the diagnosis of histoplasmosis. Then, H antigen was proven to be a beta-glucosidase which could cause a cell mediated (protective) immune response to the yeast (parasitic) phase of *H. capsulatum*.

3) Iron Acquistion

Iron is a kind of absolute growth requirement factor for *H. capsulatum* (as it is for all life forms). *Histoplasma capsulatum* acquires iron in several ways: production of hydroxamate siderophores capable of removing iron from host iron-binding proteins (transferrin, lactoterrin): expression of a hemin-binding receptor on the surface of yeast (parasitic) forms; glutathione-dependent ferric reductase which reduces Fe^{3+} to Fe^{2+}, then releasing it from host iron-binding proteins; and several uncertain iron reductases on the surface of the yeast phase.

4) M Antigen

Host immune response to M antigen was also originally used as one of diagnostic tools in the diagnosis of histoplasmosis. Soon afterwards, M antigen was found to be a catalase enzyme, which plays an important role in the survival of the yeast phase within the phagolysosome.

5) Melanin

Melanin can be produced by *H. capsulatum*. Melanin is a kind of free radical scavenger (reducing the toxicity of hydroxy radicals, superoxides, and singlet oxygen radicals found within the phagolysosome).

6) Acidification of Phagolysosome

Normal phagolysosomes have a pH<5, a pH that optimizes the activity of many digestive enzymes found in this environment. *H. capsulatum* can increase the pH of the phagolysosome to 6-6.5, thereby reducing the activity of these lysosomal enzymes.

2.3.1.3 Growth Characteristics

H. capsulatum can grow on common laboratory media over a broad temperature range. The optimum temperature for mycelial growth is 25-30℃; the cottony aerial mycelium is white, brown, or intermediate. Pigmentation parallels abundance of macroconidia. The yeast phase needs richer media (e.g., glucose cysteine blood agar) and a temperature of 34-37℃. Growth time may take a week or more before characteristic colonies are seen.

H. capsulatum can survive at ordinary temperatures for months and at refrigerator temperature for years. It can withstand freezing and thawing and tolerates heating for more than 1h at 45℃.

2.3.1.4 Variability

H. capsulatum exists as three varieties: *duboisii*, *capsulatum*, and *farciminosum*, Varieties *capsulatum* (worldwide) and *duboisii* (Africa) can produce histoplasmosis, and variety *farciminosum* can cause epizootic lymphangitis (pseudoglanders) of equids. Though *H. capsulatum* var. *capsulatum* can be found worldwide, its central focus is in the Americas. Genetically, variety *capsulatum* is divided into 6 classes: class 1 and class 2 are found in North America; class 3 is found in Central and South America; class 4 is found in Florida (North America); and class 5 and class 6 are found in human patients with acquired immunodeficiency syndrome (AIDS) from New York (North America) and Panama (Central America), respectively.

2.3.2 Ecology

2.3.2.1 Reservoir

H. capsulatum occurs sporadically worldwide. It is found in the topsoil layers, especially in the presence of bird and bat feces, which provide both enrichment and inoculum. Birds are mainly passive carriers, whereas bats undergo intestinal infectious processes. *H. capsulatum* is suitable for neutral to alkaline soil environments with annual rainfall 35-50 inches and mean temperatures 68-90°F.

2.3.2.2 Transmission

Transmission is mostly by inhalation of microconidia or hyphae fragments, possibly by ingestion, but rarely infect through wound.

2.3.2.3 Pathogenesis

(1) Mechanism and Pathology

Microconidia, hyphae fragments (from the environment), or yeast cells (from an intraphagocytic cell environment) attach to macrophages in the lung by way of a beta-2

integrin. It is immediately phagocytosed, a minimal respiratory burst occurs, and a phagolysosome result from following fusion with lysosomes. Microconidia and hyphae elements differentiate into yeast. Survival within the phagolysosome is related to modulation of phagolysosome pH (6-6.5), secretion of M antigen (a catalase), and secretion of Cbp, melanin, and successful acquisition of iron from intracellular iron stores. The yeast multiplies within the phagolysosome, ultimately killing the cell and the release of yeast cells (which continue the cycle). Intracellular multiplication continues until an effective cell-mediated immune response occurs resulting in activated macrophages, which effectively control the multiplication of the yeast.

Early events and lesions like those of tuberculosis. Thoracic lymph nodes become enlarged, and lungs may contain grayish-white nodules. The histologic response varies from suppurative to granulomatous inflammation. Caseation necrosis and calcification are usually seen.

In disseminated disease, lymph nodes and parenchymatous organs are enlarged and may contain gross nodular lesions. There may be ulcerations of skin and mucous membranes, abdominal and pleural effusions, and involvement of the central nervous system (including eyes), skin, and bone marrow. The inflammatory exudate is composed with macrophage elements colonized by yeast cells.

(2) Disease Patterns

Histoplasmosis can occur in almost any animal species, but dogs are the most commonly affected in all kinds of animals. Dogs may develop a primary pulmonary form with fever, coughing, regional lymphadenopathy, and radiographic abnormalities. The more common form in dogs is disseminated disease, marked by lethargy, weight loss, anorexia, anemia, dehydration, and diarrhea. Hepatomegaly, splenomegaly, mesenteric lymphadenitis, and ascites may cause abdominal distention.

Similar patterns have rarely been found in cats.

Disease patterns in human patients are the same to the syndromes those described above in animal.

2.3.2.4 Epidemiology

Subclinical infections with histoplasmosis are similar in dogs, cats and humans in endemic areas. Dogs aged 2-7 years are easy to be infected histoplasmosis in early autumn (September to November), and later winter to early spring (February to April). It is reported that there is no sex difference in this disease, but the pointing breeds, weimaraners, and Brittany spaniels have the greatest risk. Disseminated histoplasmosis in human patients and dogs is found in association with immunosuppression.

2.3.3 Immunologic Aspects

Recovery and resistance are managed by cell-mediated immune responses, but

circulating antibodies have no obvious protective function. Recovery from histoplasmosis appears to confer immunity, but no vaccine is available.

2.3.4 Laboratory Diagnosis

2.3.4.1 Direct Examination

Sediment of aspirates, smears of buffy coat, and tissue impressions are stained with a Romanovsky-typs (e.g., Giemsa, or Wright's) or a fungal stain [periodic acid-Schiff (PAS), Gomori methenamine silver, or Gridley] and examined for intraphagocytic yeast cells.

In sections stained with hematoxylin-eosin, *H. capsulatum* appears as tiny dots surrounded by haloes. A duplicate fungal stain [e.g., periodic acid-Schiff (PAS), Gridley, or Gomori methenamine silver] can be helpful.

Immunosenescence has been used to identify the yeast in tissue and exudates.

2.3.4.2 Culture

Specimens are inoculated onto blood agar and Sabouraud's agar (with inhibitors) and incubated in plastic bags or jars at room temperature for up to 3 months. Colonial growth may take reddish -wrinkled before the appearance of cottony brownish to white mycelium.

Microconidia and macroconidia are demonstrated in tocopherol cotton blue wet mounts. Dimorphism must be proven by conversion to the yeast phase in culture or by injection of mice. Mice will die within the following a few weeks. Their macrophages will contain the yeast forms.

A commercially available "exoantigen" test kit furnishes prepared antisera to *H. capsulatum*, and *Blastomyces dermatitidis* to be tested against extracts of suspect cultures in an immunodiffusion agar plate, where precipitation lines will appear between extracts and their homologous antisera.

2.3.4.3 Immunodiagnosis

Histoplasmin skin and CF tests using antigens of either mycelial or yeast origins have not been reliable diagnostic means in animal infections. The position of the precipitin band in immunodiffusion tests differentiates early and recovered human cases (near serum well) from active and progressive ones (near antigen well). Limited use of these tests on animals has given unstable results. A radioimmunoassay for antigen detection has been applied.

2.3.4.4 Molecular Techniques

Molecular methods are used to identify or detect *H. capsulatum* with special primers for specific sequences contained within the DNA, e.g., genes encoding ribosomal RNA,

those encoding the M protein, or other proteins. These sequences can be amplified using the polymerase chain reaction (PCR).

2.3.5 Treatment and Control

Azoles (itraconazole, ketoconazole, fluconazole) and amphotericin B have been applied successfully in the treatment of some cases of canine histoplasmosis.

Relapses are usually seen. The prognosis for disseminated cases is grave.

2.4 *Aspergillus*

Members of the genus *Aspergillus* are ubiquitous saprophytic molds, which are the opportunistic pathogenic patterns depending on impaired, overwhelmed, or bypassed host defenses. Of about 900 species, *Aspergillus fumigatus* is the most common in animal and human infections.

2.4.1 Descriptive Features

2.4.1.1 Morphology and composition

Aspergillus, are molds consisting of septate hyphae and characteristic asexual fruiting structures that are borne on conidiophores. Conidiophores are hyphae branches originating by a foot cell in the vegetative mycelium and ending in an expanded vesicle. The vesicle is covered by a layer or layers of flask-shaped phialides, from which chains of pigmented conidia (asexual reproductive units) arise. They give the fungal colony its color (Fig. 15-4).

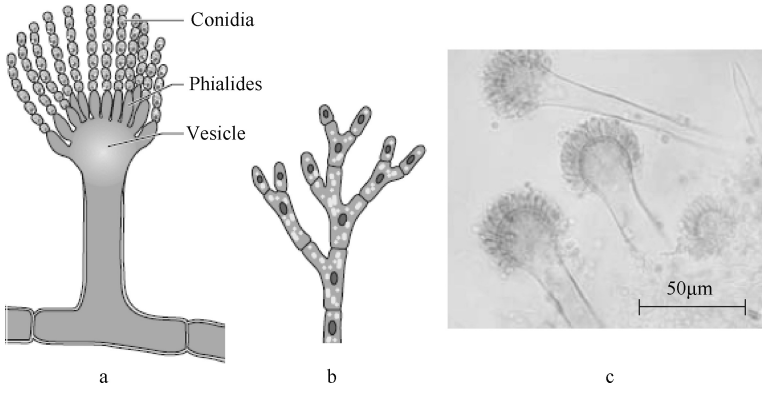


Fig. 15-4 *Aspergillus fumigatus* (Kayser *et al.*, 2005)
a. Conidiophore with conidia (2-5μm); b. Y-branched, septate hyphae (1.5-8μm);
c. Native preparation, the conidia have fallen off

In tissue, only hyphae are seen. In aerated cavities (e.g., air sacs, nasal passages, cavitary lesions), fruiting structures may be found.

Fruiting bodies are important diagnostic features of *Aspergillus* spp. by which species are identified.

2.4.1.2 Cellular Products of Medical Interest

(1) Adhesins

Members of *Aspergillus* can produce many surface proteins (conidial and hyphal) which bind to extracellular matrix proteins (fibronectin, laminin, fibrinogen, and collagen).

(2) Cell Wall

The cell wall of *Aspergillus* displays a "pathogen-associated molecular pattern" which is recognized by Toll-like receptors on the surface of host macrophage. Binding to these receptors leads to the secretion of proinflammatory cytokines.

(3) Extracellular Enzymes

Aspergillus can produce many enzymes which have the potential function *in vivo* to break down host tissue. These include phospholipases, proteases, and elastase. In addition, members of this genus can produce catalases which can reduce the effectiveness of peroxides generated by phagocytic cells.

(4) Iron Acquisition

Aspergillus produces several hydroxamate siderophores (a ferrichrome and a fusarinine) needed to acquire iron from host iron-binding proteins (lactoferrin, transferring).

(5) Pigment (Melanin)

Conidia of *Aspergillus* are pigmented. The pigment, melanin, is a free radical scavenger (reducing the toxicity of hydroxy radicals, superoxides, and singlet oxygen radicals found within the phagolysosome).

2.4.1.3 Growth Characteristics

Aspergillus can grow on all common laboratory media over a wide range at temperatures (up to 50℃). Their biochemical properties related to virulence or utilized diagnostically have not been understood.

Aspergillus thrive in the environment. Some are highly resistant to heat and drying. Most of them do not grow in cycloheximide-containing fungal media.

2.4.2 Ecology

2.4.2.1 Reservoir

Aspergillus can exist in soil, feed, vegetation, and secondarily in air, water and objects exposed to them. *Aspergillus fumigatus* become the dominant bacteria over competing microbiota in fermenting plant material (e.g., silage, hay, compost). The outbreaks of

animal disease are often traced to such sources.

2.4.2.2 Transmission

Aspergillosis comes from an environmental source, and the host is infected generally by inhalation or ingestion. Most *Aspergillus* mastitis follows intramammary inoculation. Intrauterine infections in cattle can cause dissemination of subclinical lung or intestinal infections. In poultry, egg transmission sometimes occurs, but rare.

2.4.2.3 Pathogenesis

(1) Mechanism

Following their deposition in tissue or on the surface (adhesions hinder removal) recognition (by way of "pathogen-associated molecular pattern") by phagocytic cells causes an inflammatory response. Inflammation, along with release of fungal proteases, elastase, and phospholipases, leads to tissue damage. Pigment and catalase delay destruction by phagocytic cells.

Allergenic factors, which are recognized in human aspergilloses, are insufficiently documented in animal diseases.

(2) Pathology

In pulmonary infection, suppurative exudate gathers in bronchioles and adjacent parenchyma, which surround colonies of mycelial growth, and may extend into blood vessels and produce infected vasculitis and thrombi, triggering dissemination. Infection may also spread directly into adjacent air spaces. Granulomas develop; they are visible to the naked eye as grayish white nodules and consist of mononuclear cells and fibroblasts. In older lesions, colonies are fringed by acidophilic clubs (asteroid bodies), which are similar to actinomycosis.

Lesions in avian lungs are caseous nodules. On serous membranes, caseous foci are covered by macroscopic mold colonies, accompanied by thickening of the membranes (e.g., air sacs). The cellular response is acute suppurative with chronic granulomatous.

Bovine abortion results from hematogenous seeding of placentomes, which is possibly a response to a growth factor in placental tissue. There is hyphae invasion of blood vessels producing vasculitis and a necrotizing, hemorrhagic placentitis. The fetus undergoes disseminated infection with signs of emaciation and dehydration. Lymph nodes, viscera, and brain may be involved. Ringworm-like plaques on the fetal skin are often found.

On mucosal surfaces (e.g., meatus, trachea), mold colonies form on the top of necrotic tissue, which is surrounded by a hemorrhagic zone.

2.4.2.4 Disease Patterns

Pulmonary and disseminated infections, frequently involving kidneys and the central

nervous system, can occur in most species.

(1) Avian

Avian aspergillosis, which can affect many species of birds, sometimes in epidemics, reflects heavy exposure or severe stress on domestic flocks or per bird operations, or the effects of oil spills on marine birds. The disease is usually a respiratory tract infection, sometimes, which can cause hematogenous dissemination. Signs are inappetence, weight loss, listlessness, dyspnea, sometimes diarrhea, and abnormal behavior and posture. The eyes are often affected. Mortality may approach 50%, especially in young birds. In mild cases, only gasping and hyperpnea may be seen. The course varies from a day to several weeks.

(2) Ruminant

Bovine abortions usually occur late in pregnancy and the same as abortions due to other causes. Fetal skin plaques occur also in other mycotic abortions.

Mastitis caused by *Aspergillus fumigatus* is reported at an increasing rate, especially in Europe. It is usually chronic progressive, producing abscesses in the udder.

2.4.2.5 Epidemiology

Intensity of exposure is one of the significant features in animal aspergillosis. Bovine abortion outbreaks are often related to moldy fodder or feed. Aspergillosis in chicken flocks is commonly associated with the use of heavily contaminated liner.

Stress factors are usually recognizable in outbreaks. Avian aspergillosis can be caused under conditions of poor husbandry. In oiled seabirds, their thermal regulation is severely impaired. In pregnant cattle, advanced gestation together with low-quality feed, poor weather and housing add up to a severe challenge.

2.4.3 Immunologic Aspects

Circulating antibody with no demonstrably protective role may be present in dogs with nasal aspergillosis. Cell mediated immunity may be related to resistance. Immunization procedures are not available.

2.4.4 Laboratory Diagnosis

2.4.4.1 Direct Examination

Hyphae, fruiting heads, and conidia can often be proved in samples either in wet mounts in 10% KOH or with calcofluor white. For fixed-stained smears, fungal stains [periodic acid-Schiff (PAS), Gridley, or Gomori methenamine sliver] are best; Giemsa is satisfactory, and Gram of limited use. Septate branching hyphae constitute strong evidence of aspergillosis. Other fungi (*Penicillium*, *Pseudallescheria*, *Paecilomyces*) present a similar picture but rare. Conidia may occur in air passages or other exposed

sites in the absence of infection.

In stained tissue sections, septate hyphae dividing dichotomously at acute angles are the only structures seen.

2.4.4.2 Isolation and Identification

Aspergillus is easy to culture because it is a ubiquitous contaminant. Interpretation of positive cultures is often problematic. Presence of the agent must always be correlated with pathologic and clinical findings. Identification of agent rests upon morphologic features and growth characteristics of isolates.

2.4.4.3 Immunodiagnosis

Serologic tests are useful adjuncts to the diagnosis of aspergillosis. Because the tests that are available are species specific, it is necessary to know which *Aspergillus* to expect. For example, *A. fumigatus* is most commonly seen in nasal aspergillosis of dogs, whereas disseminated aspergillosis in that species is either *A. deflectus* or *A. terreus*. An immunodiffusion kit for antibody detection is commercially available for detection of antibodies to *A. fumigatus*.

2.4.4.4 Molecular Methods

Primers designed to amplify DNA encoding ribosomal RNA (including the "internal transcribed spacer" region) are available for demonstration or identification of members of the genus. Amplification of DNA is by the polymerase chain reaction.

2.4.5 Treatment, Control, and Prevention

Aspergillosis in birds is generally not treated. The nasal form in dogs is treated topically with instillation of clotrimazole or enilconazole into the nasal passages and sinuses. Itraconazole (given orally) has been successfully used to treat nasal aspergillosis when topical treatment was not possible. Itraconazole has been beneficial in treating disseminated aspergillosis. There is no established treatment for mammary aspergillosis. For intestinal infections in pigs, foals, and calves, oral nystatin is recommended. Keratomycosis is treated topically with antimycotic ointments and solutions.

Avoidance of massive exposure requires elimination of cattle feed, particularly hay and silage that has undergone noticeable deterioration. *Aspergillus fumigatus* only reaches high concentrations under conditions of "biologic heat" generation, after other microbiota are eliminated. With poultry litter, proper storage and frequent changes of litter can prevent such buildup.

Review Questions

1. Term explanation: fungi, dimorphic fungus, yeast, mold, pseudohypha, vegetative

hyphae, reproductive mycelium, aerial hypha, asexual reproduction, sexual reproduction, toxic pathogenic fungi.

2. What are the reproductive methods of yeast and mold?
3. What is the difference between yeast and mold in morphology and structure?
4. What are the external factors that affect the growth of fungi?
5. How to diagnose and control fungal diseases?
6. What are the pathogenic effects of fungi?
7. Illustrate the toxins and their effects of pathogenic fungi with examples.
8. Try to distinguish *Penicillium* and *Aspergillus*, *Candida* and *Histoplasma*.

Ⅲ. VIROLOGY

Chapter 16 Viral Structure and Classification

Synopsis

Viruses are non-cellular forms of microorganism, and have certain morphology and structure. Viruses are very small and need to be observed by electron microscopy. Although there are different forms of the virus, they are mainly composed of protein capsid and nucleic acid core. Some viruses also have envelope and spike or peplomer outside the capsid. The capsid has three types of symmetry: helical symmetry, polyhedral symmetry and complex symmetry. The proteins of the virus can be divided into structural and non-structural proteins. Lipid and sugar are the components of envelope and spike. The virus contains only one type of nucleic acid, DNA or RNA. The nucleic acid of the virus is single-stranded or double-stranded, positive or negative, linear or annular, segmented or unsegmented. The nucleic acid is the basis of the classification of viruses, and the virus classification system is built according to the viral nucleic acid by International Committee on Taxonomy of Viruses (ICTV).

Viruses are the smallest infectious agents of 20-300nm in diameter and contain only one kind of nucleic acid (RNA or DNA) in genome. Different viruses have various shapes. The nucleic acid is encased in a protein shell which may be surrounded by a lipid-containing membrane. The entire infectious unit is termed as virion. Viruses can replicate only in living cells. The viral nucleic acid contains vital information for programming the infected host cell to synthesize virus-specific macro-molecules required for the production of viral progeny. The coat proteins assemble together to constitute the capsid, which encases and stabilizes the viral nucleic acid against the extracellular environment, and which facilitates the attachment and penetration when the virus contact with new susceptible cells.

The following criteria are consistent among viruses: ①genome of a virus has only one type of nucleic acid, RNA or DNA; ②a virus reproduces only from its nucleic acid; ③a virus does not possess genetic information for the synthesis of enzymes responsible for energy metabolism; ④a virus utilizes host ribosomal and transfer RNA

for synthesis of viral proteins.

1 Structural, Morphology and Symmetry of Virus

1.1 Terms and Definitions in Virology

Capsid: the protein shell, or coat that encloses the nucleic acid genome.

Capsomere: morphologic units on the surface of icosahedral virus particle. Capsomere is clusters of polypeptides. But, the morphologic units do not necessarily correspond to the chemically defined structural units.

Defective virus: a virus particle is functionally deficient in some aspect of replication.

Envelope: a lipid-containing membrane that surrounds some virus particles. It is acquired during viral maturation by a budding process through a cellular membrane. Virus-encoded glycoproteins are exposed on the surface of the envelope. These projections are called peplomers.

Nucleocapsid: the protein-nucleic acid complex represents the packaged form of the viral genome. The term is commonly used in cases where the nucleocapsid is a substructure of a more complex virus particle.

Structural units: the basic protein build blocks of the coat. They are usually a collection of more than one nonidentical protein subunit. The structural unit is often referred to as a protomer.

Subunit: a single folded viral polypeptide chain.

Virion: the complete virus particle. In some instances (e.g., papillomaviruses, picornaviruses), the virion is identical with the nucleocapsid. In complex virions (e.g., herpesviruses, orthomyxoviruses), this includes the nucleocapsid plus a surrounding envelope.

Viruses consist of nucleic acid (DNA or RNA) surrounded by a protein coat called as capsid. The capsid is made up of individual structural subunits called capsomeres. The combination of the nucleic acid genome enclosed in the capsid is called the nucleocapsid. In addition, various animal viruses have an envelope, which is a membranous lipid structure that surrounds the nucleocapsid.

1.2 Viruses Morphology

Viruses can be classified into several morphological types (Fig. 16-1): helical, e.g., bacteriophage M13 (Fig. 16-1a); polyhedral/cubic, e.g., poliovirus (Fig. 16-1b); enveloped-may have polyhedral [e.g., herpes simplex virus (Fig. 16-1c)] or helical [e.g., influenzavirus (Fig. 16-1d)] capsids complex [e.g., poxviruses (Fig. 16-1e)].

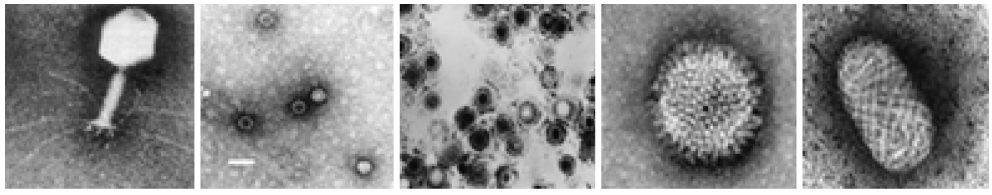


Fig. 16-1 Examples of virus morphology (MacLachlan and Dubovi, 2011)

1.3 Virus Symmetry

Electron microscope, cryo-electron microscope, and X-ray diffraction techniques have made it possible to resolve fine differences in the basic morphology of viruses (Fig. 16-2).

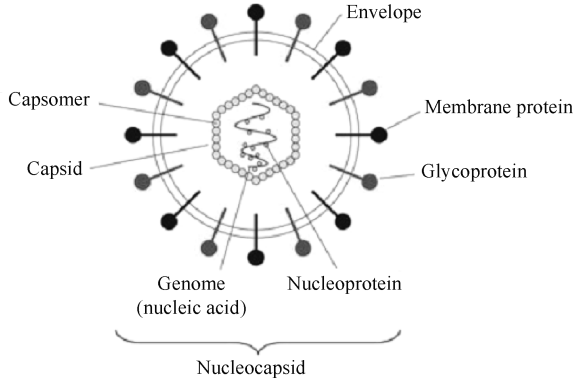


Fig. 16-2 Schematic diagram of the enveloped virus with icosahedral symmetry (Modrow *et al.*, 2013)

Viral architecture can be grouped into three types based on the arrangement of morphologic subunits: ①cubic symmetry, e.g., adenoviruses; ②helical symmetry, e.g., orthomyxoviruses; and ③complex structures, e.g., poxviruses.

1.3.1 Cubic Symmetry

All cubic symmetry of animal viruses is of the icosahedral pattern which is the most efficient arrangement for subunits in a closed shell. The icosahedron has 20 faces (each an equilateral triangle), 12 vertices, and fivefold, threefold, and twofold axes of rotational symmetry. The vertex units have 5 neighbors (pentavalent), and all others have six neighbors (hexavalent).

There are exactly 60 identical subunits on the surface of an icosahedron. In order to build a particle size adequate to encapsulate viral genomes, viral shells are composed of multiples of 60 structural units. The use of larger numbers of chemically identical protein subunits, while maintaining the rules of icosahedral symmetry, is accomplished

by subtriangulation of each face of an icosahedron (Fig. 16-3).

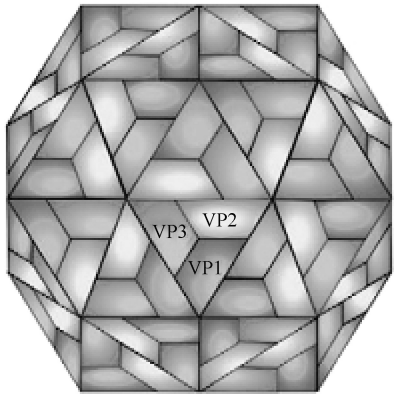


Fig. 16-3 Icosahedron symmetry: picornavirus (MacLachlan and Dubovi, 2011)

1.3.2 Helical Symmetry

In cases of helical symmetry, protein subunits are bound in a periodic way to the viral nucleic acid, winding it into a helix. The filamentous viral nucleic acid-protein complex (nucleocapsid) is subsequently coiled inside a lipid-containing envelope. Thus, there is a regular, periodic interaction between the capsid protein and nucleic acid in viruses with helical symmetry (Fig. 16-4).

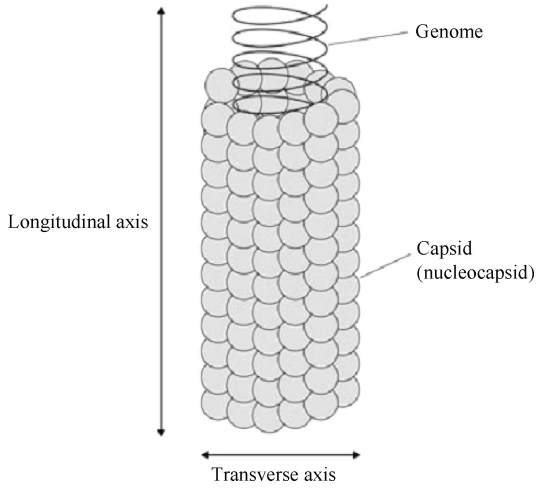


Fig. 16-4 Helical symmetry: TMV (Modrow *et al.*, 2013)

1.3.3 Complex Structures

Some virus particles do not exhibit simple cubic or helical symmetry, but are more complicated in structure. For example, poxviruses are brick-shaped, with ridges on the

external surface, while a core and lateral bodies inside (Fig. 16-5). Bacterial invader is complex symmetry (Fig. 16-5).

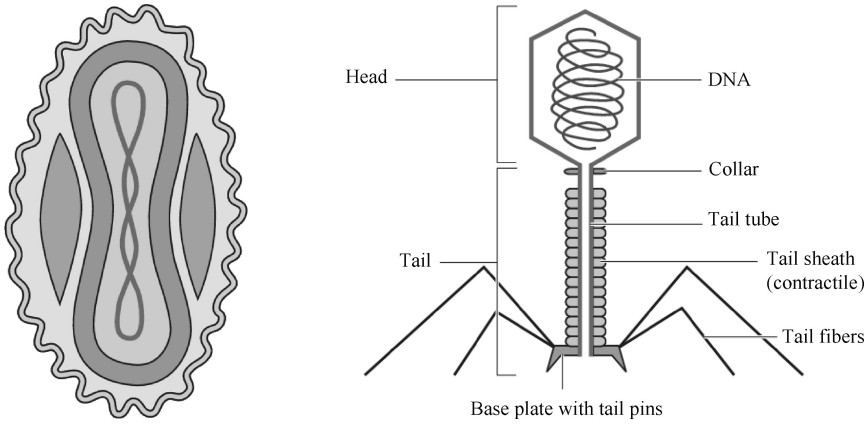


Fig. 16-5 Complex structures: parapoxvirus (left), and bacterial phage (right) (Kayser *et al.*, 2005)

2 Chemical Compositions of Virus

2.1 Viral Nucleic Acid

Viruses contain only one kind of nucleic acid, DNA or RNA, which encodes genetic information necessary for viral replication. The genome may be single-stranded or double-stranded, circular or linear, and segmented or nonsegmented.

The size of the viral DNA genome ranges from 3.2kb (e.g., hepadnaviruses) to 375kb (e.g., poxviruses). The size of the viral RNA genome ranges from about 7kb (e.g., some picornaviruses and astroviruses) to 30kb (e.g., coronaviruses).

Viral RNAs exist in several forms. The RNA may be a single linear molecule (e.g., picornaviruses). For other viruses (e.g., orthomyxoviruses), the genome consists of several segments of RNA that may be loosely associated within the virion. The isolated RNA of viruses with positive-sense genomes (e.g., picornaviruses and togaviruses) are infectious, because of its functions as mRNA within the infected cell. The isolated RNA of viruses (e.g., rhabdoviruses and orthomyxoviruses) with negative-sense genomes, are not infectious.

Viral nucleic acid can be distinguished by its G+C content. DNA viral genomes can be analyzed and compared using restriction endonucleases which can cleave DNA at specific nucleotide sequences. Each genome will yield a characteristic pattern of DNA fragments after cleavage with a particular enzyme. Using molecular cloned DNA copies of RNA, restriction maps also can be derived for RNA viral genomes. Polymerase chain reaction assays and molecular hybridization techniques (DNA to

DNA, DNA to RNA, or RNA to RNA) permit the study of transcription of the viral genome within the infected cell, in comparison of the relatedness of different viruses.

2.2 Viral Protein

The major functions of viral structural proteins are intended to facilitate transfer of the viral nucleic acid from one host cell to another. They can protect the viral genome against inactivation by nucleases, participate in the attachment of the virus particle to a susceptible cell, and provide the structural symmetry of the virus particle. The proteins determine the antigenic characteristics of the virus. The protective immune response to infected host is directed against antigenic determinants of proteins or glycoproteins exposed on the surface of the virus particle. Some surface proteins of some kinds of viruses may also exhibit specific activities, e.g., hemagglutinin of influenza virus agglutinates red blood cells from some kinds of animals.

The nonstructural protein was not included in the virus particle. These compositions were intermediate products during viral reproduction and have the enzyme-functions and other functions.

2.3 Viral Lipid Envelopes

A number of different viruses contain lipid envelopes as part of their structure (e.g., Sindbis virus). The lipid is acquired when nucleocapsid buds through a cellular membrane during viral maturation. The specific phospholipid composition of a virion envelope is determined by the specific type of cell.

Viral glycosylated proteins always protrude from the envelope and are exposed on the external surface of the virus particle. Viral unglycosylated proteins underneath the envelope, which anchor the particle together. Lipid-containing viruses are sensitive to treatment with organic solvents (e.g., ether, ethylether or trichloromethane), which indicate that disruption or loss of lipid results in loss of infectivity. Non-lipid-containing viruses are generally resistant to ether.

2.4 Viral Glycoproteins

Viral envelopes contain glycoproteins which are encoded by the virus. The sugars added to viral glycoproteins reflect that the virus is grown in a host cell. The surface glycoproteins of an enveloped virus particle attach to a target cell by interacting with a cellular receptor. The glycoproteins are also important viral antigens, which are frequently involved in the interaction of the virus particle with neutralizing antibody. While extensive glycosylation of viral surface proteins may prevent effective neutralization of a virus particle by specific antibody.

3 Classification of Virus

The primary criteria for the classification of animal viruses include morphology (size, shape, etc.), type of nucleic acid (DNA, RNA, single-stranded, double-stranded, linear, circular, segmented, etc.), and occurrence of envelopes. The ssRNA viruses possess either (+) RNA (as mRNA) or (–) RNA (as a template for mRNA). Some animal viruses exhibit a very narrow or specific host range, such as chicken anemia virus (CAV) in chickens or canine distemper virus (CDV) in dogs. But, host range is not a particularly reliable criterion for classification, because each animal species can be infected by a wide variety of viral agents, and numerous viruses can infect several different animal species. For example, West Nile virus has a primary host of birds, but it infects and causes disease in horses and humans. Some viruses, e.g., the influenza virus, can change their structure in such a way that they can shift from one primary host to another, for instance from birds to humans.

3.1 Universal System of Virus Taxonomy

By 2016, the International Committee on Taxonomy of Viruses (ICTV) had released the current edition of virus taxonomy on line. A system has been established based on the phylogenetic relationship of viruses. This system had 111 families. Twenty-nine families belong to seven orders and 82 families that are not assigned an order. Four orders related to animal virus include *Mononegavirales*, *Nidovirirales*, *Herpesvirales* and *Picornavirales*. Eighty-two families fall within the group of "virus families not assigned to an order".

The names of the virus family have the suffix *-viridae*. Within a family, genera as subdivisions are usually based on physicochemical or serologic differences. Criteria used to define genera vary from family to family. Genus names carry the suffix *-virus*. In some families (e.g., *Poxviridae*, *Parvoviridae*, *Paramyxoviridae*), a larger grouping called subfamilies has been defined, which reflect the complexity of relationships among member viruses. Virus orders may be used to group virus families that share common characteristics. Diagrams of animal virus families are shown in a later figure (Fig. 16-6).

3.2 Survey of DNA-Containing Viruses

3.2.1 Parvoviruses

Parvoviruses are 18-26nm in diameter. The virus particles have cubic symmetry, 32 capsomeres, but no envelope. The genome is linear, single-stranded DNA, 5.6kb in

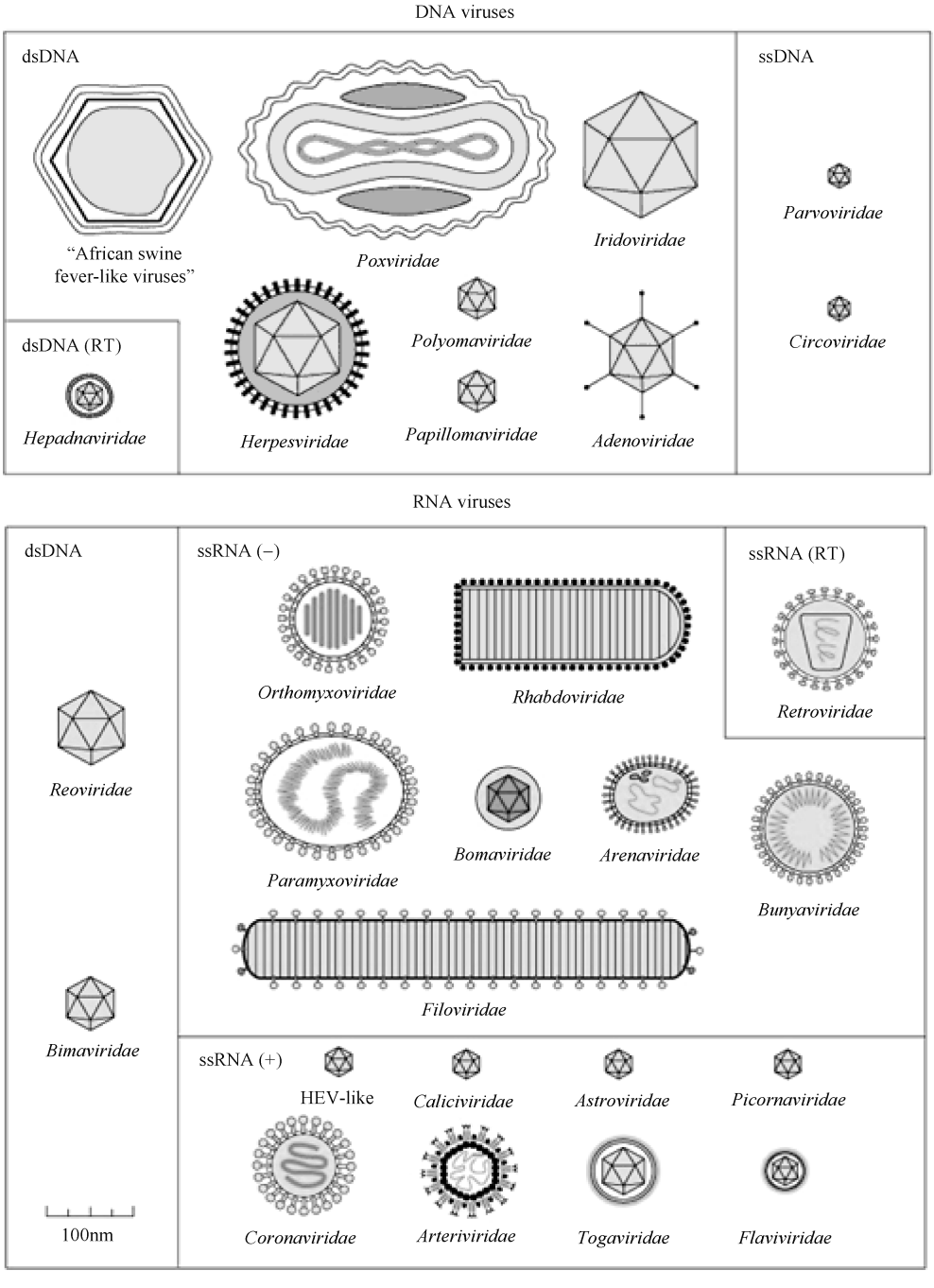


Fig. 16-6 Shapes and relative sizes of animal viruses of families that infect vertebrates (贾文祥, 2008)

size. Replication occurs only in actively dividing cells. Capsid assembly takes place in the nucleus of the infected cell. Many parvoviruses replicate autonomously, but the adeno-associated satellite viruses are defective which require the presence of an adenovirus or herpesvirus as "helper".

3.2.2 Polyomaviruses

These viruses are small (about 45nm in diameter), non-enveloped, heat-stable, ether-resistant viruses, and exhibit cubic symmetry with 72 capsomeres. The genome is circular, double-stranded DNA, 5kb in size. These agents have a slow growth cycle, stimulate cell DNA synthesis, and replicate within the nucleus. Polyomaviruses can cause infectious disease in many animals. They produce chronic infections in their natural hosts, and all can induce tumors in some animal species.

3.2.3 Papillomaviruses

These viruses are similar to polyomaviruses in some respects, but with a larger genome (8kb) and particle size (about 55nm in diameter). Papillomaviruses is very host-specific and tissue-specific. Many animal species carry papillomaviruses.

3.2.4 Adenoviruses

These viruses are medium-sized (70-90nm in diameter). Non-enveloped viruses exhibiting cubic symmetry with 252 capsomeres. Fibers protrude from the vertex capsomeres. The genome is linear, double-stranded DNA, 26-45kb in size. Replication occurs in the nucleus. Complex splicing patterns produce mRNAs. There are many serotypes of adenoviruses that infect animals.

3.2.5 Herpesviruses

These viruses are a large family of virus, 150-200nm in diameter. The nucleocapsid is 100nm in diameter, with cubic symmetry and 162 capsomeres, surrounded by a lipid-containing envelope. The genome is linear, double-stranded DNA, 125-240kb in size. The presence of terminal and internal repeated sequences results in several isomeric forms of genomic DNA. Virions contain over 30 proteins. Many herpesviruses, e.g., infectious bovine rhinotracheitis virus, avian infectious laryngotracheitis virus, Marek's disease virus, can cause serious disease in animals.

3.2.6 Poxviruses

These viruses are large brick-shaped or ovoid virus, 220-450nm long, 140-260nm wide, 140-260nm thick. Particle structure is complex, and has a lipid- containing envelope. The genome is linear, covalently closed, double-stranded DNA, 130-375kb in size. Poxvirus particles contain about 100 proteins which include many enzymatic activities (e.g., DNA-dependent RNA polymerase). Replication occurs entirely within

the cell cytoplasm. All poxviruses tend to produce skin lesions. Some are pathogenic for animals, for example, orf virus, fowlpox virus and sheeppox virus.

3.3 Survey of RNA-Containing Viruses

3.3.1 Picornaviruses

These viruses are small (28-30nm in diameter), ether-resistant viruses which exhibit cubic symmetry. The RNA genome is single-stranded and positive-sense, and is 7.2-8.4kb in size. Many groups which can infect animals are *Enterovirus* (e.g., swine vesicular disease virus), *Aphthovirus* (e.g., food-and-mouth disease virus), etc.

3.3.2 Caliciviruses

These viruses are similar to picornaviruses but slightly larger (27-40nm in diameter). Particles have cup-shaped depressions on the surface. The genome is single-stranded, positive-sense RNA, 7.4-8.3kb in size. The virion has no envelope. Rabbit hemorrhagic disease virus is an important rabbit pathogen.

3.3.3 Reoviruses

They are medium-sized (60-80nm in diameter), ether-resistant, non-enveloped viruses with icosahedral symmetry. Particles have two or three protein shells with channels extending from the surface to the core. Short spikes extend from the virion surface. The genome is linear, double-stranded, segmented RNA (10-12 segments), 16-27kb in size. Individual RNA segments range in size from 680bp to 3.9kb. Replication occurs in the cytoplasm, and genome segment reassortment occurs readily. Antigenically similar reoviruses infect many animals.

3.3.4 Arboviruses

These viruses are an ecologic grouping (not a virus family) of viruses with diverse physical and chemical properties. All of these viruses have a complex cycle involving arthropods as vectors that transmit the viruses to vertebrate hosts by their bite. Viral replication does not seem to harm the infected arthropod. Arboviruses with the help of vectors including mosquitoes and ticks can infect humans, mammals, birds, and snakes, etc.

3.3.5 Filoviruses

These viruses are enveloped, pleomorphic viruses that may appear very long and thread-like. They typically are about 80nm wide and about 1,000nm long. The envelope contains large peplomers. The genome is linear, negative-sense, single-stranded RNA, about 19kb in size. Marburg and Ebola viruses cause severe hemorrhagic fever in Africa. These viruses require maximum containment conditions (biosafety level 4) for

handling.

3.3.6 Flaviviruses

These viruses are enveloped viruses, 40-60nm in diameter, and contain single- stranded, positive-sense RNA. Genome sizes vary from 9.5kb (hepatitis C) to 11kb (flaviviruses) to 12.5kb (pestiviruses). Mature virions accumulate within cisternae of the endoplasmic reticulum. This group includes yellow fever virus and dengue viruses, etc.

3.3.7 Coronaviruses

These viruses are enveloped particles of 120-160nm in diameter, and contain an unsegmented genome of positive-sense, single-stranded RNA of 27-32kb in size. The nucleocapsid is helical, 9-11nm in diameter. Coronaviruses have petal-shaped surface projections arranged in a fringe, like a solar corona. Coronavirus nucleocapsids develop in the cytoplasm and mature by budding into cytoplasmic vesicles. These viruses have narrow host ranges. Many coronaviruses are important in human and animals, e.g., transmissible gastroenteritis virus of swine, avian infectious brochitis virus and SARS-CoV, etc. Toroviruses which cause gastroenteritis form a distinct group.

3.3.8 Orthomyxoviruses

These viruses are medium-sized particles of 80-120nm in diameter with envelop, and exhibit helical symmetry. Particles are round or filamentous, and have surface projections that contain hemagglutinin or neuraminidase activity. The genome is linear, segmented, negative-sense, single-stranded RNA, totaling 10-13.6kb in size. Each segment ranges from 900 to 2,350 nucleotides. The internal nucleoprotein helix measures 9-15nm. During replication, the nucleocapsid is assembled in the nucleus, whereas the hemagglutinin and neuraminidase accumulate in the cytoplasm. The virus matures by budding from the cell membrane. The segmented nature of the viral genome permits ready genetic reassortment when two influenza viruses infect the same cell, which may the reasons for the high rate of natural variation among influenza viruses. Transmission from other species is thought to explain the emergence of new human pandemic strains of influenza A virus.

3.3.9 Bunyaviruses

These viruses are spherical or pleomorphic, enveloped particles of 80-120nm in diameter. The genome is made up of a triple-segmented, circular, single-stranded, negative-sense or ambisense RNA, 11-19kb in size. Virion consists of three circulars, helically symmetric nucleocapsids. Replication occurs in the cytoplasm, and an envelope is acquired by budding into the Golgi. The majority of these viruses are transmitted to vertebrates by arthropods.

3.3.10 Rhabdoviruses

These viruses are enveloped virions resembling a bullet, 75-180nm in diameter. The envelope has 10nm spikes. The genome is linear, single-stranded, non-segmented, negative-sense RNA, 13-16kb in size. Particles are formed by budding from the cell membrane. Viruses have broad host ranges. Rabies virus is a member of this group.

3.3.11 Paramyxoviruses

These viruses are similar to but larger (150-300nm) than orthomyxoviruses. Particles are pleomorphic. The internal nucleocapsid measures 13-18nm, and the linear, single-stranded, non-segmented, negative-sense RNA is 16-20kb in size. Both the nucleocapsid and the hemagglutinin are formed in the cytoplasm. In contrast to influenza viruses, paramyxoviruses are genetically stable.

3.3.12 Retroviruses

These viruses are spherical, enveloped viruses (80-110nm in diameter), genome contains two copies of linear, positive-sense, single-stranded RNA of the same polarity as viral mRNA. Each monomer RNA is 7-11kb in size. Particles contain a helical nucleocapsid within an icosahedral capsid. Replication is unique. The virion contains a reverse transcriptase enzyme that produces a DNA copy of the RNA genome. Then this DNA becomes circularized and integrated into the DNA of host chromosome. The virus is subsequently replicated from the integrated "provirus" DNA copy. Virion assembly occurs by budding on plasma membranes. Hosts remain chronically infected. Retroviruses are widely distributed. There are also endogenous proviruses resulting from ancient infections of germ cells transmitted as inherited genes in most species. Leukemia and sarcoma viruses of animals and humans, foamy viruses of primates, and lentiviruses are included in this group. Retroviruses cause acquired immunodeficiency syndrome (AIDS) and made possible the identification of cellular oncogenes.

3.4 Subvirus

Subvirus including viroid and prion is a kind of microorganism, whose structure is very simple than other viruses described in this chapter.

3.4.1 Viroids

Viroids are small infectious agents that cause diseases of plants. Viroids are agents that do not fit the definition of classic viruses. They are nucleic acid molecules without a protein coat. Plant viroids are single-stranded, covalently closed circular RNA molecules consisting of about 360 nucleotides and present rod-like structure. Viroids replicate by an entirely novel mechanism. Viroid RNA does not encode any protein products. To

date, viroids have been detected only in plants; none has been demonstrated to exist in animals or humans.

3.4.2 Prions

Prions are infectious particles composed solely of protein without nucleic acid. Highly resistant to inactivation by formaldehyde, heat, and ultraviolet light. The prion protein is encoded by a single cellular gene. Prion diseases called "transmissible spongiform encephalopathies" include scrapie in sheep, mad cow disease in cattle, and kuru and Creutzfeldt-Jakob disease in humans.

Review Questions

1. Term explanation: virus, capsid, core, nuclear capsid, envelope, spike, enveloped virus, naked virus, capsomere, penton, hexon, infectious nucleic acid, structural protein, nonstructural protein, virus-like particle, viral species, subvirus, prions.
2. Try to draw the basic structure pattern of virus particles.
3. Try to describe the symmetry way of viral capsid and its characteristics.
4. Try to describe the characteristics of viral nucleic acid and protein.
5. How to determine whether the virus has a membrane?
6. Briefly describe the current status of virus classification.
7. What're the differences between the nomenclature of the virus and the bacteria?
8. Describe the system and its agencies of the virus classification.

Chapter 17 Viral Replication

Synopsis

The replication cycle of the virus mainly includes several steps: adsorption, penetration, uncoating, biosynthesis, assembly and releasing. The surface molecules of the virus have specific adsorption of receptors on the cell surface, which is the essence of hemagglutination. The penetration and uncoating of different viruses can occur in different parts of host cells, such as cell membranes, endosomes and nuclear membranes. In the biosynthesis stage, nucleic acid of parent virus is template, host component is used for transcription, translation and processing. Naked viruses often self-assemble, offspring viral particles are released after the cell lysis. Enveloped viruses often mature and are released in the form of buds.

Viral replication is a term for describing the formation of viruses during the infection process in target cells. Viruses must get into the cell, then viral replication can occur. For virus, the aim of viral replication is survival. By generating abundant copies of its genome and packaging these copies into viruses, the virus can continuously infect new hosts. Replication processes of different kind of viruses are varied and depend on gene types of virus.

1 Steps in Viral Replication

The progress of viral replication mainly include: adsorption (attachment), penetration, uncoating, viral genome replication, maturation, release (Fig. 17-1).

1.1 Adsorption

The first stage in viral replication is called the attachment (adsorption) stage. Animal viruses attach to host cells by means of a complementary combination between attachment sites on the surface of the virus and receptor sites on the surface of the host cell. This accounts for specificity of viruses with their host cells. Attachment sites on viruses (called virus receptors) are distributed over the surface of the virus coat (capsid) or envelope, and are usually glycoproteins or proteins. Receptors on the host cell

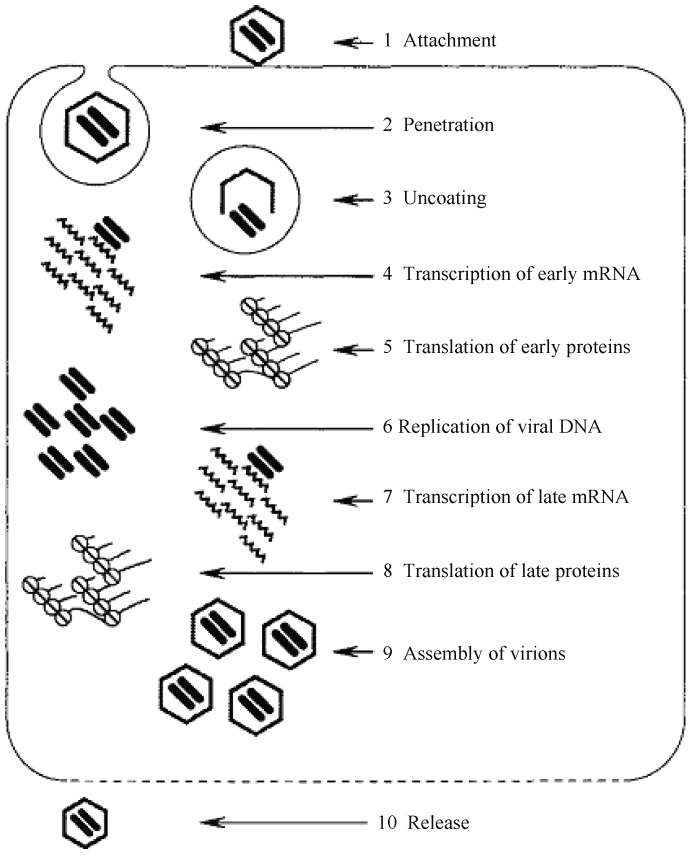


Fig. 17-1 A typical virus replication cycle (Murphy *et al.*, 1999)

(called cell receptors) are generally glycoproteins imbedded into the cell membrane. Cells lacking receptors for a kind of virus cannot be infected. Attachment can be blocked by antibodies that bind to virus receptors or cell receptors. So, the presence of these antibodies in the host is the most important basis for immunization against viral infections.

1.2 Penetration

Penetration of the virus occurs either by engulfment of the whole virus or by fusion of the viral envelope with the cell membrane allowing only the nucleocapsid of the virus to enter the cell. Animal viruses generally do not inject their nucleic acid into host cells, although occasionally non-enveloped viruses leave their capsid outside the cell while the genome passes into the cell.

1.3 Uncoating

When the nucleocapsid entry into the host cell cytoplasm, the process of uncoating

occurs. The viral nucleic acid is released from its coat. Uncoating processes are variable and only poorly understood. Most viruses enter into host cells by an engulfment process called receptor mediated endocytosis and actually penetrate the cell contained in an endosome. Acidification of endosome cause rearrangements of virus coat proteins which probably allow the viral core into the cytoplasm. Some antiviral drugs such as amantadine exert their roles by preventing uncoating of the viral nucleic acid.

1.4 Viral Genome Replication

After uncoating, the stage of viral synthesis begins immediately. In DNA viruses, such as herpesviruses, the viral DNA is released into the nucleus of the host cell where it is transcribed into early mRNA for transport into the cytoplasm where it is translated into early viral proteins. Because the early viral proteins are concerned with replication of the viral DNA, they are transported back into the nucleus where they become involved in the synthesis of multiple copies of viral DNA. These copies of the viral genome are as templates for transcription into late mRNAs which are also transported back into the cytoplasm for translation into late viral proteins. The late proteins are structural proteins (e.g., coat, envelope proteins) or core proteins (certain enzymes) which are then transported back into the nucleus for the next stage of the replication cycle.

In some RNA viruses (e.g., picornaviruses), viral genome stays in cell cytoplasm where it mediates its own replication and translation into viral proteins. In other cases (e.g., orthomyxoviruses), the infectious viral RNA enters into the nucleus where it is replicated before transport back to the cytoplasm for translation into viral proteins.

1.5 Maturation

When the synthesis of viral components is complete, the assembly stage begins. The capsomere proteins enclose the nucleic acid to form the viral nucleocapsid. The process is called encapsidation. If the virus contains an envelope, it will acquire an envelope and associated viral proteins in the next step.

1.6 Release

Release stage results in the exit of the mature virions from host cells. Virus maturation and release occur over a considerable period of time (Fig. 17-2). Some viruses are released from the cell by egestion without cell death, while others are released when the cell dies and disintegrates. In enveloped viruses, the nucleocapsid acquire envelope from the nuclear or cell membrane by a budding off process (envelopment) before egress (exit) out of the host cell. Whenever a virus acquires an envelope. It always inserts specific viral proteins into the envelope which become unique viral antigens and

be used by the virus to entry into a new host cell.

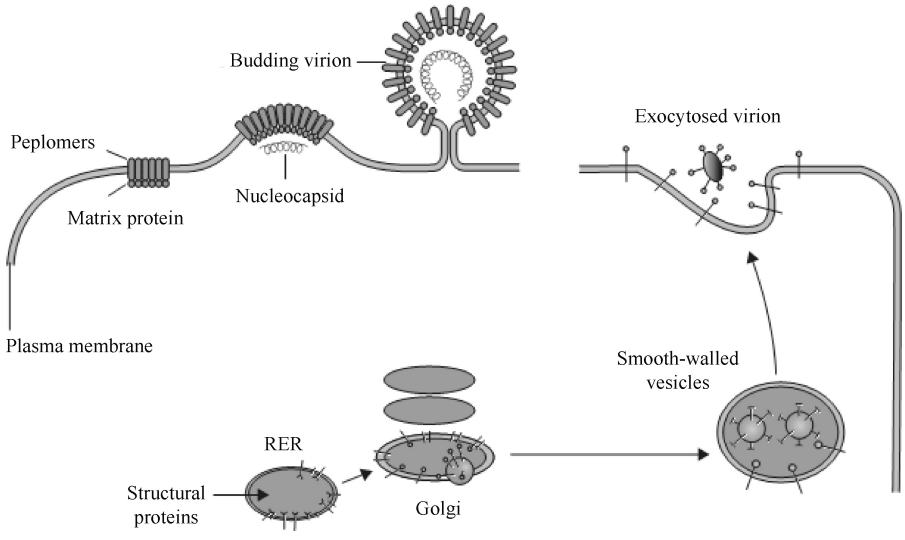


Fig. 17-2 Schematic diagram for the maturation of enveloped viruses
(MacLachlan and Dubovi, 2011)

2 Strategies of Genomic Expression

2.1 RNA Viruses

2.1.1 Positive (+) Strand RNA Viruses Coding for One Genome-Sized mRNA

This RNA serves as mRNA, encodes a single polyprotein and is packaged into virions (e.g., picornaviruses, flaviviruses). The events during the replication are following: ①the genomic RNA binds to ribosomes and is translated into a polyprotein; ②the polyprotein is cleaved; ③genomic RNA then serve as templates for the synthesis of complementary full length (–) strand RNA by a viral polymerase; ④(–) strand RNA serves as template for (+) strand RNA which then serve to produce more polyprotein, more (–) strand RNA or as part of new virions which are forming.

2.1.2 Positive (+) Strand RNA Viruses Coding for Subgenomic RNA

The events during the replication are following: ①genomic RNA binds to ribosomes, but only a portion of the 5′-end is translated into non-structural proteins; ②(–) strand RNA is synthesized, and different size classes of (+) RNA's are produced; ③one is translated into a polyprotein which is cleaved to form structural proteins, another is full length and serves as genomic RNA for new virions which are forming.

2.1.3 Single-Stranded RNA Viruses with Two Identical Strands

The events during the replication of retroviruses are following: ①genomic RNA serves as template for production of DNA copy which is produced by an RNA-dependent DNA polymerase (reverse transcriptase) contained in the virion; ②digestion of genomic RNA and synthesis of second, complementary DNA strands then proceeds; ③double-stranded DNA migrates to the nucleus and integrates into the host cell genome; ④integrated DNA may remain silent or be transcribed into genomic, full-length RNA or shorter, spliced RNA; ⑤the latter code for accessory and structural proteins; ⑥full-length RNA transcripts are packaged into forming virions.

2.1.4 Negative (–) Strand Non-segmented RNA Viruses

The events during the replication are following: ①transcription of (–) strand occurs after entry and is mediated by virion packaged transcriptase; ②(+) strand RNA are produced and proteins are synthesized; ③full-length (–) strand RNA are produced and packaged into forming virions; ④transcription and translation take place entirely in the cytoplasm of infected cells.

2.1.5 Negative (–) Strand Segmented RNA Viruses

The events during the replication are following: ①mRNAs are synthesized from each segment; ②viral proteins are synthesized; ③(+) strand RNAs are synthesized and serve as templates for (–) strand genomic RNAs.

2.1.6 Double-Stranded RNA Viruses

The events during the replication are following: ①genome is transcribed by virion packaged polymerase; ②mRNA are translated to viral proteins or transcribed to complementary strands to yield double-stranded RNA genomes for new virion formation.

2.2 DNA Viruses

2.2.1 Double-Stranded DNA Viruses Replicating in Nucleus

The events during the replication are following: ①sequential, ordered rounds of mRNA and protein production; ②regulate replication; ③structural proteins are produced during last cycle of transcription.

2.2.2 Double-Stranded DNA Viruses Replicating in Cytoplasm

Virions containing a double-stranded DNA genome, enzymes and transcription factors

attach to cells and fuse with the cell membrane to release cores into the cytoplasm. Cores synthesize early mRNAs which are translated into a variety of proteins including growth factors, immune defense molecules, enzymes, and factors for DNA replication and intermediate transcription. Uncoating occurs and the DNA is replicated to form concatemeric molecules. Intermediate genes in the progeny DNA are transcripted and the mRNA are translated to form late transcription factors. The late genes are transcripted and the mRNA are translated to form viral structural proteins, e.g., enzymes and early transcription factors. Assembly begins with the formation of membrane structures. Concatemeric DNA intermediates are resolved into unit genomes and packaged in immature virions. Maturation proceeds to the formation of infectious virions. The virions are wrapped by modified Golgi membrane and results in release of extracellular enveloped virus. Although replication occurs entirely in the cytoplasm, nuclear factors may be involved in transcription and assembly.

2.2.3 Single-Stranded DNA Viruses

The events during the replication are following: ①the synthesis of a complementary DNA strand in nucleus; ②transcription of the genome occurs.

2.2.4 Partially Double-Stranded DNA Viruses

The events during the replication of hepadnaviruses are following: ①genome is converted to a closed circular molecule by a DNA polymerase; ②two classes of RNA species are produced, which one codes for viral proteins and another serves to produce genomic DNA by a reverse transcriptase encoded by virus.

Review Questions

1. Term explanation: virus replication, replication cycle, multiplicity of infection, eclipse period, virus receptor, hemagglutination, hemagglutination inhibition, hemadsorption.
2. Describe the basic process of virus replication.
3. Describe the relationship between the adsorption of the virus and the receptor.
4. Describe the basic principles of hemagglutination, hemagglutination inhibition, hemadsorption.
5. What are the ways by which the viruses penetrate into cells, and by which the viruses are released outside the cells?
6. Illustrate the characteristics of viral biosynthesis with examples.
7. Briefly describe the gene transcription regulation and the processing of protein translation of the virus.
8. Try to draw model picture of one-step growth curve of the virus and the replication cycle of the virus.

Chapter 18 Heredity and Evolution of Virus

Synopsis

Under the influence of environment, viruses occur heredity and evolution. Viral mutations include phenotypic variation and genomic variation. Genome variations include point mutations, missing or inserting of nucleotide fragments. The mutation rate of RNA viruses is much higher than that of DNA viruses. Genetic recombination is a mutation that occurs when two or two more viruses infect the same cell, which include intramolecular recombination, reassortment, reactivation. A defective interfering mutant strain is a special mutation that requires assistance from the helper virus to replicate. The interaction between viral proteins often results in phenotypic mixing.

Viruses grow rapidly and generate millions or billions of progeny virions in cells. Therefore, mutation of viral genome can occur in a short time period, which plays a key role in the viral genetics. Mutation or recombination may genetically change viruses.

1 Mutant

Spontaneous mutations may happen naturally during viral replication, which may be due to the mistakes of polymerase or the mismatches of the bases. DNA viruses are more stable in genetics than RNA viruses. Some RNA viruses are remarkably variants in nature.

Induced mutations of viruses are often caused by physical or chemical agents. Physical agents include UV light or X-ray. Chemical agents may interact directly (e.g., nitrous acid) or indirectly with viral bases (e.g., base analogs).

1.1 Types of Mutation

The types of mutation include point mutants, insertion or deletion mutants.

Point mutations is that one base is replaced by another, which is often caused by

chemical agents or fault of DNA replication. Point mutations include transition and transversion. Common transition is that a purine is exchanged by another purine (A↔G) or a pyrimidine is exchanged by another pyrimidine (C↔T). A transition can be caused by many factors, such as nitrous acid, base mismatch, or mutagenic base analogs (such as 5-bromo-2-deoxyuridine, BrdU). Transversion is that a purine is exchanged by another pyrimidine or a pyrimidine is exchanged by another purine (C/T↔A/G).

A point mutation can be back by another point mutation. For example, a changed-base is mutated back to its original base (this is true reversion) or happen a second-site reversion (which could recover the function of changed-gene). Depending on inaccurate codons, the results of point mutations include silent mutations (the changed codon codes for the same amino acid), missense mutations (the changed codon codes for a different amino acid) and nonsense mutations (the changed codon becomes a stop and code for a truncated protein which has no function).

Insertions are that one or more bases were added into the DNA, which are often caused by transposable elements or replication errors of repeating elements. Insertions occurring in the coding region could change the splice of mRNA (this is splice site mutation) or the reading frame (this is frameshift), which could markedly change the product of a gene. However, insertions can be removed when the transposable element are deleted.

Deletions are that one or more bases were removed from the DNA. Deletion can change the reading frame of the gene, and are commonly non-reversible though it may theoretically be recovered with an insertion or transposable elements. A deletion is quite random.

1.2 Phenotypic Changes

Phenotypic changes caused by viral mutations include conditional lethal mutants, plaque size, drug resistance, enzyme-deficient mutants, hot mutants and attenuated mutants, etc.

Conditional lethal mutants grow under several specific conditions, whereas the wild-type virus can grow under both conditions. Temperature sensitive (*ts*) mutants can grow at low temperature because some irregular proteins have no functional conformation at high temperature. Host range mutants can only grow in “a partial type of cells”, whereas the wild type virus can grow in “a whole type of cells”.

Plaque size of mutants may be larger or smaller than that of wild type virus, sometimes the pathogenicity of such mutants may alter accordingly.

Drug resistant mutants may arise in the viral replication, which may block the development and application of antiviral agents.

Enzyme-deficient mutants are lack of one or more enzymes which are not always essential (e.g., thymidine kinase of herpes simplex virus is usually not required in

tissue culture).

Hot mutants can grow at high temperatures. Hot mutants may be stranger virulent because they are almost not affected by host, but could decrease the replication of wild type viruses.

Attenuated mutants could cause slight symptoms (or no symptoms). These mutants play a potential role in the development of vaccine and the pathogenic evaluation of parental virus as tools.

2 Recombination

Exchange and recombination of nucleic acid molecules of two viral genomes may occur during or after their synthesis.

Recombination of nucleic acid has three steps: breaking of covalent bonds, exchange and reforming of covalent bonds, which is common in DNA viruses or some RNA viruses (which have a DNA phase, e.g., retroviruses) because host cells have recombination systems for DNA viruses, but very rare for RNA viruses. Recombination of picornaviruses is very low efficiency through different mechanisms (probably a "copy choice" mechanism, namely the polymerase switches templates when RNA duplication) with the standard DNA mechanism.

If the distance of two genes is far, the possibility recombination between these two genes is high. A mutant virus can be rescued to a new wild type virus via recombination with DNA fragments from wild type virus, which can be applied for research of inserted foreign DNA from the same type virus or unrelated virus or host genes.

3 Reassortments

Reassortment is a kind of genetic recombination, which often occurs in RNA viruses with segmented genomes (e.g., orthomyxoviruses, reoviruses, arenaviruses, bunyaviruses). If two viruses or two variants with a segmented genome infect the same cell, offspring virion can contain several segments from one of parent, some from the other. Reassortment as a useful method has been applied for assigning the function of different segments in genome. However, reassortment is a non-classical form of recombination.

4 Complementation

Two mutants in different genes, e.g., *ts* (temperature sensitive) mutant, can not grow at a high temperature. If they infect the same cell, one mutant can provide the lost

function of the other, therefore, each can replicate. In double-stranded DNA viruses, when a lot of inactivated viruses using ultraviolet irradiation infect cells, reactivation could be occurred. This phenomenon could be called complementation. The reason of complementation may be that gene in an inactivated virion could keep active in other viruses.

These mutants with complementation can be as a basic tool in genetics to know whether mutations are in a different gene or the same gene, and to know the number of mutation genes.

5 Defective Virus

Defective virus which is lack of the genes that are necessary for a replicative cycle, e.g., deletion mutant, need other virus as a helper virus which could supply the lost functions for completing the replicative cycle. For example, some retroviruses which have sequences of host cell but lost some viral genes need a closely virus as a helper virus that has these genes. However, some defective viruses are exceptional, who can utilize unrelated virus as a helper virus. For example, delta hepatitis virus (HDV, an RNA virus) which has no genes coding for envelope proteins can utilize the envelope protein of hepatitis B virus (HBV, a DNA virus).

Defective virus can affect the replication of the helper virus, because defective virus prefers the functions provided by the helper virus. This phenomenon is called as interference. Most defective viruses which can cause interference are called as "defective interfering (DI)" virus.

6 Phenotypic Mixing

When two different viruses replicate in a same cell, offspring viruses may have coat features of two parents because they could have coat components from two parents. This phenomenon is called as phenotypic mixing. However, genetic materials of progeny viruses do not be changed. Phenotypic mixing may exist among related viruses (e.g., different members of the *Picornavirus*) or among genetically unrelated viruses (e.g., rhabdoviruses and paramyxoviruses). A viral coat could be existed in the envelope of other viruses, which is called as pseudotype (pseudovirion) formation.

Review Questions

1. Term explanation: mutation, point mutation, defective interfering mutant strain, intramolecular recombination, reassortment, reactivation, complementation, phenotypic

mixing, pseudotype virus.

2. Describe the forming cause of defective interfering mutant strains.
3. What is the biological significance of genetic recombination of viruses?
4. What is the biological significance of the interaction between viral proteins?

Chapter 19 Virus–Cell Interactions

Synopsis

Cell culture is a common method for virus proliferation, which can be used to select primary cell, diploid cell strain and established cell line. Stationary culture is a common method for viral proliferation. The method of roller culture and microcarrier culture can be used in the preparation of viral vaccine. After the virus enters into the cell, it may result in the production of the subgeneration virus or a abortive infection; it also may result in cytocidal change or non-cytocidal change. Cytopathic effect is a kind of cytocidal change. Inclusion body is a type of cytopathic effect. Cytocidal change may be caused by the inhibition role to the synthesis of nucleic acids of host cells, RNA transcription, mRNA processing, cell protein synthesis, or by the change of cell membrane and cytoskeleton. Virus infection can cause apoptosis or necrosis. The virus infected cells can produce interferon. While interferon can inhibit other virus infection to the cell, and this function is not specific.

1 Cell Culture of Virus

Viruses are obligated intracellular parasites in order to replicate they need living cells. Virus culture can use cultured cells, eggs and laboratory animals cell cultures are the sole system for virus culture in most laboratories. It is important that the development of methods for cultivating animal cells for animal virology.

1.1 Type of Cell for Viral Culture

1.1.1 Primary Cell

Primary cells can be dissociated directly from animal or human tissues and can be subcultured only once or twice for example primary monkey or baboon kidney. To prepare cell cultures, trypsin and collagenase can be used to dissociate tissue fragments. The cell suspension is then placed in a flat-bottomed glass or plastic container (petri dish, a flask, a bottle, test tube) together with a suitable liquid medium. Such as Eagle's,

and an animal serum. After a variable lag, the cells will attach to and spread on the bottom of the container and then start dividing, giving rise to a primary culture.

Primary cultures need change the fluid 2 or 3 times one week. When the cultures become too crowded, trypsin or EDTA can detach cells from the vessel wall, and portions are used to initiate secondary cultures. In primary and secondary cultures, the cells usually maintain some of the characteristics of the tissue from which they are derived.

1.1.2 Diploid Cell Strain

Human fetal tissue can derive diploid cell strain and they can be subcultured 20 to 50 times, e.g., human diploid fibroblasts. The cells of primary cultures can often be transferred serially many times. The cells can continue to multiply at a constant rate by many successive transfers. Eventually, the cells cannot be transferred any longer after a number of transfers. The growth rate declines after about 50 duplications for human diploid cells.

1.1.3 Established Cell Line

During the multiplication of the cell strain, some cells become altered because they acquire a different morphology, grow faster, and become able to start a cell culture from a smaller number of cells. These cells are not dead (Table 19-1). However, they keep contact inhibition. Human or animal tissue often derives tumours.

Table 19-1 Examples of cell lines in veterinary medicine (胡建和等, 2011)

Cell line	Organism	Original tissue	Morphology
293-T	Human	Kidney (embryonic)	Epithelium
BHK-21	Hamster	Kidney	Fibroblast
CHO	Hamster	Ovary	Epithelium
HeLa	Human	Cervical cancer	Epithelium
MDCK	Dog	Kidney	Epithelium
Sf-9	*Spodoptera frugiperda*	Ovary	Oval
Vero	African green monkey	Kidney	Epithelium

1.2 Methods of Cell Culture

1.2.1 Stationary Culture

Stationary culture is opposed to microcarrier or suspension culture.

1.2.2 Suspensive Culture

The method of cultivation of cells suspended in the medium not adhered to a surface.

When referring to mammalian cells, suspension culture is used for the maintenance of cell types, some types of blood cells not adhere, or in order to have cells express characteristics, which are not seen in the adherent form. Sometimes it need prevent adhesion by choosing a hydrophobic surface, which does not encourage cell adhesion. Serum components of medium will also help to prevent adhesion.

1.2.3 Microcarrier Culture

In microcarrier culture cells grow as monolayer on the surface of small spheres that are usually suspended in the culture medium by gentle stirring. It is possible to achieve yields of several million cells per milliliter by using microcarrier in simple suspension culture systems.

2 Effects of Viral Infection to Host Cell

Understanding of the nature of a viral infection in the individual host animal is a key to for understanding infection in the whole host population, so understanding of the nature of infection in the individual cell may understand infection such as in complex tissues, organs, and the whole host animal. Viruses often encode genes which induce, mimic, or shut down host cell functions for their own benefit and, of course, the host has elaborated systems in order to shut down viral functions. The result of infection may have many kinds such as essentially benign and undetectable, to tolerate, to lethal (Table 19-2). Viral and cellular factors which influence the outcome of infection are often in small balance, easily shifted one way or the other.

Table 19-2 Types of virus–cell interaction (胡建和等, 2011)

Type of infection	Effects on cell	Production of infectious virions	Example
Cytocidal	Morphologic changes in cells (cytopathic effects); inhibition of protein, RNA and DNA synthesis; cell death	Yes	Alphaherpesviruses, enteroviruses, reoviruses
Persistent, productive	No cytopathic effect; little metabolic disturbance; cells continue to divide; may be loss of the special functions of some differentiated cells	Yes	Pestiviruses, arenaviruses, rabies virus, most retroviruses
Persistent, nonproductive	Usually nil	No, but virus may be induced	Canine distemper virus in brain
Transformation	Alteration in cell morphology; cells can be passaged indefinitely; may produce tumors when transplanted to experimental animals	No, oncogenic DNA viruses Yes, oncogenic retroviruses	Polyomavirus, adenoviruses murine, avian leukosis, and sarcoma viruses

The kinds of viral infections have cytocidal (cytolytic, cytopathic) or noncytocidal. Further, not all viral infections are productive, or lead to the production and release of

new virions. Host cell leading to cell death in some instances and cell transformation in others, may also result in nonproductive (abortive) infections. Certain kinds of cells are free, i.e., they keep complete replication of a particular virus, whereas others are nonpermissive, i.e., viral replication may be blocked at any point from viral attachment through to the final stages of virion assembly and release. Permissive and nonpermissive cells can occur cytopathic changes. Often a virus which replicates perfectly well in a particular cell type finds a similar cell type nonpermissive or nonproductive; in these cases, it may be not to trace the defect to the cell or the virus. It is often the combination that is unproductive. For instance, if there is a defect in the viral genome, replication may be non-productive even within an otherwise fully permissive cell. Deletion mutants known as defective interfering (DI) mutants and the point mutants known as conditional lethal mutants are two particular examples of such viral defects.

Some of the most important of all non-productive virus cell interactions are those related to persistent infections or latent infections. The term persistent infection simply describes an infection which lasts a long time. The term latent infection describes an infection which "exists but is not exhibited", i.e., an infection in which infectious virions is not formed. In two cases, the virus or its genome is maintained uncertainty in the cell, either by the integration of the viral nucleic acid into the host cell DNA or by carriage of the viral nucleic acid in the form of an episome. In these cases, the cell survives, indeed may divide repeatedly. In some instances, such cells never release virions; in others, the infection may become productive when induced by an appropriate stimulus may be infected. Persistent or latent infections may also be associated with cell transformation.

2.1 Cytocidal Changes in Virus-infected Cells

Cytopathic viruses kill the cells when they replicate. When a monolayer of cultured cells is inoculated with a cytopathic virus, the first round of infection yields progeny virus that spreads by the medium to infect adjacent as well as distant cells eventually all cells in the culture may become infected. The resulting cell damage is named as a cytopathic effect (CPE). Cytopathic effect can usually be observed via low-power light microscope of unstained cell cultures. The nature of the cytopathic effect is usually characteristic of the particular virus involved and is therefore an important preliminary clue in the identification of clinical isolates. It can reveal further diagnostic details by fixation and staining of infected cell monolayers.

So many pathophysiologic changes lead to cells infected with cytopathic viruses which the death of the cell usually cannot redound any particular event; rather, cell death may be the final result of the cumulative action of many insults. However, several specific mechanisms have been discovered in recent years, some of which have become targets of therapeutic drugs. Particular viruses can cause host cell damage

though many different means. Mechanisms of cell damage are followed.

2.1.1 Inhibition of Host Cell Nucleic Acid Synthesis

Viral infections usually inhibition of host cell DNA synthesis. The result of viral inhibition of host cell protein synthesis and its effect on the machinery of DNA replication, but some viruses use more specific mechanisms. For instance, poxviruses produce a DNase that degrades cellular DNA, and herpesviruses specifically replacement the synthesis of host cell DNA with their own synthetic processes.

2.1.2 Inhibition of Host Cell RNA Transcription

Many different kinds of viruses, including poxviruses, rhabdoviruses, reoviruses, paramyxoviruses, and picornaviruses, inhibit host cell RNA transcription. In some cases, this inhibition possible the indirect result of viral effects on host cell protein synthesis, which decreases the availability of transcription factors required for RNA polymerase activity. In other cases, viruses encode specific transcription factors for the purpose of regulating the expression of their own genes; these factors modulate of the expression of cellular genes as well. Such as herpesviruses encode proteins which bind directly to specific viral DNA sequences, so regulating the transcription of viral genes.

2.1.3 Inhibition of the Processing of Host Cell mRNAs

Many viruses, comprising of vesicular stomatitis viruses, influenza viruses, and herpesviruses, obstruct the splicing of cellular primary mRNA transcripts that are needed to form mature mRNAs. In some cases, spliceosomes are formed, while subsequent catalytic steps are inhibited. Such as a protein synthesized in herpesvirus-infected cells suppresses RNA splicing and resulting in reduced amounts of cellular mRNAs and the accumulation of primary mRNA transcripts.

2.1.4 Inhibition of Host Cell Protein Synthesis

The characteristic of many virus infections is that shutdown of host cell protein synthesis, while viral protein synthesis continues. In picornavirus infections that shutdown is particularly rapid and profound, while it is also pronounced in togavirus, influenza virus, rhabdovirus, poxvirus, and herpesvirus infections. Compared with some other viruses, the shutdown occurs after the course of infection and it is much gentler, whereas with noncytocidal viruses, for instance pestiviruses, arenaviruses, and retroviruses, there is no shutdown and no cell death. It has many different kinds of the mechanisms underlying the shutdown of host cell protein synthesis: some are as mentioned earlier, however others include: ①the production of viral enzymes that reduce cellular mRNAs, ②the production of factors which bind to ribosomes and inhibit cellular mRNA translation, and ③the modification of the intracellular ionic

environment favoring the translation of viral mRNAs over cellular mRNAs. Most importantly, some viral mRNAs simply outcompete cellular mRNAs for cellular translation machinery via mass action; i.e., the large excess of viral mRNA exceed cellular mRNA for host ribosomes. Viral proteins may also inhibit the processing and this inhibition may lead to their degradation. This effect is seen in lentivirus and adenovirus infections.

2.1.5 Cytopathic Changes Involving Cell Membranes

Cellular membranes participate in many phases of viral replication, including viral attachment and entry, the formation of replication complexes, virion assembly. Viruses may alter plasma membrane permeability, affect ion exchange and membrane potential, induce the synthesis of new intracellular membranes, and cause the rearrangement of previously existing membranes. Entry into cells of normally excluded macromolecules or escapes of intracellular molecules can detect membrane permeability, occurs early during picornavirus, alphavirus, reovirus, rhabdovirus, and adenovirus infections. Most importantly, enveloped viruses also direct the insertion of their surface glycoproteins, containing fusion proteins, into host cell membranes as a part of their budding process, usually leading to membrane fusion and syncytium formation.

2.1.5.1 Cell Membrane Fusion and Syncytium Formation

Lentiviruses, paramyxoviruses, morbilliviruses, pneumoviruses, some herpesviruses, and some other viruses have a conspicuous feature that infected cell monolayers which is apt to form syncytia, which due to the fusion of an infected cell with neighboring infected or uninfected cells. Such multinucleate syncytia may also in the tissues of animals infected with these viruses; for instance, in horses fatally infected with the Australian equine morbillivirus, a prominent characteristic of interstitial pneumonia has been alveolar epithelial syncytia (also called multinucleate giant cells). This syncytia may represent an important mechanism of viral spread in tissues: fusion bridges may allow subviral entities, for exemple viral nucleocapsids and nucleic acids, to spread while escaping the effects of host defenses. Viral fusion proteins or fusion domains on other viral surface proteins can mediate cell membrane fusion. For instance, the fusion activity of influenza viruses is carried on hemagglutinin peplomers (spikes). However, the fusion activity of many paramyxoviruses, such as the separate peplomers composed of fusion (F) protein can carry parainfluenza virus 3.

For high multiplicity of infection, paramyxoviruses may cause a rapid fusion of cultured cells don't have any requirement for viral replication. This occurs simply because of the action of fusion protein activity of input virions when they interact with plasma membranes. Fusing different types of cells can produce functional heterokaryons. Milstein and Kohler's experiments that produced the first monoclonal antibodies, parainfluenza virus inactivated via irradiation with ultraviolet light was used to produce

hybridoma cells through the fusion of antibody-producing B lymphocytes with myeloma cells.

2.1.5.2 Hemadsorption and Hemagglutination

Orthomyxoviruses, paramyxoviruses, and togaviruses are used to infect cells which grow monolayer, all of which bud from the plasma membrane, acquire the ability to adsorb erythrocytes. This phenomenon, known as hemadsorption, as the result of the incorporation of viral glycoprotein peplomers into the plasma membrane of infected cells where they hold a post receptors for ligands on the surface of erythrocytes. The same glycoprotein peplomers are in charge of hemagglutination, *in vitro*, i.e., the agglutination of erythrocytes. In this case, virions added to an erythrocyte suspension form cell–virus–cell bridges involving lots of erythrocytes. Although hemadsorption and hemagglutination are not known to work in the pathogenesis of viral diseases, both phenomena are used widely in laboratory diagnostics.

2.1.5.3 Cytolysis by Immunologic Mechanisms

Viral proteins (antigens) break in the host cell plasma membrane may constitute targets for specific humoral and cellular immune responses which may cause the lysis of the cell. This may occur before significant progeny virus is produced, so slowing or arresting the progress of infection and hastening recovery. As an option, in some instances the immune response may precipitate immunopathologic diseases and, in cells which are transformed via viruses, viral antigens incorporated in the cell membrane may behave as tumor-specific transplantation antigens.

2.1.6 Cytopathic Changes Involving the Cytoskeleton

The changes of the common characteristics of a virus infection in cultured cells are the shape of cells. Such changes are caused via damage to the cytoskeleton, which consists of several filament systems, for instance microfilaments (e.g., actin), intermediate filaments (e.g., vimentin), and microtubules (e.g., tubulin). The cytoskeleton is responsible for the structural integrity of the cell, for the transport of organelles via the cell, and for certain cell motility activities. Particular viruses are known to damage specific filament systems. For instance, a depolymerization of actin-containing microfilaments and enteroviruses induce extensive damage to microtubules by canine distemper virus, vesicular stomatitis viruses, vaccinia virus, and herpesviruses cause. Such damage helpful to the drastic cytopathic changes which precede cell lysis in lots of infections. The foundation of the cytoskeleton is also employed through many viruses in the course of their replication.

2.2 Noncytocidal Changes in Virus-infected Cells

Noncytocidal viruses often do not kill the cells in which they replicate. In contrast, they

often occur persistent infection, where infected cells produce and release virions but all cellular metabolism is affected slightly. In lots of cases, infected cells even continue to grow and divide. This style of virus–cell interaction is discovered in cells infected with several kinds of RNA viruses: pestiviruses, arenaviruses, retroviruses, and some paramyxoviruses, especially. However, with several exceptions (e.g., some retroviruses), there are slowly progressive changes which ultimately result in cell death. In the host animal, cell replacement case so rapidly in most organs and tissues which the slow fallout of cells because of persistent infection may have no effect on overall function; yet neurons, once destroyed, are not replaced and persistently infected differentiated cells may lose their capacity to perform specialized functions.

Some viruses, for instance pestiviruses, arenaviruses, and retroviruses, which do not shut down host cell protein, RNA, or DNA synthesis and do not kill their host cells produce important pathophysiologic changes in their hosts via affecting crucial functions which are neither associated with the complete of cells nor their basic housekeeping functions. Harm to the specialized functions of differentiated cells may influence the regulatory and homeostatic functions of endocrine organs, the digestive and metabolic functions of exocrine organs, "fight-or-flight" behavioral functions of neuroendocrine systems, and the protective functions of the immune system. The influence of viral infections on these organs and tissues, affecting hormonal levels, chemical and electrical neurotransmitter functions, and other higher functions, are just beginning to be appreciated, while there is already a sense which will be a major extension from research into clinical practice in the future.

So far, lots of the basis for considering the importance of this subject stem from experimental models in laboratory rodents. For instance, lymphocytic choriomeningitis virus replicating in somatotropic cells of the pituitary gland of the persistently infected mouse lowers the production of the mRNA for growth hormone in infected cells, so impeding the growth and development of the animal. The same to lymphocytic choriomeningitis virus replicating in B cells of the islets of langerhans in the pancreas of the mouse can induce hyperglycemia which is not dissimilar to insulin-dependent diabetes in dogs or humans. Neuropsychiatric effects may follow persistent viral infection of particular neuronal tracts. For instance, Borna disease virus induces bizarre changes in the behavior of rats, cats, and horses, and is now being researched as the possible occurrence depression and other bipolar neuropsychiatric illnesses in humans.

While the pathophysiologic effects of persistent viral infection are manifested via influences on the immune system, we do not often consider the infection of individual cells, but rather about infection all organ system, the whole host animal. Viruses which infect lymphocytes may induce a generalized immunosuppression or other subtle dysfunctions in particular immune responses. The complexity of the infection caused though feline immunodeficiency virus is a case interrelated. Similarly, when persistent

infection involves muscle cells, we often concentrate system dysfunctions, for example, changes in cardiac capacity or rhythm or gastrointestinal motility. When infection touch upon respiratory epithelial cells, we not concerned these cells while on cilia stasis, and the likelihood of bacterial superinfection and secondary pneumonia. However, in different cases it is hard to specialize functions of differentiated cells which underlie the complex pathophysiologic effect and the disease.

2.3 Inclusion Bodies

A typical morphological change in cells infected through certain viruses is the formation of inclusion bodies (or inclusions), which may be approbated via light microscope following fixation and staining. According to the virus, inclusion bodies may be single or multiple, large or small, round or irregular in shape, and acidophilic or basophilic. Most notably, viral inclusion bodies are the intracytoplasmic inclusions discover in cells infected with poxviruses, reoviruses, paramyxoviruses, and rabies virus and the intranuclear inclusion bodies found in cells infected with herpesviruses, adenoviruses, and parvoviruses. Some viruses, such as canine distemper virus and porcine cytomegalovirus, may produce both nuclear and cytoplasmic inclusion bodies in the identical cell.

Inclusion bodies are variety in nature. Some inclusions are accumulations of viral components. For instance, the intracytoplasmic inclusions in cells infected with rabies virus is called Negri bodies, are actually large number viral nucleocapsids, and the intracytoplasmic inclusions found in cells infected with poxviruses are actually sites of viral synthesis (viroplasm, also called viral factories). Other inclusions are consist of crystalline aggregates of virions, such as adenovirus contains the nucleus and reovirus inclusions in the cytoplasm of infected cells perform large flock virions. The consequence of some inclusion bodies due to degenerative cellular changes. In fixed, stained cells, herpesvirus intranuclear inclusions are usually noticeable appearance, they often appear like “owl’s eyes”. It is because of that viral induced chromatin condensation and a fixation artifact which lead to the formation of a clear zone in centrally condensed nucleoplasm and marginated chromatin.

2.4 Virus-induced Cell Death: Apoptosis and Necrosis

Has long been hypothesized that viruses kill cells by direct means, via usurping cellular machinery, disrupting membrane integrity, etc. That is to say, it was considered that the level of insult was so that the cell could not keep on living which in the words of the virologist Kay Holmes, the cell dies though necrosis or “murder”. In recent years, but it has been discovered that although some viruses cause the necrosis of their host cell; triggering cellular “suicide”, or programmed cell death is the way of the viruses to kill

their host cell. Cells which have activated their programmed cell death may undergo characteristic morphologic changes mentioned as apoptosis. Noticeable morphologic changes which occur during apoptosis are differentiated from those of necrosis. Cells dying via apoptosis display chromatin condensation and margination and eventually split into membrane-bound bodies which contain dense cytoplasmic and nuclear debris. Also, typically, a cellular endonuclease is activated during apoptosis which segment cellular DNA into 180-200bp fragments. This endonuclease-induced change is not seen in cellular necrosis.

There are some potential mechanisms that cells are induced to activate their apoptotic route. The direct action of a specific protein in adenoviruses, alphaviruses, and circovirus (chicken anemia virus) can induce apoptosis of some viruses. In some cases, viruses induce apoptosis indirectly by their influence cellular processes. In contrast, some viruses have obtained one or more antiapoptotic genes and gene products to extend cell survival until their replication cycle is complete and progeny virions have been released.

In short, programmed cell death, i.e., apoptosis, similar to an important host defense mechanism. When a virus induces cell death through necrosis, it usually does very late, after progeny virus production is thorough. In other cases, e.g., with picornaviruses, lysis of the host cell via necrosis portion of the viral strategy to effect virion release. But when a cell induces itself-death, though apoptosis, it often does so early, before progeny virus production is intact. Contradictorily, this early clear up of virus-infected cells which take place before the release of progeny virus may arrest or at least slow the spread of virus extends everywhere the body enough so that other host defenses can be marshaled.

2.5 Interferons

It is said that viral interference occurs when a virus-infected cell resists superinfection with the same or a different virus. The interfering virus does not necessarily have no choice but to replicate to induce interference, and the capability of the challenge virus to replicate perhaps completely or only partially inhibited. Two main mechanisms have been proved: ①interference mediated via defective interfering mutants, manipulate only against the homologous virus, and ②interference mediated though interferons.

2.5.1 Properties of Interferons

It has been reported that cells of the chorioallantoic membrane of embryonated hen's eggs infected with influenza virus release into the medium a non-viral protein, "interferon", which protects uninfected cells against infection with the same or unrelated viruses in 1957 by Isaacs and Lindenmann. This finding gives hope that this

substance would be a safe, nontoxic, broad-spectrum antiviral chemotherapeutic agent. Although a lot of work since then, and the capacity to produce large amounts via recombinant DNA technology, it's practicality conduct a therapeutic agent use for the treatment of viral diseases remains an unfulfilled dream (Table 19-3).

Table 19-3 Properties of interferon (胡建和等, 2011)

Propert	Interferon α	Interferon β	Interferon γ
Principal source	Leukocytes, many other cells	Fibroblasts Epithelial cells	T lymphocytes, NK cells
Inducing agent	Virus infection	Virus infection	Antigen (or mitogen)
Number of subtypes	At least 22 in humans, fewer identified in animals	1	1
Glycosylation	No (most subtypes)	Yes	Yes
Functional form	Monomer	Dimer	Tetramer
Principal activity	Antiviral	Antiviral	Immunomodulation
Mechanism of action	Inhibits protein synthesis	Inhibits protein synthesis	Enhances MHC antigens; activates cytotoxic T cells, macrophages, and NK cells

Today we are well known that there are about 24 interferons in humans, and fewer in animals probably only owing to less research. We know that interferons are typical components of the large family of normal cellular regulatory proteins called cytokines. They fall into three chemically different types, they are interferon α (which occurs as at least 22 subtypes in humans), interferon β, and interferon γ.

Interferons are not synthesized though cells constitutively. Any virus, especially RNA viruses can multiply in almost any type of cell and any vertebrate species, induces interferons α and β. T lymphocytes (and natural killer cells) and only following antigen-specific or mitogenic stimulation can make interferon γ; it is a lymphokine which has immunoregulatory functions. Some interferons, especially β and γ display a degree of host species specificity; such as mouse interferons are ineffective in humans, and vice versa. However, there is a few or no viral specificity, i.e., interferons α, β, or γ induced via a paramyxovirus infection is fully effective against a togavirus infection. Secreted interferons are distributed locally or carried through the circulation penetrates the body. Usually, the course of infection of virus can produce interferons in early, as early as the time viral progeny are first freed by infected cells; so, they can have a very important effect in protecting neighboring cells and suppressing the early propagate of virus locally and the early dissemination of virus throughout the body.

Interferons have an important role as a defense mechanism which has been documented via three types of experimental and clinical observations: ①in many viral infections, interferon production related to recovery; ②inhibition of interferon production, for example in knockout mice, improves the severity of infection; and ③treatment with

interferons protects fight infection.

2.5.2 Antiviral Actions of Interferons

Following their induction via viral infection, the infected cell can release interferons and bind to specific receptors on the plasma membrane of other cells. It seems that one receptor for interferons α and β and another for interferon γ binding of interferons α and β to their receptor activates a perplexed transcriptional cascade though signal transduction, which in turn causes the synthesis of many cellular proteins, over 20 cellular genes are up-regulated. Binding of interferon to its receptor triggers different cascades, a set of different genes and proteins. Replication of virus is inhibited by many of the induced proteins, each in a different way. The mechanisms related are beyond the scope of this book, but one example will be used for illustrating the complexity of mechanisms contains interferon-mediated interference.

P1/eIF-2α kinase is made constitutively at low levels in untreated cells, is up-regulated via interferon α, β, or γ. Following binding of double-stranded RNA, it is an intermediate or by-product formed in the process of RNA virus replication, this protein kinase phosphorylates its own P1 subunit, so activating the enzyme to phosphorylate the α subunit of the eukaryotic protein synthesis initiation factor eIF-2, thereby inactivating it. Due to eIF-2 is required to initiate the synthesis of all polypeptides, interferon-induced P1/eIF-2α kinase inhibits the synthesis of all proteins of any virus which has stimulated its activation.

2.5.3 Interferons and Resistance/Susceptibility to Viral Infections

It is hard to determine what cell types are responsible for interferon production in particular viral infections *in vivo*. Certainly, interferons can be discovered in the fluids bathing most infection sites, such as in the mucus bathing airway epithelial surfaces during respiratory infections and in the blood in systemic infections. Epithelial, mesenchymal cells and T cells can produce interferons.

It shows that interferons can be instrumental in deciding the fate of the animal following viral infection was provided in the early 1970s though Gresser and colleagues, who showed that any of several nonlethal viruses, or with sublethal doses of more virulent viruses can infect mice, die if anti-interferon globulin is administered. In studies with transgenic mice carrying multiple copies of the gene for human interferon β, improved resistance to pseudorabies virus was discovered that was proportional to the resulting concentration of circulating interferon evoked via the transgene.

Generally considered that interferons have an important feature in recovery from viral infections, data are less certain. In case interferons were keys, one might expect that infection occurred by any virus or indeed immunization through any attenuated virus vaccine might protect an animal against challenge with an irrelevant virus, yet

this cannot be illustrated. The evidence is a little stronger which infection of the respiratory tract with one virus will provide temporary, local protection against others. This distinction may provide a clue which the antiviral effect of interferons is limited in both time and space. Their main antiviral effect which natural infections may be local protect cells in the immediate vicinity of the initial center of infection, and decelerate the movement of virus during crucial early stages of infection.

2.5.4 Actions of Viruses to Combat Effects of Interferons

Due to its early induction and broad effectiveness, many viruses have developed defense mechanisms for circumventing the host's interferon system. Such as adenoviruses encode RNA which bind to P1/eIF-2α kinase, preventing its activation via double-stranded RNA. Reoviruses might be expected to be exceptionally easily affected interferons since they have double-stranded RNA genomes, are not because one of their capsid proteins binds more strongly to viral double-stranded RNA than P1/eIF-2α kinase.

Review Questions

1. Term explanation: cell culture, primary cell, diploid cell strain, established cell line, stationary culture, roller culture, suspension culture, microcarrier culture, abortion infection, persistent infection, cytopathic effect, median cell infection dose, apoptosis, antibody-dependent enhancement of viral infection, plaque, plaque forming unit, viral load, inclusions, occlusion body, RNA interference, interferon.
2. Describe the methods of cell culture and their characteristic.
3. How to count the number of the virus?
4. What are the mechanisms of cytocidal change caused by viral infection.
5. What are the consequences of viral infection?
6. What is the significance of non-cytocidal change caused by viral infection?
7. What's the point of RNA interference?
8. What are the functions of interference?

Chapter 20 Pathogenesis of Viruses

Synopsis

The virus can invade host tissues and organs through respiratory tract, gastrointestinal tract, skin or wound, and then spread and reproduce, which can lead to tissue or organ damage resulting in the presenting of clinical disease. Virus infection can cause subclinical infection or overt infection, local infection or systemic infection. Most viral infections can lead to persistent infections, latent infections, slow infections or acute infections with late clinical manifestations. Virus infection can lead to host immunity or immunosuppression. The injury of the immune system is an important pathogenesis of virus infection.

1 Tissular and Organic Damage Caused by Viral Infection

Viruses enter into a host via several major routes that include the respiratory, alimentary, urogenital routes and direct transmission (e.g., bite by an insect or animal). Successful infection of virus depends on the presence of receptors on the susceptible host cell and the physics-chemical nature of the virus. When viral infection has succeeded, infected animals may present clinical signs.

1.1 Subclinical and Symptomatic Infection

The basic process of viral infection includes the viral replication (partial or complete) in a host cell. The responses of host cell to viral infection may vary, which include cytopathology with accompanying cell death, hyperplasia and non-apparent effect. Based on the process of viral infection and the manifestation of the host, viral infection can be divided into subclinical infection and symptomatic infection.

Subclinical infection is that a virus-infected host has no clinical symptom or obvious damage, resulting that the virus can not eventually arrive the target organ. In fact, many viral infections are subclinical. The outcome of any viral infection is determined by the interaction between the virus and the host, and affected by the genetics of virus and host.

Symptomatic infection is that a virus-infected host appears overt symptoms. A viral strain is more virulent than other strains if it can cause more severe disease in a certain host. Viral virulence in normal animals is often confused with cytopathogenicity in cultured cells.

1.2 Local and Systemic Infection

Viral infection also can be divided into two basic patterns: local and systemic infection which have several important features compared in Table 20-1.

Table 20-1 Important features of viral infection (胡建和等, 2011)

Features	Local infection	Systemic infection
Example of specific disease	Respiratory (rhinovirus)	Measles
Site of pathology	Portal of entry	Distant site
Incubation period	Relatively short	Relatively long
Viremia	Absent	Present
Duration of immunity	Variable (may be short)	Usually lifelong
Role of secretory antibody (IgA) in resistance	Usually important	Usually not important

Local infection is that viral replication and cellular injuries are localized near the site of viral entry (e.g., the skin, the mucous membranes of the respiratory, gastrointestinal, and genital tract). For example, rhinovirus infections in animals are often confined to the nasal epithelium. Parainfluenza and respiratory syncytial viruses (RSV) replicate in the lungs, but tissue injuries caused by these viruses typically are restricted to the respiratory tract.

Systemic infection is that the viruses are widely distributed in the host body and have the maximum viral titer compared with the results of overt disease. The development of systemic infection has several sequential steps: ①the primary viral replication occurs at the entry site or/and in local lymph nodes; ②offspring virus diffuse through blood and lymphatics to other target organs; ③viral replication occurs again; ④virus spread to other target organs; ⑤viral replication occurs again in target organs with the results of cell degeneration and/or tissue damage, and clinical disease. For example, canine distemper virus (CDV) replicates at the entry site, spreads via blood or lymphatic with the results of systemic infection which involves of more target organs (Fig. 20-1). After incubation period, different clinical signs occur in various organ systems infected by CDV.

Viral infections without overt disease (also called as inapparent infections), which are very common, are important in the viral spread, and can provide protective immunity against the following the attack of virulent strains. Several factors which can affect

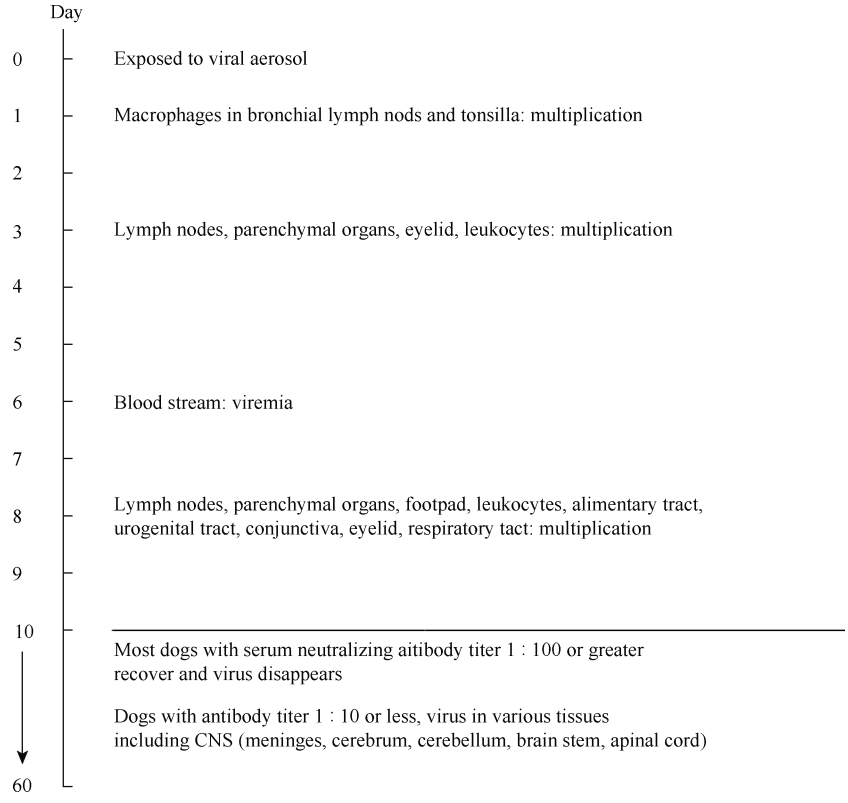


Fig. 20-1 Pathogenesis of canine distemper virus in dogs (Scott McVey *et al.*, 2013)

inapparent infections are the nature of the virus (e.g., virulent or attenuated strains), host immunity, viral interference, and the ability of virus to arrive the target organ (e.g., the block of the blood-brain barrier).

Viruses can cause damage of host cell. The injuries of virus-infected cells can cause functional disorder and different pathological changes (e.g., plasmatorrhexis, deliquescence, diminution, confluence into giant cells and cytorrhyctes). This kind of viral pathological effect is called as cytopathic effect (CPE). The types of viral CPE include: cytocidal effect (some virus replication in cells cause deliquescence and death), cytorrhyctes, confluence to multinucleated giant cell (often caused by paramyxovirus and coronavirus), cell transformation (some viruses DNA are integrated into genome DNA of host cell and then the genetic information of host cell may be changed), and hemagglutination (hemagglutinin on the surface of some viruses such as orthomyxovirus and paramyxovirus can bind red blood cells of some animals).

1.3 Pathogenesis of Virus–Host Infection

Steps related to viral pathogenesis include viral entry and primary replication, viral

spread and cellular injury, immune response of host, viral clearance or establishment of persistent infection, and viral shedding.

After the attachment, a virus must enter into cells of one kind of body surfaces (e.g., skin, respiratory tract, gastrointestinal tract, urogenital tract, or conjunctiva). Most viruses enter host cells through the mucosa of several organs, some viruses enter directly into bloodstream by needles, blood transfusions, or sting of insect vectors (e.g., arboviruses). After the entrance, viruses such as influenza viruses (respiratory infections) and rotaviruses (gastrointestinal infections) may replicate at the primary entry site and cause diseases at the entry portal.

Many viruses can cause diseases at distant sites from entry point. For example, enteroviruses enter the gastrointestinal tract, but may cause disease of central nervous system. After primary replication at the entry site, offspring viruses spread in host. Mechanisms of viral spread vary, but the most common route is via the bloodstream or lymphatics. The presence of virus in blood is called viremia. Virions may be free in the plasma (e.g., enteroviruses, togaviruses) or associated with particular cell types (e.g., measles virus). Some viruses even multiply within those cells. The viremic phase is short in many viral infections. In some instances, neuronal spread is involved; this is apparently how rabies virus reaches the brain to cause diseases and how herpes simplex virus moves to the ganglia to initiate latent infections. Viruses tend to exhibit organ and cell specificities. Such tissue and cell tropism by a given virus usually reflects the presence of specific cell surface receptor for that virus. Receptors are components of the cell surface with which a region of the viral surface (capsid or envelope) can specifically interact and initiate infection. Receptors are cell constituents that function in normal cellular metabolism but also happen to have an affinity for a particular virus. The chemical nature of viral receptors is unknown in many cases. Factors affecting viral gene expression are important determinants of cell tropism. Enhancers regulate transcription of viral genes. Another mechanism dictating tissue tropism involves proteolytic enzymes. Certain paramyxoviruses are not infectious until an envelope glycoprotein undergoes proteolytic cleavage. Multiple rounds of viral replication will not occur in tissues that do not express the appropriate activating enzymes. Viral spread may be determined in part by specific viral genes. Studies with reovirus have demonstrated that the extent of spread from the gastrointestinal tract is determined by one of the outer capsid proteins.

1.3.1 Cell Injuries and Clinical Illness

Destruction of virus-infected cells in the target tissues and physiologic alterations produced in the host by the tissue injuries are partly responsible for the development of disease. Some tissues, such as intestinal epithelium, can rapidly regenerate and withstand extensive damage better than others, such as the brain. Some physiologic

effects may result from nonlethal impairment of specialized functions of cells, such as loss hormone production. Clinical illness from viral infection are the result of a complex series of events, and many of the factors that determine degree of illness are unknown. General symptoms associated with many viral infections, such as malaise and anorexia, may result from host response elements such as cytokine production. Clinical illness is an insensitive indicator of viral infection; inapparent infections by viruses are very common.

1.3.2 Recoveries from Infection

The host either succumbs or recovers from viral infection. Recovery mechanisms involve humoral and cell-mediated immunity, interferon and other cytokines, and possibly other host defense factors. The relative importance of each component differs with the virus and the disease.

In acute infections, recovery is associated with viral clearance. However, there are times when the host remains persistently infected with the virus.

1.3.3 Viruses Shedding

Shedding of infectious virions is crucial to the maintenance of infection in populations. The last in pathogenesis is the shedding of infectious virus into the environment. This is a necessary step to maintain a viral infection on populations of hosts. Shedding usually occurs from the body surfaces involved in viral entry (lymph nod→blood: primary viremia→bone marrow, liver, spleen, blood vessel: endothelium→blood: secondary viremia→nasal and oral mucous membranes, skin, brain, lung, salivary gland, kidney). Shedding occurs at different stages of disease depending on the particular agent involved. It represents the time at which an infected individual is infectious to contacts. In some viral infections, such as rabies, humans represent dead-end infections, and shedding does not occur.

2 Persistent Viral Infections

Persistent and latent virus infections are characterized by the fact that virus is not eliminated from the host. Disease may or may not occur in such chronically infected animals. Potential mechanisms of viral persistence include non-cytocidal infection of host cells, destruction of immune effector cells or growth within these cell types, evasion of protective host responses including cytokines and antibodies, and integration of the viral genome into that of the host cell. Persistent infections are those in which virus continuously is present, with or without expression of disease. Disease, when it does occur in persistently infected animals, often is a result of immunopathologic mechanisms. Latent infections are those in which virus is demonstrated only when reactivation (recrudescence) occurs; this is highly characteristic of herpesvirus infections.

Viral persistence: chronic and latent virus infections. Viral infections are usually self-limiting. Sometimes, however, the virus persists for long periods of time in the host. Long-term virus–host interaction may take several forms. Chronic infections are those in which virus can be continuously detected, often at low levels; mild or no clinical symptoms may be evident. Latent infections are those in which the virus persists in an occult, or cryptic, form most of the time. There will be intermittent flare-ups of clinical disease; infectious virus can be recovered during flare-ups. Inapparent or subclinical infections are those that give no overt sign of their presence.

Chronic infections occur with a number of animal viruses, and the persistence in certain instances depends upon the age of the host when infected. In humans, for example, rubella virus and cytomegalovirus infections acquired in utero characteristically result in viral persistence that is of limited duration, probably because of development of the immunologic capacity to react to the infection as the infant matures. Infants infected with hepatitis B virus frequently become persistently infected (chronic carriers); most carriers are asymptomatic. In chronic infections with RNA viruses, the viral population often undergoes many and antigenic changes.

Herpesviruses typically produce latent infections. Herpes simplex viruses enter the sensory ganglia and persist in a noninfectious state. There may be periodic reactivations during which lesions containing infectious virus appear at peripheral sites (e.g., fever blisters). Chicken pox virus (varicella-zoster) also becomes latent in sensory ganglia. Recurrences are rare and occur years later, usually following the distribution of a peripheral nerve (shingles). Other members of the herpesvirus family also establish latent infections. All may be reactivated by immunosuppression.

Persistent viral infections may play a far-reaching role in animal disease. Persistent viral infections are associated with certain types of cancers as well as with progressive degenerative diseases of the central nervous system. In pigs that have eaten virus-bearing lung worms, swine flu is occult until the appropriate stimulus induces viral production and, in turn, clinical disease. Lymphocytic choriomeningitis (LCM) virus may be established in mice by in utero infection. A form of immunologic tolerance develops in which virus-specific T cells are not activated. Antibody is produced against viral proteins; this antibody and circulating lymphocytic choriomeningitis virus form antigen-antibody complexes that produce immune complex disease in the host. The presence of lymphocytic choriomeningitis virus in this chronic infection (circulating virus with little or no apparent disease) may be revealed by transmission to an indicator host, e.g., adult mice from a virus-free stock. All adult mice develop classic acute symptoms of lymphocytic choriomeningitis and frequently die.

Spongiform encephalopathy is a group of chronic, progressive, fatal infections of the central nervous system caused by unconventional, transmissible agents called prions. Prions are thought not to be viruses. The best example to this type of slow infection is

scrapie in sheep; Kuru disease and Creutzfeldt-Jakob disease occur in humans.

3 Immune Systems Damage of Virus–Host Infection

Viruses are antigenic and typically induce strong immune responses after infection. The immune responses are mainly humoral and cellular immunity. Humoral immune responses lead to the production of antibodies that can be demonstrated by the usual serologic procedures, such as complement-fixation, agglutination, precipitation, and gel diffusion techniques. When preparations of virus are inoculated in susceptible hosts, infection will not occur if the antisera contain virus-neutralizing antibody. Three classes of immunoglobulins, IgG, IgM and IgA, can serve as neutralizing antibodies. The interaction of virus and antibody, particularly antibodies specific to the viral antigens responsible for attachment to specific cell receptors, results in a virus-antibody complex formation that prevents attachment of virus to cell receptors, and to a lesser extent prevents the penetration of virus into the susceptible cell. The interaction between virus and antibody does not physically alter viral structure; however, the complement system and antiviral antibody can induce lysis of enveloped viruses as well as destroy virus-infected cells.

3.1 Cellular Immunity

Cellular immunity in viral infection is another important factor in host resistance to some viral infections. The destruction of virus-infected cells by immune lymphocytes can limit the dissemination of virus, particularly in instances where virus is transmitted from infected to noninfected cells through cell fusion. The macrophages play a role in host resistance to viral infections. Macrophages are key participants in the inflammatory responses, and they can be activated either by interaction with viruses or by the soluble products produced by virus reacting with lymphocytes. Activated macrophages have been shown to participate in a wide range of host responses to viral infections, including phagocytosis of virus-antibody complexes, production of interferon, cytotoxicity for virus-infected cells, and immunoregulatory functions.

3.2 Viral Immunosuppression

Viral immunosuppression is an important character for immune system damage of virus–host infection. Several important viruses in veterinary can infect lymphocytes, including canine distemper virus, feline panleukopenia virus, feline leukemia virus, bovine viral diarrhea virus, hog cholera virus, Newcastle disease virus, and infectious bursal disease virus of chickens. The destruction of lymphocytes and resultant atrophy of lymphoid tissues by the viruses can suppress or compromise immune responses,

predisposing the affected host to other opportunistic bacterial or viral infections.

A variety of spontaneous primary immune deficiency diseases occur in domestic animals (equine, bovine, ovine, porcine, canine, feline) that can predispose them to infectious diseases. A good example is the fatal respiratory tract infection of Arabian foals with combined immunodeficiency disorder (lack of production of functional T and B lymphocytes) by equine adenovirus.

Both humoral and cellular components of the immune response are involved in control of viral infections. Viruses elicit a tissue response different from the response to pathogenic bacteria. Whereas polymorphonuclear leukocytes form the principal cellular response to the acute inflammation caused by pyogenic bacteria, infiltration with mononuclear cells and lymphocytes characterizes the inflammatory reaction of uncomplicated viral lesions.

Virus-encoded proteins, usually capsid proteins, serve as targets for the immune responses. Virus-infected cells may be lyzed by cytotoxic T lymphocytes as a result of recognition of viral polypeptides on the cell surface. Humoral immunity protects the host against reinfection by the same virus. Neutralizing antibody blocks the initiation of viral infection, probably at the stage of attachment or uncoating. Secretory IgA antibody is important in protecting against infection by viruses through the respiratory or gastrointestinal tracts.

In addition to specific immunity, some nonspecific host defense mechanisms may be elicited by viral infection. The most prominent among the nonimmune responses is the induction of interferons.

3.3 Effects of the Immune Responses

Special characteristics of certain viruses may have profound effects on the host's immune responses. Some viruses infect and damage cells of the immune system. The most dramatic example is the human retrovirus associated with acquired immunodeficiency syndrome that infects T lymphocytes and destroys their ability to function.

Adverse effects of the immune response to viral infection are also known. Certain viruses do not invariably kill the cells they infect. The immunologic response of the host in such situations may be involved in the development of pathologic changes and clinical illness.

Another potential adverse effect of the immune response is the development of auto-antibodies. If a viral antigen is to elicit antibodies that fortuitously recognized an antigenic determinant on a cellular protein in normal tissues, cellular injury or loss of function unrelated to viral infection might be the result. The magnitude of this potential problem is currently unknown.

Viruses have evolved a variety of ways that serve to suppress or evade the host immune and thus avoid being eradicated. These mechanisms are becoming recognized

through detailed studies of the function of specific viral gene products. Oftentimes, the viral proteins involved in modulating the host response are not essential for growth of the virus in tissue culture, and their properties are realized only in pathogenesis experiments in animals. They may infect cells of the immune system and abrogate their function, or they may infect neurons that express little or no less class Ⅰ MHC (herpesvirus), or they may encode immunomodulatory proteins that inhibit MHC function (adenovirus). Viruses may mutate and change antigenic sites on virion proteins (influenza virus) or may down-regulate the level of expression of viral cell surface proteins (herpesvirus).

Review Questions

1. What is a superinfection, and the meaning of superinfection?
2. Illustrate the damage of viral infection to respiratory tract, digestive tract, urogenital tract, immune system, etc.
3. Try to describe the characteristics of various persistent infections.
4. Try to describe the basic process of virus invasion, diffusion and emission.

Chapter 21 Detection of Virus Infection

Synopsis

The detection of the virus mainly includes the collection and preservation of samples, the separation and cultivation, the observation of morphology, the assay of physical and chemical properties, the identification of serology and molecular biology methods. The sample should not be contaminated and keep cool. Samples should be pretreated in the separation of virus. Viral morphological observations can be performed by electron microscopy or immunoelectron microscopy. Viral antigens can be detected with several immunological methods including immunofluorescence assay, immunohistochemical method, ELISA, immunochromatography, radioimmune, latex agglutination and immunological diffusion. Antibodies against virus can be detected with several immunological methods including ELISA, serum neutralization test, immunoblotting, immunofluorescence, hemagglutination inhibition test, immunological diffusion. Viral nucleic acid can be detected with several methods including dot hybridization, *in situ* hybridization, PCR, sequencing of gene, oligonucleotide fingerprinting and restriction enzyme mapping.

Generally, there are two ways to diagnose a virus infection: ①presence of corresponding virus, antigen, or the nucleic acid and ②presence of corresponding viral antibody. Too many traditional methods are still widely used, some of them may influence the clinical management of a particular case, and took much time for display the result. This highlights the need of rapid methods that diagnose virus in less than 24h or even during the course of initial examination of the animal. These methods must fulfill five prerequisites: speed, simplicity, sensitivity, specificity, and cheap. For many viruses, ①standardized diagnostic method and components of good quality are available commercially, ②assays have been miniaturized to conserve reagents and lower costs, ③tests have been developed to automate instruments, and lower costs, and ④computerized analyses and printouts of results facilitated reporting and record keeping or billing developed.

Because of economic return and cross test, there is less number of commercially available rapid diagnostic kits in veterinary medicine in compared with human medicine. These methods detect viral antigens or antibody taken from the animal

during acute phase of the illness. Solid-phase enzyme immunoassays (EIAs), in particular, has revolutionized the field of viral diagnostics, could detect both antigen and antibody in many situations. For laboratory-based diagnosis, the polymerase chain reaction (PCR) is being widely explored to detect viral nucleic acids in clinic which is a very rapid alternative to other virus detection methods.

To supply a comprehensive service of diagnose viral infections for domestic animals in a single laboratory, is a formidable undertaking. Over 200 individual viruses, belonging to some 25 viral families, cause obvious infections in eight major domestic animal species (cattle, sheep, goat, swine, horse, dog, cat, and chicken). If consider viral subtypes or variants or animal species such as turkey, duck, laboratory animals, and wildlife species, then the number of known viruses could enlarge indeed. The international reference centers and culture collections track over 30,000 viral variants. It is therefore that no single laboratory has the necessary available specific reagents or the skills to identify of all viruses. For this reason, veterinary diagnostic laboratories have tendency of specialization (e.g., in diseases of food animals, companion animals, poultry or laboratory species, or in diseases caused by exotic viruses). Within these specialized laboratories there is considerable scope for the development of rapid diagnostic methods that simplify virus isolation and identification, all of which are expensive, time consuming, and, in some cases, insensitive.

1 Specimens and Preparation in Laboratory

1.1 Collections, Packaging, and Transport of Specimens

The chance of isolating a virus critically depends on the attention of veterinarian to collected specimen. Clearly, such specimens must be taken from the right place and at the right time. The right time is as soon as possible after the animal first appeared which usually accompany a maximum amount of viral load at about this time, often rapidly, during the ensuing days. After days or weeks of a failing empirical therapy, specimens taken as a last resort at this time are almost useless and will usually consume more laboratory time than an early well-collected specimen.

The specimen collected position will be influenced by the clinical signs, together with knowledge of the pathogenesis of the suspected disease. As a general rule, the epithelial surface which constitutes the viral entry and the primary viral replication site should be collected. The most important specimen, respiratory infections as well as other generalized infections, is a nasal or throat swab or a nasopharyngeal aspirate in which mucus is sucked from the back of nose and throat. The second important specimen, enteric and many generalized infections, is feces. Swabs may be taken from the genital tract, eye, or from vesicular skin lesions. Skin scrapings may be obtained

with a scalpel blade from pock-like skin lesions. Some viruses caused systemic infections can be isolated from blood leukocytes (buffy coat). Biopsy or necropsy specimens may be collected from any proper site of the body. Obviously, tissue taken for the purpose of virus isolation must not be put in formalin or other fixative.

Because of the stability of many viruses, specimens collected for virus isolation must always be cold and moist. The collected plain cotton or dacron swab should be swirled around in a small screw-capped container containing virus transport medium immediately. This medium consists of a salt solution buffered has been added protein and antibiotics (e.g., gelatin, albumin, or fetal calf serum) to protect the virus against inactivation, and bacteria or fungi infection. Probably, the specimen may also be used for bacteria, *Rickettsiae*, chlamydiae, or mycoplasmas isolation, the collection medium must not contain antibiotics (treated with antibiotics later) or a completely separate set of specialized impregnated swabs should be obtained for the isolation of these organisms. The swab stick is then broken or cut off aseptically into the fluid with cap fastened tightly and adhesive tape to prevent leakage. Then the tube is labeled with the identity of the animal/owner, date of collection, and nature of specimen, and immediately send to the laboratory. A properly completed laboratory submission form (seal in a plastic sleeve to prevent it getting wet) that including an informative clinical history, a provisional diagnosis, and a request for a particular test(s) must according to the sample. The specimens should be protected from breaking and refrigerated (but not frozen) in transit, with "cold packs" (4℃ or ice in a thermos flask or styrofoam box. International or transcontinental transport generally requires that specimens be packed in the dry ice (solid CO_2). Governmental and IATA regulations relating to these specimens transport require precautions such as double-walled containers with absorbent padding in case of breakage. A permit should be obtained from the appropriate authorities for interstate and international transportation.

1.2 Initial Processing in the Laboratory

The specimen must be processed immediately in the laboratory at the arrival or refrigerated when this is not practically. For cell culture inoculation, swabs or feces are shaken in fluid medium, and tissue specimens are homogenized in a blender with high-speed. Cell debris and bacteria are deposited in low speed centrifugation, then the supernatant is usually passed through a 0.45μm membrane filter to remove remaining non-viral contaminating organisms. Some of the original sample and of the filtrate is retained at 4℃ or frozen at –70℃ at least until virus isolation assay are completely over. The filtrate is inoculated into cell cultures and/or sometimes into chicken embryos or newborn mice. Clinical specimens managed with this way are suitable to detect viral antigens/DNA/RNA by *in vitro* tests.

The virus titer, the concentration of antigen, or gene copy number, is usually expressed per milliliter of the virus transport material into which the specimen was collected and processed within the laboratory; the dilution factor relative to the high concentrations in which viruses and antigens can occur in secretions/excretions and tissues is generally of minor significance; further the weight/volume of clinical material collected is seldom known, making the correction difficult. For pathogenesis research, the titer in conformed dilution reflect concentration per measured/weighed milliliter of secretion/excretion or gram of tissue collected.

2 Direct Identification of Virus

2.1 Detection of Viruses by Electron Microscopy

Perhaps the most valid method of virus identification is direct visualization of the virus itself. Mostly, an unknown virus is sufficiently characteristic to assign to the correct family according to its morphology, e.g., detection of parapoxvirus in a scraping from a pock-like lesion on a cow's teat; the method may present an immediate definitive diagnosis. Noncultivable viruses could also be detectable by electron microscope. Started in the late 1960s, electron microscope was the means to the discovery in feces of several new groups of previously noncultivable viruses, especially rotaviruses, caliciviruses, astroviruses, and previously unknown adenoviruses and coronaviruses.

As a diagnostic tool, low sensitivity is the biggest limitation of electron microscope. Too much time is required (15min or more) for a skilled microscopist, using a very expensive machine, to scan the grid adequately and detect viruses when the specimen contains less than 10^7 virions per milliliter. Such levels are often surpassed in feces and vesicle fluid instead of respiratory mucus. Feces are first clarified by low-speed centrifugation; the supernatant is then subjected to an ultra-centrifugation, which often through a sucrose cushion, to deposit the virions. Or salts and water can be removed from a drop of virus suspension hanging from a carbon-coated plastic support film by diffusion into agar, leaving the concentrated virions on the film. Specimens are then stained negatively with phosphotungstate, or sometimes uranyl acetate, and scanned by electron microscope.

2.2 Identification of Viruses by Immunoelectron Microscopy

To further identification, the virions may be achieved by adding specific antibody to the specimen, and then the virus-antibody complexes was detected by electron microscope.

To improve the detection, antibody may be labeled with gold. The solid-phase immunoelectron microscopy techniques have been developed via virus-specific antibody bound to the plastic supporting film on the copper grid firstly. Sensitivity can

also be enhanced by a double layering procedure, through staphylococcal protein A (which binds the Fc fragment of IgG) is first bound the film, to which virus-specific antibody and the unknown sample are added sequentially.

2.3 Virus Isolations

The rapid development of new techniques has produced the "same-day diagnosis" which can present virus, viral antigen, or viral nucleic acid in specimens collected directly from the animal. However, most of them could not achieve proper sensitivity of virus isolation in cell culture. Theoretically, a single viable virion from specimen can be grown in cultured cells, thus million folds expanding could produce enough antigen to be characterized at least. Virus isolation still remains the "gold standard", which newer methods must be compared. There is an excitement in isolating viruses. Moreover, it is the only technique that can detect the unexpected, i.e., identify a totally unforeseen virus, or even discover an entirely new agent. Accordingly, even those laboratories well equipped for rapid diagnosis sometimes also inoculate cell cultures in an attempt to isolate the virus. Culture is the only way to supply live virus for further examination, such as antigenic variation. Research and reference laboratories, usually explore for new viruses within the context of emerging diseases; such viruses require a comprehensive characterization to defined. Moreover, large quantities of virus must be cultured in cells to produce diagnostic antigens and monoclonal antibodies for distribution to other laboratories. In nearly cases, vaccine development is currently dependent on the availability of viruses grown in culture. However, the production of the vaccine may not be dependent on culturing because of recombinant DNA technology.

Many viruses may grow well in chick embryos or newborn mice but neither is now commonly used because cell culture is generally the simpler alternative. Mice can be used for arboviruses and rabies virus isolation. The suckling mice less than 24h old are injected intracerebrally and/or intraperitoneally, and then observed for pathognomonic signs up to 2 weeks before euthanizing them for different process such as histopathology, immunofluorescence, immunohistochemistry, or serology.

When all other methods have failed, applying the putative natural host animal to "isolate" viruses is also an option in veterinary medicine. Natural host animals are also applied in other purposes such as pathogenesis and immunity research.

Embryonated hens' eggs are used for the isolation of influenza viruses and many other avian viruses. Indeed, there are several important pathogens which derived from chick embryo tissues that replicate much better in eggs than in cell cultures. According to the viral tropism, the diagnostic specimen is inoculated amniotic cavity, allantoic cavity and yolk sac, or on the chorioallantoic membrane. Evidences of viral growth may be seen on the chorioallantoic membrane (e.g., characteristic pocks caused by pox-viruses)

or to used for viral growth detection (e.g., hemagglutination, immunofluorescence).

3 Immunological Diagnoses

3.1 Identification of Viral Antigen

3.1.1 Immunofluorescence

If antibody is labeled with a fluorochrome, such as fluorescein isothiocyanate, the antigen-antibody complex, when excited with short wavelength light, emits with particular longer light wavelength, which can be visualized upon fluorescence in an optical microscope when light of all other wavelengths is filtered out. Generally, the sensitivity of this method is too low to detect complexes of fluorescent antibody with virions or soluble antigen, hence, the antigen in the test typically takes the form of virus-infected cells. There are two main variants of the technique, direct immunofluorescence and indirect immunofluorescence.

3.1.2 Immunohistochemistry (immunoperoxidase staining)

An alternative method of locating and identifying viral antigen in tissues is to employed antibody coupled to horseradish peroxidase. Then hydrogen peroxide together with a benzidine derivative forms a colored insoluble precipitate. Advantages of this method are that the preparations are transient and it requires much more cheaper equipment than immunofluorescence. A disadvantage is that endogenous peroxidase is present in the cells of many tissues, particularly leukocytes. This may produce false positives, but this problem can be conquered by meticulous technique and adequate controls.

3.1.3 Enzyme-Linked Immunosorbent Assay

Enzyme immunoassays, usually termed as enzyme-linked immunosorbent assays (ELISAs), have markedly used to diagnose virology. Assays can be designed to detect viral antigens or antibodies. This method enables detect less than 1ng of viral antigen per milliliter in specimens collected from the animal. A wide variety of different process is applied, including direct, indirect, and competitive assays. The assays may be processed on a single sample in the veterinarian's clinic or on many hundreds at a time using automated systems in centralized laboratories. Most enzyme immunoassays are solid-phase enzyme immunoassays; the "capture" antibody is attached by adsorption at pH 9.3 to a solid substrate, accompany with polystyrene or polyvinyl microtiter plates. The simplest format is a direct enzyme immunoassay. For most applications, enzyme immunoassays have already replaced the complement fixation test including those for the differentiation of the serotypes of foot-and-mouth disease.

Virus and soluble viral antigens collected from specimen are allowed to bind to the capture antibody. After unbound components are washed away, an enzyme-labeled antiviral antibody (the "detector" antibody) is added in (various enzymes can be linked to antibody; horseradish peroxidase and alkaline phosphatase are the most commonly used). After a washing step, an appropriate organic substrate according to the particular enzyme is added and absorption is based on the color change that follows. The colored production of the action of the enzyme on the substrate is visible by eye. The test can be made quantitative by serially diluting the antigen to obtain an end point or by spectrophotometry to determine the amount of enzyme-conjugated antibody bound to the captured antigen.

A further refinement of the extraordinarily high binding affinity of avidin for biotin. The antibody is conjugated to biotin, a reagent that gives reproducible labeling and does not alter the antigen-binding capacity of antibody. The antigen-antibody complex is recognized with high sensitivity easily by adding avidin-labeled enzyme and substrate. Other modifications of enzyme immunoassays, such as high-energy substrates that release fluorescent, chemiluminescence, or radioactive products, further increase its sensitivity.

Indirect enzyme immunoassays are widely used because of their greater sensitivity and avoid to label each specific antiviral antibody in all of the sample in the laboratory. Here, the detector antibody is unlabeled and a second labeled (species-specific) anti-immunoglobulin is adjoined as the "indicator" antibody; obviously, the antiviral antibodies constituting the capture and detector antibodies, must be raised from different animal species. Alternatively, labeled staphylococcal protein A, which binds to the most mammalian species Fc fragment of IgG, can be used as an indicator in indirect immunoassays.

Monoclonal antiviral antibodies widely used as capture and/or detector antibodies in enzyme immunoassays. Their obvious advantages are that they presented a highly pure, mono-specifically combination, recognizing only one single epitope, are free of "natural" and other extraneous antibodies against host antigens or adventitious agents concurrently infecting the animal, and can be made available in large amounts as reference reagents. It is important to select high affinity monoclonal antibodies, for the low specificity leading to missed adhere some strain in the assay. Indeed, the specificity of this assay can be predetermined by selecting a monoclonal antibody which directed confined to a particular viral serotype or common to all serotypes within a given species or genus.

Enzyme immunoassays have also been applied to in veterinary clinics on single animal specimens.

3.1.4 Immunochromatography

Now the immunochromatography tests become a modeled at home pregnancy tests

used by women in many countries. They differ from traditional systems of measuring antigen-antibody reactions is that the specimen from the indicate animal is made to flow through a filter after which it is immobilized in a membrane at a site where antigen (or antibody) in the specimen comes into contact with a previous antibody (or antigen) already present in the membrane. All controls are included in the membrane as well and results are seen as colored spots or bands, as one of the test reagents is conjugated to colloidal gold or a chromogenic substance. Because all this is done in a plastic device, often with only one step required to activate the reaction, tests are simple and obvious to do and view. Of course, the demonstrate result depends on the quality of the reagents and the conditions of the test. In any case, these tests are usually properly expensive.

3.1.5 Radioimmunoassay

Radioimmunoassay happening before enzyme immunoassay but is progressively being superseded by it. The one significant difference is that the label is not an enzyme but a radioactive isotope such as ^{12}Si and the bound antibody or antigen is measured in a gamma counter. It is a highly sensitive and reliable assay that lends itself well to automation, but the cost of the equipment and the health hazard of working with radioisotopes argue against its use in small laboratories.

3.1.6 Latex Particle Agglutination

Perhaps the simplest of all immunoassays is the agglutination by antigen of small latex beads previously coated with antiviral antibody. The test can be read by eye within a minute or so. Surprisingly, diagnostic kits based on this method have become popular with small laboratories and with some veterinary practitioners. Currently, tests suffer from low sensitivity and low specificity. Thus, false negatives appear unless large numbers of virions are present, therefore this assay for antigen tends to be restricted to examination of feces. False positives, however, appear quite commonly with fecal specimens. If these problems can be overcome, latex agglutination may develop a better reputation for reliability.

3.1.7 Immunodiffusion

In agar gel diffusion, also termed as immunodiffusion assays, a sample suspected to contain viral antigen is placed in a well cut in agar, in the opposite a similar well containing antibody; the reactants diffuse toward each well and form a visible line of precipitation if antigen is present. These assays are seldom used, only just a few examples with antibody is unknown are still used.

3.2 Detections and Quantitation of Antiviral Antibodies

Innovative assay strategies based on know more about the nature of immune response

to viral infection, miniaturization of assay systems upon affecting the cost of reagents (e.g., multiwell plates), automated equipment, and integrated communication systems for fastly import diagnostic laboratory data have all contributed to the revolution diagnose in clinical veterinary and zoonotic virology. Innovative enzyme immunoassays, often based on monoclonal antibodies or viral antigens that produced by recombinant DNA technologies, have led to widespread developing of diagnostic kits by commercial vendors particularly. Thus, assays that are extremely complex in design, engineering, materials, and readout are all reduced to the most straightforward, simple procedures in the clinical setting. These same assays are now incorporated into epidemiologic surveys, disease control programs, and even regional eradication programs based on its sensitivity and specificity. Nowhere has this revolution had more impact on the measurement of the host's immune response to viral infection instead in laboratory serology methods.

For the serologic diagnosis of a viral disease in an individual animal, the classic approach was to test paired sera, i.e., an acute and convalescent serum harvest from the same animal, for the presence of a specific antibody. The acute-phase serum sample is collect as early as possible during the illness, the convalescent-phase sample usually at least 2 weeks later. At this given time line, diagnosis based on this approach is said to be "retrospective". In recent years, this approach has been complemented by serologic methods for detecting specific IgM antibodies in many viral diseases. A presumptive diagnosis may be made on it in a single acute-phase serum specimen in many cases.

3.2.1 Serum Specimens for Serologic Assays

Blood, collected in plain tubes, is needed to clot and the serum is separated. Paired acute and convalescent sera have to be tested simultaneously. For certain tests such measure inhibition of some biologic function of the virus, e.g., virus neutralization or hemagglutination inhibition, serum must be "inactivated" by heating at 56℃ for 30min firstly. Sometimes sera must also be treated additionally, e.g., by absorption with kaolin or treatment with neuraminidase, to remove the nonspecific inhibitors. Prior treatment of the serum is usually not required for assays that simply measure antigen-antibody binding, such as enzyme immunoassays, immunofluorescence, or immunodiffusion. Paired sera are then titrated for antibodies using any of a wide range of available serologic techniques.

3.2.2 Enzyme Immunoassay Enzyme-Linked Immunosorbent Assay

Enzyme immunoassays (EIAs), also termed as enzyme-linked immunosorbent assays (ELISAs), are the serologic assays to choose for the qualitative (positive or negative) or quantitative determination of viral antibodies. Enzyme immunoassays display the advantage that quantitative assays can be based on a single dilution of serum in the colorimetric (optical density) at end point being interpolated by the spectrophotometric

instrument and attached computer ("the reader" in the usual vernacular usage). The most common format of serologic enzyme immunoassays is that 96-well microtiter plates are coated with purified antigen to reduce nonspecific antibody binding positive and negative serums are included in every run as the control to adjusted cutoff limits by the activity in the given run. Kits have been designed for in-clinic testing of single serum samples need three wells for each case: the test sample and a positive and negative control serum.

A more widely used format is that the test serum flows through a membrane filter has three circular areas impregnated with antigen, two of them have already interacted with a positive and a negative serum, respectively. As the test serum flows through the membrane and a washing step is completed, then a second anti-species antibody with an enzyme linked to it is added and the membrane is again rinsed before the added enzyme substrate. The result is read as a color change in the test sample circle, which is compared with the color change in the positive control and no change in the negative control. As a single sample are relatively expensive compared with economies of testing hundreds of sera in a single run in a fully automated laboratory. And it is a great savings in time and effort to send samples to the laboratory. However, the fact that decisions can be made while both client and patient are still in the consulting room, make single tests is more attractive and useful in the immediate clinical management of sometimes critically ill animals.

3.2.3 Serum Neutralization Assay

As virus isolation is considered a gold standard for detection of which other assays must be compared, so that serum neutralization has been the gold standard, when available, for the detection and quantitation of antiviral antibodies. Neutralizing antibody also attracts great interest as it is considered a direct correlate of protective antibody *in vivo*. For the assay of neutralizing antibody, two general procedures are available: the alpha or constant serum-variable virus method and the beta or constant virus variable serum method.

In the alpha procedure, two 10-fold dilution series of virus are set up in a 96-well microtiter plate. To one of the series a constant amount of the test serum, usually diluted 1∶10 or 1∶100, is added and to the other series a negative or perhaps an acute-phase serum from the test animal is added. The assay is based on the difference in virus titer between the two titrations. The amount to which the virus titer is reduced by the test (or convalescent) serum compared with the normal or acute-phase serum is a measure of the amount of antibody in the test serum. In the beta procedure, a constant amount of virus, usually 100 $TCID_{50}$, obtained by previous titration and dilution of a stock virus, is mixed with, twofold serial dilutions, of the test serum. The highest dilution of serum which neutralizes the test dose of virus is the titer of the serum. In

both the alpha and the beta versions, end points may be indicated by cytopathology, immunofluorescence, or even by enzyme immunoassay.

In both alpha and beta systems, the serum-virus mixtures are inoculated into disposable, nontoxic, sterile, with 96 plastic flat-bottomed wells in each of which a cell monolayer has been established. Plates are then incubated until the wells containing the "virus only" controls present evidence of infection. By neutralizing the infectivity of the virus, that protects the cells against viral structure, with highest dilution of antibody that protects cells from the virus demonstrate the titer of neutralizing antibody contained in the serum specimen. Again, statistical methods are used to convert raw end point data into a precise unit of neutralizing antibody.

3.2.4 Immunoblotting (Western Blotting)

Western blotting as a simultaneously tests, but independently measure antibodies against all of the proteins present in a particular virus. There are four key steps of Western blotting. First, purified virus is solubilized with the anionic detergent sodium dodecyl sulfate (SDS) and the constituent proteins are separated into discrete bands by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular mass (Mr). Second, the separated proteins are transferred and immobilize electrophoretically ("blotted") onto nitrocellulose. Third, the test serum is allowed to bind to the viral proteins on the membrane. Fourth, their presence is usually demonstrated by using a radiolabeled or an enzyme-labeled antispecies antibody. Thus, immunoblotting are permits to show antibodies to some or all of the proteins of any given virus and can be used not only to discriminate infection with closely related viruses sharing certain antigens, but also to monitor the presence of antibodies during different stages of infection. The versions of dip stick in which membrane strips containing separated viral proteins are exposed to the serum and the strips are "developed" for consulting room use.

3.2.5 Indirect Immuno-Fluorescence Assay

Indirect immune-fluorescence assays for the detection and quantitation of antibody have been widely used in many clinical and research laboratories for many years. This assay, which is similar to enzyme immunoassays and radioimmunoassays in principle, involves two steps: first the serum is layered over the substrate that containing the virus (usually virus-infected cells fixed on glass microscope slides); following incubation of the slide to allow binding antibodies in the serum to the viral substrate and washing to remove unbound antibody, then fluorescein isothiocyanate-conjugated antiglobulin (matched to the species from which the serum specimen was obtained) is added. Slides are examined in a microscope-equipped with an ultraviolet light and filters that transmit only light emitted from the fluorescent label. A positive reaction is view as apple-green color localized where viral antigen is present in the infected cell substrate, only when the serum sample contains antibodies to the virus in question. This method

is rapid and may be useful in detecting antibodies that are difficult or even impossible to demonstrate by any other means, but the nonspecific fluorescence frequently confuses interpretation of results.

3.2.6 Hemagglutination-Inhibition Assay

For those viruses that could hemagglutinate one or another species red blood cells, such as many of the arthropod-borne viruses, influenza and parainfluenza viruses, hemagglutination-inhibition assays are widely used. For detecting and quantitating antibodies in the serum of animals, these methods are sensitive, specific, simple, reliable, and quite inexpensive. Assays are procedured in 96-well microtiter plates similar to that of a constant virus-variable serum dilution neutralization assay. Serum is serially diluted in the wells of the microtiter plate, usually in twofold steps, and to each well a constant amount of virus, usually four or eight hemagglutinating units, is added. The highest dilution of serum that could inhibit the agglutination of the red blood cells by the standardized amount of virus represents the hemagglutination-inhibition titer of this serum.

3.2.7 Immunodiffusion

Agar gel diffusion assays were used for the specific diagnosis of a number of diseases, including hog cholera, equine infectious anemia (Coggins test), and bovine leukemia for several years. This because the assays require only very simple, readily available materials. A further advantage of this assays was that the antigen used could be a relatively crude preparation (e.g., spleen pulp from an infected animal). As a commonly used format is six peripheral wells and a single central well, all 3mm in diameter, were cut in agar with a punch. Antigen was placed in the central well, a known positive serum was placed in every other peripheral well, and three suspect sera from animals were placed in the remaining wells. Precipitin lines were formed by combination of antibodies diffusing from the wells containing positive control sera and antigen diffusing from the central well. Precipitin lines produced by any of the test sera and the antigen indicated that it contained antibody; precipitin lines from test sera that fused with lines produced by the positive control sera were considered as a confirmed presence of specific antibody.

3.2.8 IgM Class-Specific Antibody Assay

A rapid antibody-based diagnosis of a disease can be made based on the single acute-phase serum by demonstrating virus-specific antibody of the IgM class. As IgM antibodies appear early after infection but drop to low levels within 1-2 months and generally completely disappear within 3 months, they are usually as indicator of recent (or chronic) infection. Moreover, if they were found in a newborn animal, they are the diagnostic of intrauterine infection, because maternal IgM could not cross the placenta.

The most common method is IgM antibody capture assay, in which the viral antigen is bound to a solid-phase substrate a microtiter well. The test serum is allowed to react with the substrate and then specific IgM antibodies is "captured" by the antigen and detected with labeled anti-IgM antibody matched to the species from which the sample was obtained. All of the immunoassays described earlier can easily be rendered in IgM class-specific enzyme immunoassays, radioimmunoassays, and indirect immunofluorescence assays have proven generally useful. The class-specific immunoassays can also be designed to measure IgG subclasses (useful in measuring colostral antibodies in cattle and sheep), IgA (useful in measuring mucosal immunity), and IgE (useful in measuring IgE mediated hypersensitivity states). Such immunoglobulin class-specific antibody capture assays have a much more important place in the armamentarium of diagnostic laboratories.

4 Identification of Viral Nucleic Acid

4.1 Hybridization Methods

The detection of specific viral nucleic acid by hybridization using a labeled viral DNA and RNA probes to detect nucleic acid has been widely applied for rapid diagnosis, although the methods have already been superseded in many instances by the polymerase chain reaction.

The principle of nucleic acid hybridization is single-stranded DNA will hybridize by hydrogen-bonded base pairing to another single strand of DNA (or RNA) of a complementary base sequence. Thus, these two strands of the target DNA molecule are first separated by heating, then following cooling, allowed to hybridize with a labeled single-stranded DNA or RNA probe present in excess. The reaction can be completed in solution, which is useful for determining kinetics of annealing or stoichiometry of this reaction, from which can be calculated the percentage identity between the two sequences calculated from the kinetics of annealing. The value set for annealing, especially temperature and ionic strength determine the degree of discrimination (stringency) in the test. Under conditions of a low stringency, a number of mismatched base pairs are tolerated, whereas at high stringency such a hetero-duplex is unstable.

Another major factor determining the specificity of test is the nature of the probe itself. This may correspond in length to a whole viral genome or single gene or much shorter nucleotide sequence which deliberately chosen to represent a variable or a conserved region of the genome, and depending on whether it is intended that the probe be type specific or more wider. The oligonucleotide sequence that intended as a probe is produced by chemical synthesis or by cloning in a bacterial plasmid or bacteriophage.

Traditionally, radioactive isotopes such as ^{32}P and ^{35}S were used to label nucleic

acids or oligonucleotides and as probes for hybridization tests, with the signal being read by counting in a spectrometer or autoradiography. The trend is now toward nonradioactive labels. Some of these (e.g., fluorescein or peroxidase) can produce a signal directly, whereas others (e.g., biotin or digoxigenin) act indirectly by binding to another labeled compound, which then also emits a signal. Biotinylated probes can be adjoined with various types of readout, including an avidin-based enzyme immunoassay. The luminal, as chemiluminescent substrates, are also being widely exploited. Indeed, we are witnessing a development of diagnostic kits for various diseases, many of them based on novel labels and/or methods of readout.

4.2 Dot Bot (filter hybridization) Methods

The most popular hybridization methods are two-phase systems, usually known as filter hybridization. In the simplest format, dot (blot) hybridization, DNA or RNA was extracted from virus or infected cells, is denatured and then spotted directly onto a charged nylon or nitrocellulose membrane whereas it binds tightly on baking. The single-stranded DNA or RNA probe is then hybridized to the target nucleic acid *in situ* on the membrane, and the unbound probe is washed away. The signal generated by bounding probe is measured by auto-radiography as its radioactive or by the formation of a colored precipitate if an enzyme-labeled probe is used. By choosing RNA as a probe, sensitivity can be optimized and the incidence of false positives reduced by treating the filters with RNase before counting.

4.3 *In situ* Hybridization Methods

In situ hybridization has been widely used by pathologists to screen animals with a persistent infections or viral-induced cancers for appearance of integrated or non-integrated copies of the viral genome. As previously demonstrate, frozen sections on slides are probed, and the intracellular location of viral nucleic acid sequences is marked by auto-radiography or immunoperoxidase cytochemistry.

4.4 Southern Blot Hybridization Methods

The Southern blotting has been of great significance and wide application. The restriction is used to cleave DNA into fragments in various size and number which depending on the location and number of restriction endonucleases. The fragments are then separated in electrophoresis on an agarose or polyacrylamide gel. After staining with ethidium bromide (gold view) to reveal the position of fragments, the gel is treated successively with acidic and basic solutions to depurination and denatures the DNA, which is then transferred by electrophoresis, diffusion, or other methods

("blotting") onto nylon or nitrocellulose membrane. Individual bands (fragments) are appeared by hybridization of a labeled DNA or RNA probe followed by auto-radiography or a color development process. While Northern blotting for detection of the RNA follows a similar principle.

4.5 Polymerase Chain Reaction (PCR)

PCR has been applied in many areas, including genetics, evolutionary biology, forensic medicine, parentage determination, and the diagnosis of infectious diseases. The great potential of the PCR to specifically amplify minimal amounts of target DNA was particularly appealing for diagnose of the viral diseases. It is referenced that "the polymerase chain reaction detects a needle in a haystack and makes a haystack out of the needle". It must remind that polymerase chain reaction technology amplifies the target gene sequence, as itself is not a diagnostic test; specific detection of the amplified product is then by one of the techniques that described later.

4.6 Viral Genomic Sequencing

Direct viral genomic sequencing has become a major mean in viral diagnostics, especially in protocols that based on viral nucleic acid amplification via the polymerase chain reaction. Sequencing methods in viral diagnostics are the same as in the applied of molecular biology; in the diagnostic setting, where large numbers of suspect specimens and where diagnostically unique sequences must for practical reasons, be detected quickly. The critical primers for PCR, automated sequencers, and computer analysis of partial sequence information is the key to overall value.

4.7 Oligonucleotide Fingerprinting and Restriction Endonuclease Mapping

For most routine diagnostic purposes, to "type" the isolate is usually not necessary. Sometimes, however, important epidemiologic information can be obtained much more by identify differences between subtypes within a given type. This may be completed using the PCR and partial sequencing, oligonucleotide fingerprinting of viral RNA, or the determination of a restriction endonuclease fragment patterns (fingerprints) of viral DNA. Viral DNA originated from virions or infected cells can be cut with appropriately restriction endonucleases and the fragments separated by agarose gel electrophoresis. When stained, a restriction endonuclease fingerprints are obtained. The method has been applied in all DNA virus, particularly in epidemiologic studies. Depending on the viral family, the advantage of these methods is that different isolates of the same virus are distinguishable. Minor degrees of genetic drift, often not appeared in serologic differences, can sometimes be detected in this method.

Review Questions

1. Try to describe the basic principles of the diagnosis of viral diseases.
2. How to collect samples for virus detection?
3. What are the direct detection methods of viruses?
4. What are the immunological methods for viral detection?
5. What are the detection methods for viral nucleic acid?
6. What are the rapid detection methods for viral detection?
7. How to determine the unit of viral infection?
8. What are the high-throughput methods for viral detection?

Chapter 22 Double-Stranded DNA Viruses

Synopsis

The viral genome of *Poxviridae*, *Asfarviridae*, *Iridoviridae* and *Adenoviridae* are linear double strands of DNA, while the viral genome of *Nimaviridae*, *Baculoviridae*, *Polyomaviridae* and *Papillomaviridae* are circular double strands of DNA. The structure of poxvirus is more complex. There is no cross infection between the mammalian poxvirus and the bird poxvirus. Variola virus can cause smallpox in humans. Vaccinia virus has pathogenicity to many kinds of animals. Sheeppox virus and goatpox virus can cause disease in sheep and goat. Fowlpox virus main infect chickens. Many adenoviruses have agglutinin, which can agglutinate red blood cells. Infectious canine hepatitis virus and egg drop syndrome virus are important pathogenicity to the host. The genome of herpes virus is also doublestrands of DNA, but herpes virus is list in *Herpesvirales*.

1 *Poxviridae*

Poxviruses are large DNA viruses that are capable of infecting both vertebrates and invertebrates. Diseases caused by poxviruses often affect the skin, although some cause systemic infections in which clinical signs of disease may or may not be apparent. Poxvirus infections often cause proliferative epithelial lesions in birds, whereas papular and/or pustular epithelial lesions are characteristic of poxvirus-infected mammals and only in some infections do these become proliferative.

Poxviruses are large and complex and replicate in the cell cytoplasm. Of the two subfamilies of *Poxviridae*, members of the *Chordopoxvirinae* infect vertebrates, whereas viruses within the *Entomopoxvirinae* infect insects. There are eight distinct genera in the *Chordopoxvirinae*, and there is considerable antigenic cross-reactivity between viruses in the various genera. *Chordopoxvirinae* have pleomorphic, roughly brick-shaped virions [(220-450) nm × (140-260) nm] that include a lipoprotein surface membrane and a biconcave or cylindrical core. The genome is enclosed in the core and consists of a single, very large (up to 130kb) molecule of double-stranded DNA (dsDNA). The viral genome encodes 150-300 proteins, approximately 100 of these are

contained within virions, and many enzymes involved in virus replication are included.

Significant Genera (Subfamily: *Chordopoxvirinae*) in veterinary medicine include *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Suipoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Yatapoxvirus*.

1.1 *Orthopoxvirus*

1.1.1 Disease

Orthopoxvirus infections cause a variety of papular human and animal diseases. Papules are raised by epithelial proliferation that often ulcerates rapidly. Lesion development may be confined to specific areas of the skin or the infection may be generalized. Skin lesions often appear first as a rash or papule followed by pustule formation, and then rapidly crust and scab.

1.1.2 Etiologic Agent

Orthopoxviruses induce diseases including vaccinia, cowpox, camelpox, monkeypox, ectromelia, and variola (human smallpox). They are morphologically indistinguishable, and there is considerable serological cross-reactivity between the viruses within this genus (Fig. 22-1). Orthopoxviruses contain a hemagglutinin. The various orthopoxviruses can be distinguished on the basis of their genomic DNA sequence, and by pock formation on chick embryo chorioallantoic membranes (pock appearance and the highest temperature at which they develop). The animal host-species is sometimes predictive of the type of orthopoxviruses, but not invariably so.

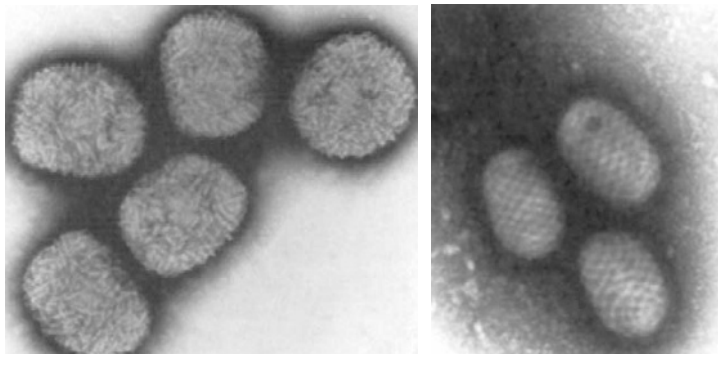


Fig. 22-1 Electronic photograph: vaccinia virus (left), a member of the genus *Parapoxvirus* (right) (MacLachlan and Dubovi, 2011)

1.1.3 Host-Virus Relationship

Transmission of orthopoxviruses occurs by contact, aerosol, arthropod bites, or exposure to fomites. The poxviruses tend to be very stable in the environment.

Both cellular and humoral responses are critical to immunity against poxviruses. Cellular immunity facilitates destruction of virus-infected cells and is important in confining the virus to a localized area. Defects in cellular immunity permit widespread distribution of virus, resulting in generalized disease. Neutralizing antibodies are important in recovery from infection. Immunity appears to be of long duration.

1.1.4 Laboratory Diagnosis

Diagnosis of orthopoxvirus infection can be made by electron microscope of virus extracted from lesions or by viral isolation. Isolation may be achieved by inoculation (scarification) of laboratory animals (especially rabbits), of cell cultures, or of the chorioallantoic membrane of embryonated chicken eggs. The latter procedure has been widely used to differentiate viral types.

1.1.5 Vaccinia

Vaccinia virus is the genus prototype and was used to vaccinate humans against smallpox (variola) which was a devastating disease prior to its eradication in world. Vaccination of humans with vaccinia was responsible for the global elimination of smallpox. Vaccinia was originally believed to be cowpox, but its origin is uncertain since it is quite distinct from its purported ancestor. Buffalopox and rabbit pox (an infection of laboratory rabbits) are caused by viruses that are closely related or identical to vaccina.

Vaccinia may infect cattle via handling (milking) by infected personnel; infected cattle can also infect personnel. Vaccinia infection of cattle can produce lesions that are indistinguishable from those caused by cowpox virus; these occur on the teats and udder and appear as small papules that progress to pustules, and finally, exudative scabs. Like vaccina, buffalopox virus can infect humans.

1.1.6 Cowpox

Cowpox virus is closely related to vaccinia virus, but antigenically distinct. Cowpox in cattle is characterized by papular, pustular, and crusty exudative lesions on the udder and adjacent areas of skin. Rodents serve as reservoir hosts of the virus, which also is contagious to humans, cats (domestic and large wild cats), and a variety of other animal species.

1.1.7 Camelpox

Camelpox is a severe, generalized pustular disease of the skin of affected camels. It is of economic importance in countries with an indigenous camel population, including those of the Middle East, Africa, and parts of Asia. Disease occurs principally in younger animals and is characterized by fever, generalized rash, and sequential development of papular, pustular, and scabbing lesions on the limbs, neck, and head.

1.1.8 Mousepox (Ectromelia)

Mousepox occurs in laboratory mice, although the virus is excluded readily from appropriately managed facilities. Mousepox is a severe, rapidly fatal disease characterized by extensive necrosis of the liver of affected mice. A more chronic form also occurs, characterized by necrosis of the distal extremities (feet, tail and snout) of affected mice.

1.2 *Parapoxvirus*

Parapoxviruses infect a wide variety of animals, principally ungulates and domestic livestock, although several are also contagious to humans (zoonoses). Parapoxviruses typically produce localized papular and proliferative cutaneous lesions; examples include bovine papular stomatitis virus, orf virus, and pseudocowpox virus.

Parapoxviruses are morphologically unique in that an organized tubular, threadlike structure forms a crisscross pattern on the virion surface. The viruses are all antigenically related, but the envelope contains antigenically distinct epitopes. The viruses are stable for long periods of time at ambient temperatures.

1.2.1 Bovine Papular Stomatitis

1.2.1.1 Disease

Bovine papular stomatitis is characterized by the presence of papules, which are raised epithelial proliferations, on the muzzle, nares, lips, buccal cavity, dental pad, palate, and tongue. Hyperemic papules, with central necrosis and concentric colored rings are highly characteristic. The disease is generally of minor importance, although it occasionally can mimic important vesicular diseases of cattle like foot-and-mouth disease and vesicular stomatitis.

1.2.1.2 Host-Virus Relationship

Bovine papular stomatitis virus infection of cattle occurs worldwide; humans sometimes are infected with the virus. It is believed to persist in a latent state in cattle. Virus is present in oral and nasal secretions and transmission may occur by direct contact.

1.2.1.3 Laboratory Diagnosis and Control

Bovine papular stomatitis must be differentiated from important vesicular diseases. Virus may be isolated on cell cultures or directly visualized in skin scrapings or biopsy material by electron microscope. Vaccines have not been developed.

1.2.2 Contagious Ecthyma

1.2.2.1 Disease

Contagious ecthyma virus is the etiologic agent of contagious ecthyma of sheep and

goats (synonyms include scabby mouth, contagious pustular dermatitis of sheep, sore mouth, infectious labial dermatitis, and orf). Orf is the term used to describe the disease in humans. Contagious ecthyma of sheep and goats initially is characterized by the appearance of papules and vesicles on the lips, mouth, interdigital skin, genitalia, and udder. Papules and vesicles rapidly progress to pustules, followed by scab formation. Lesions on the lips interfere with suckling or grazing, resulting loss of condition of affected animals. Young animals are most likely to be affected, and the virus rapidly spreads among susceptible animals.

A related parapoxvirus causes ulcerative dermatosis of sheep, a disease characterized by the presence of ulcerated papules on the lips, face, legs, feet, and external genitalia. Genital infections are transmitted by sexual contact.

1.2.2.2 Host-Virus Relationship

In addition to sheep and goats, contagious ecthyma virus may also infect humans (zoonoses), deer, and perhaps other species. Related viruses infect chamois and seals. The virus is distributed worldwide and is maintained in nature by persistent infections of sheep, as well as survival of the virus for prolonged periods in dried scabs. The virus is readily transmitted on rough feed that causes erosions and ulcers in the oral cavity of affected sheep.

1.2.2.3 Laboratory Diagnosis and Control

Presumptive diagnosis is based upon clinical observations and pathology (e.g., epithelial proliferation with intracellular edema leading to cytoplasmic vacuolation which is often with the presence of cytoplasmic inclusion bodies). Virus can be identified by electron microscopy and isolated from embryonic sheep skin or testicular cells.

Vaccination is used to control the disease in endemic areas, and immunity is considered to be long term in duration. Virulent virus is used in vaccination by scarification of the skin in areas not affected by the disease, a potential adverse impact of this approach is that it does ensure perpetuation of the virus in nature.

1.2.2.4 Pseudocowpox

Pseudocowpox is a mild disease of cattle that particularity affects lactating animals. Teat lesions develop as papules with an ulcerated center that becomes encrusted. This results in a pathognomonic scab with a ring or horseshoe appearance. The causative parapoxvirus is closely related to bovine papular stomatitis virus, and can infect humans via direct contact, producing so-called milker's nodules.

Pseudocowpox occurs in most countries but has little economic significance, because most infections are either asymptomatic or very mild. Immunity is of short duration and recurrent infections are common. Cattle occasionally develop chronic infections.

1.3 *Avipoxvirus*

Avipoxviruses infect many avian species. Fowlpox virus is the genus prototype.

1.3.1 Disease

Pox is a common disease in commercial chickens and many different species of pet and wild birds. Fowlpox causes decreased egg production and increased mortality affected on commercial premises. The lesions in affected birds are designated as either cutaneous or diphtheritic. Cutaneous lesions are characterized by nodular, wart-like proliferations of hyperplastic epithelium that involve head skin (comb, wattles, corner of the mouth, nostrils and eyes). The diphtheritic form is characterized by proliferative lesions on the mucous membranes and may extend into the sinuses; involvement of the larynx and trachea results in dyspnea and rales. The lesions are characterized by inflammation, epidermal hyperplasia and development of eosinophilic intracytoplasmic inclusion bodies.

Mortality in affected commercial chickens and turkeys, pigeons, and psittacines is typically low, whereas infection in canaries is almost always fatal.

1.3.2 Etiologic Agent

The avipoxviruses are antigenically related but are differentiated by host range, serological tests, plaque formation in cell cultures, and pock formation on the chorioallantoic membrane of embryonated chicken eggs. The viruses are also distinguished by analysis of their genomic DNA. Like other poxviruses, avipoxviruses are highly resistant to desiccation.

1.3.3 Host-Virus Relationship

Avipoxviruses have a worldwide distribution. Viral transmission can occur by direct contact or mechanical transmission. Virus can survive for long periods in scabs, leading to cutaneous or respiratory infection. The viruses also can be transmitted by biting insects, especially mosquitoes and mites. Chronic infections of individuals may contribute to viral persistence on a given premise.

Humoral and cellular immune responses develop following infection and apparently confer long-term immunity. Maternal antibody does not confer protection to hatched chicks.

1.3.4 Laboratory Diagnosis

Diagnosis of avian poxvirus infections is based on clinical signs and histopathology and electron microscope. Virus can be isolated by inoculating susceptible birds, the chorioallantoic membrane of embryonated chicken eggs and cell cultures (chicken and

duck embryo fibroblasts).

1.3.5 Treatment and Control

Control of avian poxvirus infections can be aided by providing adequate nutrition, housing, and insect control among birds. Live virus vaccines are used to immunize birds against pox; these vaccines induce a mild disease that leads to protective immunization. Recombinant vaccines recently have been developed and provide a vector for the incorporation of heterologous genes for protective immunization.

1.4 *Capripoxvirus*

The genus *Capripoxvirus* includes sheeppox virus, goatpox virus, and lumpy skin disease virus (Neethling virus). Sheeppox virus is the genus prototype. The viruses are more elongated than other poxviruses, and measured about 115nm×194nm. The viruses are closely related but differ antigenically. Infection with any virus produced cross-protection to heterologous virus, with the exception of certain strains of goatpox virus. The viruses are predominantly host-specific, though some strains produce lesions in sheep, goats and/or humans.

1.4.1 Sheeppox and Goatpox

1.4.1.1 Disease

Sheeppox and goatpox are important poxvirus diseases of livestock, although infection with these viruses can produce clinical signs that range from inapparent to a severe generalized condition. Animals of all ages are susceptible but disease is more severe in younger animals, especially in endemic areas. Breed and immune status also affect disease severity. Both sheeppox and goatpox viruses cause systemic infections of susceptible hosts, and viremia occurs soon after infection. Virus is disseminated to the skin, lymphnodes, and multiple organs, including the spleen, kidneys and lungs. The first clinical signs of disease include pyrexia, rhinitis and conjunctivitis, followed by varying degrees of lesion development on external nares, lips, tongue, gums and skin (especially where wool or hair is minimal). Skin lesions are initially papules and vesicles that rapidly become pustular and necrotic with scab formation. Infected epithelial cells may contain eosinophilic cytoplasmic inclusion bodies. Lesions may also develop in the respiratory and alimentary tracts, liver, kidneys and other organs. A nodular form of the disease has been referred to as stone pox. Increased mortality rates are associated with secondary bacterial infections and disseminated internal lesions.

1.4.1.2 Host-Virus Relationship

Virus is transmitted through aerosol, direct contact and possibly arthropod vectors. Viral persistence is probably due to the survival of virus in scabs and its transimission

to susceptible animals within an endemic area. Antibodies, including those with viral-neutralizing activity, develop within one week of lesion development. Immunity is considered to be lifelong.

1.4.1.3 Laboratory Diagnosis

Histopathology, fluorescent antibody staining, electron microscopy and serology can help confirm clinical diagnosis. Virus can be isolated in cell cultures of ovine, bovine and caprine origin. The virus grows relatively poor in embryonated chicken eggs as compared to orthopoxviruses and parapoxviruses.

1.4.1.4 Treatment and Control

Preventative measures such as import restrictions are practiced by countries free of the virus. Attenuated and inactivated viral vaccines are used in endemic areas.

1.4.2 Lumpy Skin Disease

1.4.2.1 Disease

Lumpy skin disease of cattle and buffalo is caused by Neethling virus closely related to sheeppox virus and goatpox virus. Infection is followed by viremia and fever, with the subsequent formation of nodular lesions on the skin (predominantly on the neck, face, muzzle, brisket, flank legs, perineum, and scrotum) and internally in the respiratory, digestive and reproductive tracts. The cutaneous lesions are firm, raised and circumscribed, and they often undergo central necrosis and ulceration. Similar lesions occur within the epithelium lining the upper respiratory and alimentary tracts. The virus causes a disseminated infection that result in vasculitis, lymphangitis and lymphadenopathy.

1.4.2.2 Host-Virus Relationship

Neethling virus is confined to Africa and Madagascar. Viral transmission probably occurs by direct contact, aerosol and insect vectors. Persistence of virus in nature is probably similar to that described for sheeppox and goatpox, but it is proposed that buffalo may serve as viral reservoirs in some areas.

1.4.2.3 Laboratory Diagnosis

Definitive diagnosis requires fluorescent antibody staining or electron microscopy of tissues, viral isolation in cell cultures (lamb and calf kidney) or embryonated chicken eggs (development of pocks on the chorioallantoic membrane), or serology.

1.4.2.4 Treatment and Control

Treatment is confined to supportive therapy. Vaccination results in long-term immunity. Countries free of lumpy skin disease have imposed import restrictions on stock from infected countries.

1.5 *Suipoxvirus*

Swinepox virus is the only member of the genus *Suipoxvirus*. It is the cause of swinepox.

1.5.1 Disease

Swinepox occurs in pigs of all ages, although younger animals are more commonly affected. The disease is very mild usually, with virtually no mortality. Lesions typically develop on the abdomen and inner thighs and sometimes on other areas of the skin. Lesion development progresses from papular through pustular and scabbing stages. Microscopic lesions are similar to other poxvirus lesion and include hyperplasia of epidermal cells with hydropic degeneration and the formation of eosinophilic intracytoplasmic inclusion bodies. Inflammatory cells are found in the dermis. Vaccinia can cause an identical disease in pigs.

1.5.2 Host-Virus Relationship

Swinepox has a worldwide distribution. Virus may be spread by direct contact or mechanically by lice; transplacental transmission may also occur. The virus persists for long periods in dried scabs. Swine develop immunity in the absence of detectable neutralizing antibody, suggesting that local humoral immunity or cell-mediated immunity is important in viral clearance and protection against reinfection.

1.5.3 Laboratory Diagnosis

The skin lesions of swinepox are highly characteristic, and often are associated with lice infestation. Laboratory confirmation may be required to rule out other important vesicular diseases. Definitive diagnosis can be obtained by fluorescent antibody staining, electron microscopic evaluation of lesions for the presence of poxviruses, or virus isolation in porcine cell cultures.

1.5.4 Control

The best way to control of swinepox is the elimination of external parasites. Vaccination is not commonly used.

2 *Adenoviridae*

The *Adenoviridae* have five genera: *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus* and *Siadenovirus*. Many members of *Adenoviridae* can infect human and animals, and some members can cause tumor in infected host.

Adenoviruses have been isolated from many species of animals, but it is likely that additional animal adenoviruses exist that have not yet been identified. The host range

of individual adenoviruses is highly restricted. Although adenovirus infections of animals are often asymptomatic or subclinical, some adenoviruses are pathogenic and cause respiratory and/or systemic diseases.

Two important genera of *Adenoviridae* are *Mastadenovirus* which includes adenoviruses that infect mammals and *Aviadenovirus* which includes adenoviruses that infect birds. Members of these two genera do not share a common group antigen. Adenoviruses are non-enveloped icosahedrons (Fig. 22-2), which are 70-90nm in diameter and composed of 252 capsomers. Extended fibers project from the virion surface (Fig. 22-2). The genome of adenoviruses is a large molecule (26-45kb) of double-stranded DNA. Approximately 40 different proteins are encoded by the adenovirus genome. Adenoviruses replicate in the nucleus of infected cells.

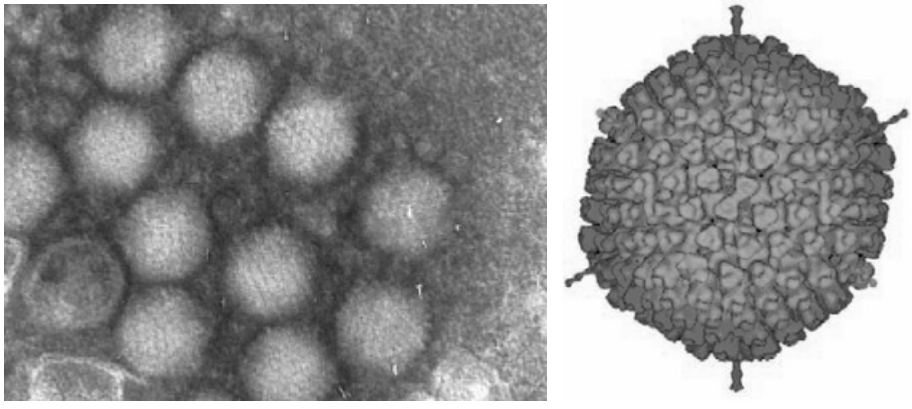


Fig. 22-2 Electron micrograph of adenoviruses (left) (Markey *et al.*, 2013) and reconstruction of human adenovirus 2 from cryo-electron micrograph (right) (MacLachlan and Dubovi, 2011)

2.1 Canine Adenovirus 1

2.1.1 Disease

Infectious canine hepatitis (ICH) is a disease of dogs caused by canine adenovirus 1 (CAV-1). Although once as an important disease of dogs, ICH is increasingly rare in most countries, perhaps as a result of widespread vaccination. The majority of infections are asymptomatic, but the disease in susceptible dogs is characterized by fever, hepatic necrosis and widespread hemorrhage as a consequence of vascular injury. Affected dogs may exhibit increased thirst, anorexia, tonsillitis, petechial hemorrhages on the mucous membranes, diarrhea and be reluctant to move. During the acute phase of illness, dogs may also develop conjunctivitis and photophobia. Severe ICH is most likely to occur in pups that are not immune to the disease.

Most dogs that survive acute ICH recover uneventfully, but transient corneal edema may occur in some convalescent animals after acute signs disappear. The CAV-1 also

has been implicated as a cause of chronic progressive hepatitis and interstitial nephritis, but its role in spontaneous occurrence of these disorders in dogs is highly conjectural (CAV-1 is most unlikely to be a significant cause of either of these two common diseases of dogs).

2.1.2 Etiologic Agent

CAV-1 is antigenically related but distinct from canine adenovirus 2 (CAV-2). CAV-1 is morphologically similar to other adenoviruses, but antigenically distinct.

2.1.2.1 Resistance to Physical and Chemical Agents

CAV-1 is resistant to ether, alcohols and chloroform. It is stable for at least 30min at a wide range of pH 3-9, and is also stable in soiled material at room temperature for several days. Viral infectivity is lost after heating for 10min at 50-60℃. Steam cleaning and treatment with iodine, phenol, sodium hydroxide, or lysol are effective means of disinfection.

2.1.2.2 Infectivity for Other Species and Culture Systems

CAV-1 causes clinical disease in dogs and other canids (wolves, foxes and coyotes). Skunks and bears are also susceptible. Infection in foxes can manifest as encephalitis. CAV-1 replicates well in canine kidney cells.

Although some strains of CAV-1 and CAV-2 are oncogenic in inoculated hamsters, these viruses have not been associated with neoplastic disease in dogs.

2.1.3 Host-Virus Relationship

2.1.3.1 Distribution, Reservoir and Transmission

Infectious canine hepatitis has a worldwide distribution, although clinical disease is increasingly rare. The infection is spread through the urine of infected dogs. Dogs may retain virus in their kidneys and shed it in urine for months after infection.

2.1.3.2 Pathogenesis and Pathology

Following aerosol infection, the virus localizes in the tonsils and spreads to regional lymph nodes and then to the systemic circulation. Viremia results in rapid dissemination of virus to all body tissues and secretions, including saliva, urine and feces. The virus has a particular tropism for hepatocytes and endothelial cells, which produces the characteristic signs of the disease. Virus-induced injury to endothelial cells leads to consumptive coagulopathy (disseminated intravascular coagulation) and a generalized bleeding tendency (hemorrhagic diathesis) that is reflected by abnormal clotting parameters.

Dogs that die during the acute phase generally have edema and hemorrhage of superficial lymph nodes and cervical subcutaneous tissue. The abdominal cavity often

contains fluid, which may vary in color from clear to bright red. Hemorrhages are present on all serosal surfaces. A fibrinous exudate may cover the liver, which can be swollen and congested. The gall bladder is characteristically edematous. Large characteristic intranuclear inclusion bodies may be present in hepatocytes, vascular endothelium and macrophages.

The ocular lesions that develop in some dogs that recover from ICH are the result of deposition of immune complexes within the ciliary body of the eye.

2.1.3.3 Host Response to Infections

Recovery from ICH, regardless of the severity of illness, results in long-lasting immunity that is likely lifelong. Recovered animals have high titers of neutralizing antibody to CAV-1.

2.1.4 Laboratory Diagnosis

The diagnosis of ICH can be confirmed by serologic testing (complement fixation, HI test and ELISA) to demonstrate rising titers of antibody to CAV-1, PCR detection of viral nucleic acid or virus isolation from affected tissues, or immunohistochemical staining of tissues with CAV-1 specific antibodies.

2.1.5 Treatment and Control

Therapy for dogs that develop ICH involves supportive and symptomatic treatment. Control is by vaccination and strict sanitation of affected premises with quarantine of exposed dogs. Available vaccines include both inactivated and modified live virus varieties, including CAV-2 vaccines that induce heterologous protection against CAV-1. CAV-2 vaccines do not induce immune complex uveitis in dogs. Care must be exercised to ensure that maternal antibodies do not interfere with active immunization of pups, because vaccination success is directly related to the level of neutralizing antibody.

2.2 Canine Adenovirus 2

Canine adenovirus 2 (CAV-2) has been isolated from dogs with acute cough and is one of several infectious agents implicated in infectious tracheobronchitis. Experimental infection produces mild pharyngitis, tonsillitis and tracheobronchitis, and virus persists in the respiratory tract for up to 28 days. Unlike CAV-1, CAV-2 does not produce generalized disease, is not excreted in the urine, and does not produce renal or ocular lesions. CAV-2 is antigenically related to CAV-1, and CAV-2 vaccines have been developed as ICH vaccine since they do not produce postvaccinal ocular lesions.

2.3 Avian Adenoviruses

Adenoviruses infect poultry and other bird species worldwide. Although adenoviruses

are often isolated from apparently normal birds, specific diseases are also associated with adenovirus infections. These include egg-drop syndrome, a disease of both wild and domestic birds that is characterized by production of eggs that lack shells or have abnormally soft shells, and hemorrhagic enteritis of turkeys and marble-spleen disease of pheasants, which are similar diseases characterized by intestinal hemorrhage and enlargement of the spleen of affected birds.

Review Questions

1. What are the viruses with double-stranded DNA?
2. Try to draw the structural model of the poxvirus and adenovirus.
3. What are the important poxvirus and adenovirus that have pathogenicity?
4. Try to describe the differences in the host spectrum of the poxvirus.
5. Try to describe the common characteristics of poxvirus.
6. Try to describe the common characteristics of adenovirus.
7. Try to describe the microbiology diagnose of poxvirus disease.
8. Try to describe the microbiology diagnose of adenovirus.

Chapter 23 *Herpesvirales*

Synopsis

Herpesviruses are distributed in terrestrial and aquatic animals. In 2012, *Herpesvirales* was confirmed in ninth report of the ICTV. Currently, *Herpesvirales* has three families, *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*, which have the characteristics of persistent infection. *Herpesviridae* has three subfamilies, many of which have important pathogenicity to humans and animals. Pseudorabies virus, duck plague virus, avian infectious laryngotracheitis virus, infectious bovine rhinotracheitis virus and Marek's disease virus are important pathogenic agents of animals. These diseases caused by these virus are noted disease in OIE rules.

Herpesvirales is a new established order, have three families: *Alloherpesviridae*, *Herpesviridae*, *Malacoherpesviridae*. Many members of *Herpesvirales* are important in humans and animals.

The *Herpesviridae* consists of viruses that have been isolated from a wide range of animal species, humans, fish and invertebrates such as oysters. Within this group of viruses, there is a wide variation in biological properties including pathogenicity, a propensity to form latent infections, and oncogenic potential. Herpesviruses are morphologically similar, with a double-stranded DNA core and an icosahedral capsid consisting of 162 capsomeres, surrounded by a granular zone composed of globular proteins (tegument) and encompassed by a lipid envelope (Fig. 23-1). The genome of herpesviruses is large, 125-135kb, and encodes many different proteins; functions of the proteins encoded by the viral genome include virus replication, virus structural proteins, and a variety of proteins that regulate cell growth and modulate the host's antiviral response.

The *Herpesviridae* consists of three major subfamilies, *Alpha Herpesvirinae*, *Beta Herpesvirinae*, and *Gamma Herpesvirinae*, which were initially distinguished by host range, duration of reproductive cycle, cytopathology and latent infection characteristics, and an unassigned genus. The members of *Alphaherpesvirinae* have a variably-restricted host range, are generally highly cytopathic in cell culture, have a relatively short replicative cycle (24h), and frequently cause latent viral infections in sensory ganglia. The members of *Betaherpesvirinae* have a variable host range and a long

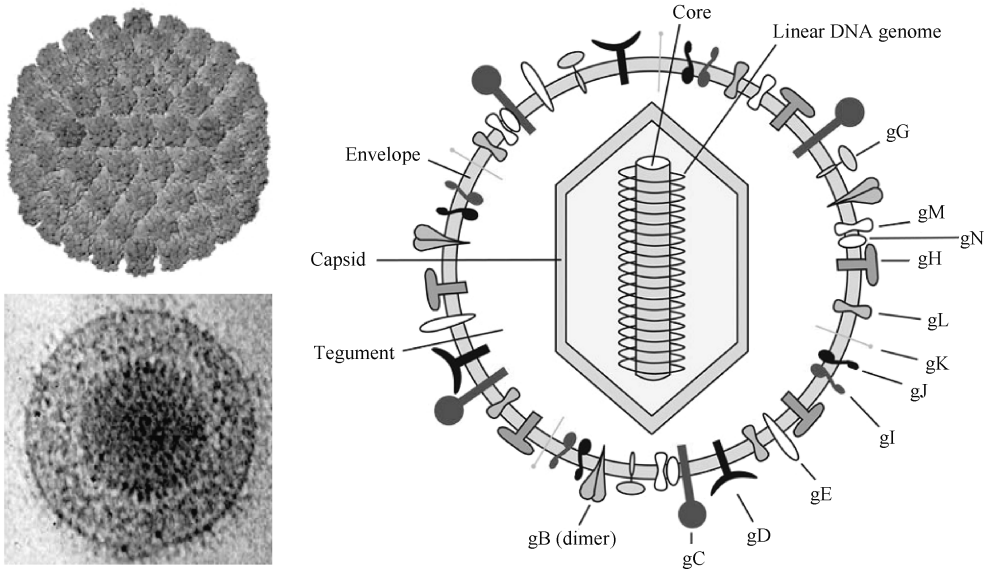


Fig. 23-1 Human herpesvirus 1: reconstruction structure (left top) and cryo-electron microscope image (left bottom) (MacLachlan and Dubovi, 2011); structure of a herpes simplex virus particle (right) (Modrow *et al.*, 2013)

replicative cycle; infected cells often become enlarged (cytomegalic, thus their designation as cytomegaloviruses). Latency can be established in numerous tissues. The members of *Gammaherpesvirinae*, with some exceptions, tend to be tropic for B or T lymphocytes (lymphotropic), replicate in lymphoblastoid cells, and may cause lytic infections in certain types of epithelial and fibroblastic cells. Infection is frequently arrested at a prelytic stage with persistent and minimum expression of viral genome in the cell. Latency is established in lymphoid tissue frequently. Host range is narrow with experimental hosts usually limited to the order of the natural host.

1 Pseudorabies Virus

1.1 Disease

Pseudorabies in swine is most severe in younger animals. The virus commonly affects the nervous system and the mortality rate varies from 5-100%. Infection of sows during mid- to late pregnancy can result in abortion, fetal death, mummification or stillbirths. In adult pigs, severe nervous disorders are rare, and pseudorabies usually presents as a rather vague illness of transient pyrexia, dullness, inappetence, incoordination and ataxia. Respiratory disease can also be seen in pigs of various ages but is most common in grower and finishing pigs. Inapparent or mild disease may be missed or misdiagnosed

in older swine. Pseudorabies also occurs in a number of other species, including cattle, sheep, dogs, cats and raccoons, in which the clinical signs are usually neurologic and manifested by an intense pruritis.

1.2 Etiologic Agent

Pseudorabies virus (PRV) is an alphaherpesvirus that is designated suid herpesvirus 1 (SuHV-l). Only one serotype has been identified. However, strain variability has been shown by restriction endonuclease digestion of viruses from different geographic areas. Attenuated strains have been demonstrated to have a deletion in their genome, suggesting that specific regions are associated with virulence.

1.2.1 Sensitivity to Physical and Chemical Agents

The PRV is fairly sensitive to high temperatures and is stable in cell culture fluid at a pH of 6-8 at cooler temperatures. Virus has been observed to survive in unchlorinated water for 7 days and for 2 days in an anaerobic lagoon. Chemicals that cleave chlorine appear to be the most effective disinfectants.

1.2.2 Infectivity for Other Species and Culture Systems

The disease occurs naturally in cattle, sheep, dogs, cats and rats. In all but adult swine, the disease is almost always fatal; hence, other animals are essentially “dead-end” hosts. Although there is a report of human infection, PRV is not readily transmitted to humans.

The virus replicates readily in cell cultures from many species and tissues, including cat, dog, cattle, badger, coyote, deer, buzzard, chicken and goose.

1.3 Host-Virus Relationship

1.3.1 Distribution, Reservoir and Transmission

Pseudorabies is early recognized as a severe, highly fatal disease of newborn pigs. The principal reservoir of PRV appears to be the pig and transmission is frequently from pig to pig. The virus is transmitted by ingestion and inhalation, and during coitus the virus can be transmitted from boar to sow or vice versa. Transmission can occur in a contaminated environment under crowded conditions.

Feral swine can transmit the virus to domestic swine and among wild animals. Infected raccoons may transmit by close contact with swine and swine may be exposed by consuming infected raccoon carcasses. The pig is the primary source of viral spread to other species. Cases in dogs have been linked to consumption of feral swine tissues. The cat appears to be more sensitivity, and infection in cats was in 51% of PRV-infected farms where cats were present.

1.3.2 Pathogenesis and Pathology

The virus replicates primarily in the upper respiratory epithelium including the tonsillar tissue. Virus can be isolated from the brain 24h following infection, which suggests that the route of infection is via the axoplasm. It is difficult to demonstrate viraemia, however, viral shedding may persist in nasal secretions for up to 14 days. Lower airway infection often occurs, and cardiac and splanchnic ganglia become involved.

The virus produces a nonsuppurative meningoencephalomyelitis with extensive damage to neurons, widespread perivascular cuffing and gliosis. The brain stem is particularly affected, but lesions also occur throughout the cerebral cortex and cerebellum. There may be intranuclear inclusion bodies in all types of cells. In the respiratory form of the disease, a case of necrotizing tracheitis and pneumonia occur that result in loss of epithelium in airways and necrosis of alveolar cells.

Microscopic lesions in aborted fetuses include necrosis of many organs, but primarily liver, spleen, visceral lymphnodes and adrenal glands. Intranuclear inclusion bodies are often present in degenerating hepatocytes, cells of the adrenal cortex, and occasionally mononuclear phagocytic cells of the spleen and lymph nodes. Placental lesions are characterized by degeneration and necrosis of the trophoblasts and mesenchymal cells of the chorion.

1.3.3 Host Response to Infection

IgM antibodies are first detectable about the fifth day after infection followed by measurable IgG antibodies about the seventh day, reaching maximum levels from the twelfth to fourteenth day.

1.4 Laboratory Diagnosis

Because signs of the disease in swine vary widely with the age of the animal, the dose of virus received, the strain of virus and the route of exposure, clinical diagnosis is often difficult.

In the laboratory, a definitive diagnosis of pseudorabies can be made by viral isolation. Immunofluorescent staining of frozen tonsil or brain tissue can provide a rapid diagnosis. Serologic tests for pseudorabies antibodies include solid phase radioimmunoassay, immunodiffusion tests, ELISA, complement-fixation test, counter immunoelectrophoresis, serum virus-neutralizing test (SVN) and indirect hemagglutination. ELISA tests are used to differentiate antibody response to gene-deleted vaccines and field infection. In an acute outbreak, serology may not be helpful because of the time needed for antibodies to develop. In the United States, the most commonly used tests are latex agglutination (LAT), ELISA and SVN. In eradication efforts, the sensitive LAT, which is quick and easy to perform, is commonly used as a screening assay. For

confirmation, the SVN and ELISA are used with specific ELISA tests, which are especially useful in detecting animals vaccinated with gene-deleted vaccines.

1.5 Prevention and Control

In an effort to avoid the disease in a breeding herd, a producer should: ①purchase animals from sources free of PRV; ②require testing prior to purchase; ③isolate new arrivals and test for antibodies a minimum of 12 days after receipt and isolation; ④restrict human traffic among the swine and practice hygienic measures; and ⑤make efforts to restrict contact of the swine with other animals. Feed is a potential source of virus and appropriate measures should be used.

In infected herds, quarantine is the most urgent obligation and it is recommended that the movement of swine be limited for slaughter only. Porcine origin antiserum with titers of at least 1∶256 has proven effective in reducing death losses if administered to neonatal pigs. However, none is commercially available.

Attenuated live vaccines are available and have been successful in reducing death losses in endemic areas. These vaccines do not prevent reinfection with virulent wild virus or the shedding of virulent virus for variable periods. Latent infected and vaccinated animals may shed the virus for indeterminate periods while asymptomatic.

Inactivated vaccines are commercially available. Their principal use has been in susceptible sows in endemic areas to provide antibodies in colostrum for protection of newborn pigs during the first few weeks of life. Genetically engineered (gene-deletion) vaccines are currently used in designated states in the United States since control programs utilize differential serology as part of a federal eradication program.

2 Marek's Disease

2.1 Disease

Marek's disease (MD) is a lymphoproliferative disease of chickens that may involve numerous tissues. Most frequently peripheral nerves are affected. Prior to vaccine development, MD was responsible for heavy losses, and increased losses of MD in vaccinated flocks have suggested an evolution toward greater virulence. There are two species of Marek's disease virus (MDV): gallid herpesvirus-2 (GaHV-2), which is synonymous with MDV type 1, includes isolates that cause mild to severe signs of disease; gallid herpesvirus-3 (GaHV-3), which is synonymous with MDV type 2, includes strains that are nononcogenic. The third species is meleagrid herpesvirus-1 (MeHV-l), which includes viruses from turkeys.

Progressive paralysis of one or more extremities, incoordination, drooping wings

and lowered head position are the most common signs of MD. Mortality varies from 10% with mild MD to over 50% in unvaccinated birds.

2.2 Etiologic Agent

GaHV-1 and GaHV-2 and MeHV-l are alphaherpesviruses.

2.2.1 Resistance to Physical and Chemical Agents

Cell-free virus is readily inactivated at temperatures greater than 37℃ and is only relatively stable at 25℃ (4 days) and 4℃ (2 weeks). MDV can be maintained for long periods at 27℃. Virus is inactivated by pH 3 and pH 11. Infectivity of dried MDV-infected feathers is destroyed by chlorine, organic iodine, quaternary ammonium compound, cresylic acid, synthetic phenol and sodium hydroxide.

2.2.2 Infectivity for Other Species and Culture Systems

The chicken is the primary natural host for the MDV, and disease is rare in other species except for quail. Marek's disease virus has not been shown to affect any non-avian animals. No etiologic link has been demonstrated between MDV and human cancer. The virus is most often cultivated on chicken or duck embryo fibroblast cells (Fig. 23-2). Chicken kidney cells have also been used.

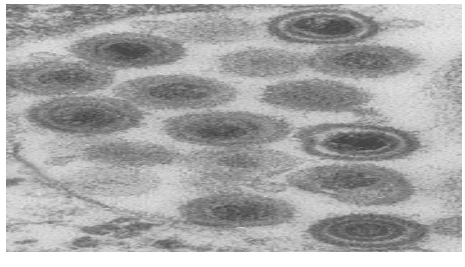


Fig. 23-2 Electron micrographs of MDV in duck embryo fibroblast (Swayne, 2013)

2.3 Host-Virus Relationships

2.3.1 Distribution, Reservoir and Transmission

Marek's disease is a major disease of domestic chicken flocks worldwide. The virus can persist in excreta, litter and poultry house dust, and horizontal infection via aerosols of chickens appears to be the main method of transmission. Egg transmission is doubtful. Marek's disease virus matures to its enveloped infectious form only in the feather follicles and can then be spread to the environment via desquamated cells. Whole live cells from blood or tumor material, or infected whole cell cultures, are infectious experimentally. Cell-free fluid does not appear to be infectious. Certain

chicken lines are genetically resistant to MDV, and resistance in most birds is associated with the development of serum-neutralizing antibody.

2.3.2 Pathogenesis and Pathology

The incidence of MD is variable, depending on the strain of the virus, the breed and age of the chicken. It usually occurs in chickens between 2 and 5 months old and is commonly felt not to be seen in birds older than 22 weeks. However, disease has been observed in birds as young as 3-4 weeks and in 60-week-old laying hens. The virus primarily affects the nervous system, although visceral organs and other tissues may also be involved. Lesions are present in the nervous system and involve peripheral nerves and spinal roots. The principal nerve trunk involved shows gross lesions consisting of a grayish-white swelling, which are histologically characterized by extensive lymphocytic infiltrations. Edema may be present and myelin degeneration of nerve sheaths may be apparent.

Ocular lymphomatosis is another possible outcome of MDV infection with blindness resulting due to iris involvement. Histologically, a similar infiltration of lymphocytes is present, which can also occur in the optic nerve.

In the visceral form, lymphoid tumors of varying degrees of severity infiltrate the gonads, liver, lungs and skin. Affected chickens have enlarged visceral organs with white nodular or miliary foci. Occlusive atherosclerosis has been observed experimentally.

2.3.3 Host Response to Infection

The immune response to MDV is complex, in both the humoral and cell-mediated immunity (CMI) in normal birds. MDV infection can be immunosuppressive. In addition, the immune response may be involved in tumor formation. Bursectomized birds survive experimental infection, suggesting that CMI is important. In chicks, passively acquired antibody is thought to limit the extent of infection rather than prevent it or clear the virus. Viral specific antibodies appear within 1-3 weeks following infection and neutralizing antibodies persist for the life of the bird. Following infection, transient CMI suppression is common; it may persist in birds that develop neoplasms. Both B and T lymphocytes have been identified in tumors, and thymectomy has been shown to reduce the level of lymphomas in affected birds. It has been suggested that MD might have an autoimmune component based on antibody responses to myelin and peripheral nerves.

2.4 Laboratory Diagnosis

On the necropsy, gross lesions are common in peripheral nerves, root ganglia, and the spinal roots. Lymphomatous lesions are characteristically composed of small lymphocytes, lymphoblasts and reticulum cells. Arterial lesions of atherosclerosis are often present.

Confirmatory diagnosis is made by viral isolation or by antigen detection using fluorescent antibody, immunoperoxidase, or ELISA on feather follicle cells. Antibodies can be detected by agar gel immunodiffusion, indirect immunofluorescence, and viral neutralization and ELISA assays.

2.5 Prevention and Control

Experimentally, MDV-free flocks can be maintained by strict isolation, constant surveillance, and frequent monitoring for virus and antibody, but these techniques have been of limited commercial use. Commercial vaccines are available and effective in reducing the incidence of MD. The three live virus serotypes have been used for vaccines. HVT vaccine (serotype 3) is the most economical to produce and is most effective when exposure is not heavy. Vaccination does not prevent infection or shedding of virulent MDV, but it does prevent tumor formation. Bivalent and polyvalent vaccines have been used successfully where monovalent vaccines were not effective. Embryo vaccination is currently used in at least 55% of broiler embryos. Genetically resistant lines of chickens have been maintained experimentally.

3 Infectious Laryngotracheitis Virus

3.1 Disease

Infectious laryngotracheitis virus (ILTV) usually occurs as an acute disease in chickens and represents a serious problem in areas of intense poultry husbandry. The virus produces signs of respiratory distress and coughing that often produce a bloody discharge. Mild enzootic forms of ILTV infection may result in reduced egg production, conjunctivitis and persistent nasal discharge.

3.2 Etiologic Agent

ILTV is a typical alphaherpesvirus that is also designated as gallid herpesvirus-1 (GaHV-1).

3.2.1 Sensitivity to Physical and Chemical Agents

ILTV can be inactivated by 3% cresol, 1% sodium hydroxide, and 1% lye, 24 hours' exposure to ether, and 10-15min at 55℃. The virus can be stored for lengthy periods by lyophilization and freezing.

3.2.2 Infectivity for Other Species and Culture Systems

ILTV primarily occurs in chickens, but has also been reported in pheasants and peafowl. Young turkeys have been infected experimentally, and the virus replicates in embryonated

turkey eggs. Starlings, sparrows, crows, doves, ducks, pigeons and guinea fowl have been found to be resistant to ILTV.

Chicken kidney monolayer cell cultures, embryonated chicken eggs, and chicken embryo (kidney, liver and lung) cell cultures have been used to culture ILTV.

3.3 Host-Virus Relationship

3.3.1 Distribution, Reservoir and Transmission

ILTV has been identified in almost every country in the world; it occurs primarily in areas with high concentrations of chickens and occasionally occurs in pheasants. Chickens are assumed to be the primary reservoir and mode of transmission, which occurs by direct contact through droplet infection of the ocular and respiratory secretions. Mechanical transmission can occur via contaminated equipment and litter. Egg transmission of ILTV has not been demonstrated. A carrier state can develop in birds with sublethal disease, and ILTV has been isolated from chickens 2 years after infection. Unvaccinated birds are susceptible to infection from vaccinated birds, and vaccinated birds may become carriers. Acutely infected birds represent a greater source of virus than clinically recovered carrier birds.

3.3.2 Pathogenesis and Pathology

ILTV in natural conditions enters through the upper respiratory tract and ocular tract. In the natural disease, the greatest consent ration of ILTV is found in the trachea, since the virus replicates only in the nasal cavity, trachea and lower respiratory tract. Latent virus has been demonstrated in the trigeminal ganglion. Viremia has not been reported.

In lethal infections of ILTV, thickening of tracheal mucosa, hemorrhage, congestive heart failure, and severe congestion of all internal organs have been found. Histopathology demonstrates fibrinous tracheobronchitis, with detachment of the tracheal epithelium and large intranuclear inclusion bodies in detached cells that are the basis for a strong presumptive diagnosis.

3.3.3 Host Response to Infection

The first signs of ILTV infection usually appear 6-12 days following natural exposure. Resistance to disease followed the infection or vaccination usually persists for approximately 1 year. Infected birds develop precipitating and serum-neutralizing antibodies; however, the cell-mediated response appears to be important in resistance. Full immunity can be demonstrated in bursectomized chickens in the absence of a humoral response.

3.4 Laboratory Diagnosis

Virus can be isolated from tracheal and lung tissue in embryonated chicken eggs, cell

culture, and ILTV DNA can be demonstrated by PCR. Immunofluorescent staining of trachea can demonstrate presence of viral antigen up to 14 days postinfection. ELISA-based serology is widely used.

3.5 Prevention and Control

Use of live ILTV vaccine results in carriers which can shed viruses to nonvaccinated susceptible birds. Since the virus can survive for 10 days at temperatures of 13-23℃, the cleaning of infected premises is very important. Complete depopulation and disinfection of premises have been used to control the disease.

Vaccination has been successful via the cloaca, infraorbital sinuses, intranasal instillation, feather follicles and drinking water. Birds younger than 2 weeks do not respond, as well as older birds. Water administration of ILTV vaccine does not give as complete or long-lasting immunity as other methods. For effective water vaccination, the virus has to penetrate into the nasal cavity or trachea. Cloacal administration of the vaccine leads to rapid absorption of the virus into the bursa of Fabricius and results in an immune response. However, this route is undesirable, since ILTV damages the bursa of young birds. Adequate ILTV vaccination occurs via aerosol procedures. Modified live ILTV vaccines have been associated with disease and chicken embryo origin vaccines have been demonstrated to increase in virulence after *in vivo* passage. The development of genetically engineered vaccines holds promise for improved control strategies.

Review Questions

1. Try to describe the classification status of *Herpesvirales*.
2. What are the common herpesviruses which have pathogenicity?
3. Try to describe the common characteristics of herpesvirus infection.
4. Try to describe the mechanism of the pseudorabies virus.
5. Try to describe the pathogenesis of Marek's disease virus.
6. Try to describe the microbiology diagnosis of pseudorabies.
7. Try to describe the microbiology diagnosis of Marek's disease.
8. Try to describe the prevention and control measures of Marek's disease.

Chapter 24 Single-Stranded DNA Viruses

Synopsis

The single-stranded DNA viruses that are pathogenic to animals are mainly list in *Parvoviridae* and *Circoviridae*. The genome of viruses in *Parvoviridae* is a single line strand. The replication of viruses in *Parvoviridae* depends on the division of the host cells. Parvoviruses of pigs, dogs, cats, geese, and ducks have great pathogenic significance to host. The vaccine can effectively control the infection of parvovirus. The genome of viruses in *Circoviridae* is a single ring strand. The typically representative of circovirus are chicken anemia virus and porcine circovirus. Chicken anemia virus can cause the apoptosis of T cell of chicken resulting in immunosuppression. But, there is no effective vaccine against chicken anemia virus. Porcine circovirus 2 has great damage to pigs, but there is effective vaccine for control the infection of porcine circovirus 2.

1 *Parvoviridae*

The *Parvoviridae* have two subfamilies: *Densovirinae* and *Parvovirinae*. Parvoviruses are small (18-26nm), nonenveloped icosahedral viruses (Fig. 24-1), which contain a linear sing-stranded DNA genome (molecular weight approximately 6×10^6Da). Members of the *Parvoviridae* are the causative agents of specific disease.

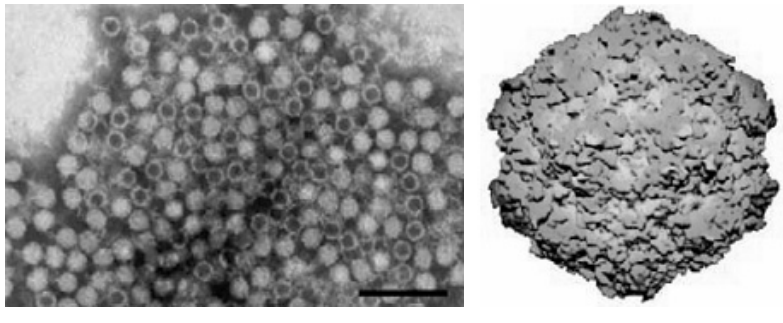


Fig. 24-1 Canine parvovirus: electron micrograph (left), space-filling models of the capsid structures (right) (MacLachlan and Dubovi, 2011)

1.1 Feline Panleukopenia

1.1.1 Disease

Feline panleukopenia (syn. feline infectious enteritis) is a highly contagious, acute viral disease of cats characterized by high fever, anorexia, depression, vomiting followed by dehydration, diarrhea and death. Leukopenia is characteristic of feline panleukopenia, and the severity of clinical disease often mirrors the severity of leukopenia. Cats of all ages are susceptible to infection, but mortality is highest among kittens. Cats can be infected by either the oral or respiratory routes, and the incubation period after infection is short. Intrauterine infection with feline panleukopenia virus may lead to neonatal death or congenital abnormalities of the central nervous system (CNS) manifested by cerebellar ataxia in kittens after birth; kittens up to 2 weeks of age are susceptible to the same teratogenic effects of feline panleukopenia virus infection.

1.1.2 Etiologic Agent

Feline panleukopenia virus is a typical parvovirus. The virions are nonenveloped (18-26nm in diameter) with icosahedral symmetry. The genome is a single-stranded DNA molecule that includes two open reading frames, one of which encodes at least four proteins that is required for transcription and DNA replication, and the other, encodes the capsid proteins of the virus. VP3 constitutes the major capsid protein, and it appears to control tissue/cell tropism of the virus. Virus replication occurs within the nucleus of host cells and, because the virus lacks its own DNA polymerase, its replication requires cells that are cycling (late S phase or early G2 phase of the cell cycle) so that they can utilize host cell enzymes for their own replication.

Feline panleukopenia virus is closely related to canine parvovirus and mink enteritis virus, although the three viruses can be distinguished by sequence analysis.

1.1.2.1 Resistance to Physical and Chemical Agents

Feline panleukopenia virus is strongly resistant to environmental factors and many commercial disinfectants. 0.175% sodium hypochlorite solution [Clorox (1∶30)] is the most effective and practical virucidal disinfectant.

1.1.2.2 Infectivity for Other Species and Culture Systems

All members of the Felidae are likely to be susceptible to infection with feline panleukopenia virus. A very closely related virus that is antigenically indistinguishable from feline panleukopenia virus causes enteritis in ranch mink, and the same virus can cause disease in raccoons and in coatimundi. Canine parvovirus 2 has emerged relatively recently, and it is ancestrally closely related to feline panleukopenia virus.

Feline panleukopenia virus grows in primary or continuous feline kidney cell cultures but not in canine cell cultures.

1.1.3 Host-Virus Relationship

1.1.3.1 Distribution, Reservoir and Transmission

Feline panleukopenia occurs worldwide, and infected cats are the principal reservoir. Both infected cats suffering from acute disease and those having clinically inapparent infection excrete virus in their urine, feces and various secretions. The infection spreads rapidly by contact with contaminated utensils, cages and bedding, and the virus is highly stable in the environment.

1.1.3.2 Pathogenesis and Pathology

Cell-free viremia occurs for several days in kittens experimentally infected intranasally or orally with feline panleukopenia virus, during which the virus is disseminated throughout the body and infects cells with the necessary receptors. The virus then replicates in those cells that are in S phase of the cell cycle, particularly hematopoietic cells within the bone marrow and lymphoid tissues (thymus, spleen and lymph nodes), leading to severe and protracted leukopenia that affects all white blood cell types and atrophy of lymphoid tissues. The rapidly dividing cells of the intestinal crypts also are highly susceptible to infection, which leads to marked destruction of the intestinal epithelium with resultant malabsorption diarrhea. Histologic lesions are characterized by necrosis of the epithelium of the intestinal crypts and marked destruction and depletion of lymphocytes in the lymph nodes, thymus and spleen. Regenerative lymphoid hyperplasia may be present in the later phase of the disease.

In late term fetuses and very young kittens, the virus infects and destroys the cells within the external granular layer of the cerebellum, leading to cerebellar hypoplasia and atrophy as a consequence of failure of the internal granular layer to develop, and to degeneration and loss of Purkinje cells. Similar congenital lesions can be produced by in-utero infections of rats, hamsters, ferrets and mice after infection at critical stages of gestation with the appropriate species of parvovirus.

1.1.3.3 Host Responses to Infection

Neutralizing antibodies first appear in cats at approximately one week after infection, and high titers of antibody are present after 10-12 days generally. Hemagglutination-inhibiting antibodies rise from the fourth day after infection, reaching a peak on the seventh day. These antibodies can persist in cats for several years. Maternal antibody with neutralizing titer of 30 or greater against feline panleukopenia virus protects kittens against viral infection, but it also interferes with active immunization by modified live or inactivated feline panleukopenia viral vaccines.

1.1.4 Laboratory Diagnosis

There is no effective treatment for feline panleukopenia; thus, control is achieved by vaccination, quarantine of cats that survive from infection, and rigorous sterilization of premises that have housed affected cats. Highly effective inactivated and modified live feline panleukopenia viral vaccines are commercially available, although maternal antibodies can interfere with the immunization of young kittens.

1.1.5 Treatment and Control

Clinical signs, the presence of leukopenia, and histopathological examination are useful for the presumptive diagnosis of feline panleukopenia. The diagnosis can be confirmed by one of the following laboratory methods: ①isolation of feline panleukopenia virus from the feces, urine or from the feline kidney cells; ②detection of viral antigen in infected tissues by capture ELISA or immunofluorescence staining with a feline panleukopenia virus-specific antisera conjugate; ③PCR amplification of viral nucleic acid; ④serologic diagnosis is accomplished by ELISA or indirect immunofluorescence, although paired sera is required to confirm the diagnosis.

1.2 Canine Parvovirus Disease

1.2.1 Disease

Parvovirus disease in dogs is characterized by the sudden onset of diarrhea, vomiting, anorexia, fever, depression, lymphopenia and dehydration. Mortality is higher in puppies than in adults, very young puppies sometimes develop myocarditis without clinical signs of enteritis.

The disease occurs worldwide. Canine parvovirus disease is caused by canine parvovirus 2, which is a variant of feline panleukopenia virus. Canine parvovirus 2 has continued to evolve since it emerged in dogs, with the appearance of new variants that have been designated as canine parvovirus 2a and 2b. The minimum virus of canines that does not produce disease in dogs has been designated as canine parvovirus 1.

1.2.2 Etiologic Agent

Canine parvovirus 2 is very similar to feline panleukopenia virus.

1.2.2.1 Resistance to Physical and Chemical Agents

Parvovirus is strongly resistant to environmental factors, such as extremes of temperature, pH and some disinfectants. The virus can persist for long periods in premises where infected dogs are kept and can be transmitted to other areas by fomites. It can be inactivated by common bleach such as Clorox (1∶30).

1.2.2.2 Infectivity for Other Species and Culture Systems

Canine parvovirus 2 infects dogs of all breeds and other members of the Canidae, such as wolves, foxes and coyotes. Domestic cats without antibodies to the virus are susceptible to experimental infection but remain asymptomatic.

Canine parvovirus 2 can be propagated in primary cell cultures of canine or feline fetal lung and kidney, as well as continuous cell lines such as canine cell line A72 and feline cell lines NLFK and CRFK.

1.2.3 Host-Virus Relationship

1.2.3.1 Distribution, Reservoir and Transmission

Canine parvovirus 2 infection of dogs and other members of the Canidae is prevalent in many areas of the world. Parvovirus infected dogs continue to excrete infectious virus in their feces for up to 10 days after the onset of infection, and the virus is readily transmitted between dogs by the fecal-oral route.

1.2.3.2 Pathogenesis and Pathology

The pathogenesis of canine parvovirus 2 infection of dogs is similar to that of panleukopenia virus infection of cats, although cerebellar hypoplasia and atrophy is not recognized as a consequence of in utero infection of dogs with canine parvovirus. Similarly, myocarditis is a potential consequence of parvovirus infection of young pups, whereas it is not described in panleukopenia-virus infected kittens. Cell-free viremia precedes infection of the intestinal epithelium and lymphoid tissues, including thymus, tonsils, retropharyngeal and mesenteric lymph nodes, and spleen of infected puppies, and widespread infection of the intestinal mucosa occurs on about the sixth day after experimental inoculation. Fecal excretion of virus begins as soon as the third day after infection and peaks come soon thereafter. Most infected dogs stop excreting virus by the twelfth day.

The most striking lesion of parvovirus enteritis in dogs is hemorrhage within the lumen of the small bowel and accompanying enlargement and edema of the mesenteric lymph nodes. Mottled white streaks within the myocardium are indicative of cardiac involvement in young puppies.

Microscopic lesions associated with canine parvovirus infections are confined to organs with large populations of rapidly proliferating cells, such as the small intestine, lymph nodes and bone marrow. The most frequent findings in the small intestine are necrosis of crypt epithelium and atrophy of epithelial villi. Regeneration of intestinal epithelium occurs in dogs surviving the acute phase of enteric infection. Lymphocytolysis in the thymic cortex and germinal centers of lymph nodes is common and results in cellular depletion. In the myocardial form of parvovirus infection, the

ventricular myocardium shows myofiber degeneration and necrosis that is accompanied by infiltration by mononuclear cells.

1.2.3.3 Host Response to Infection

Dogs infected with parvovirus rapidly develop high, long-lasting titers of virus neutralizing antibodies. The immunity that develops after natural infection appears to be lifelong in survived dogs. Cellular immune responses also are generated during infection, and are likely to be important in limiting virus replication during acute infection.

1.2.4 Laboratory Diagnosis

Clinical signs, history, contrast radiography and histopathological examinations are useful in the presumptive diagnosis of canine parvoviral disease. Laboratory procedures used to confirm the diagnosis include the followings: ①isolation of canine parvovirus 2 from the feces or tissues of infected animals on susceptible cell cultures, or by identification of parvoviral nucleic acid in infected tissues by PCR; ②detection of parvoviral antigen in the histological sections of intestine by the immunofluorescent or immunohistochemical staining with virus-specific antibodies; ③demonstration of parvovirus in feces or infected tissue by electron microscope or immunoelectron microscope; ④identification of parvovirus in feces by hemagglutination test using swine or rhesus monkey red blood cells and the specific hemagglutination-inhibition by anti-canine parvovirus antiserum; ⑤detection of parvovirus in feces by ELISA using monoclonal antibodies to the canine parvovirus 2 hemagglutinating protein; ⑥demonstration of anti-canine parvovirus antibody in serum by such serologic tests as hemagglutination inhibition, virus neutralization or ELISA. The IgM-capture ELISA can be used to confirm recent infection of dogs.

1.2.5 Treatment and Control

Severe cases of canine parvovirus disease are characterized by marked dehydration and metabolic acidosis, thus supportive treatment relies on replacing lost body fluids and correcting disturbed electrolyte balance and acidosis. Broad-spectrum antibiotics are used to prevent secondary bacterial infections. Vaccination of susceptible canine populations is the best prophylaxis for canine parvoviral infection. Antibody levels correlate directly with the degree of protection. Effective inactivated and modified live parvoviral vaccines are available, although the presence of maternal antibodies interferes with active immunization of puppies. Canine parvovirus is strongly resistant to environmental factors and can persist under adverse conditions for a long period, thus prompt disinfection of premises where infected animals are being kept and vaccination of puppies prior to their introduction to such premises are important in preventing this disease.

1.3 Porcine Parvovirus Infection

1.3.1 Disease

Infection of swine with porcine parvovirus occurs worldwide and sometimes leads to reproductive failure in swine and cutaneous lesions in piglets. Transplacental infection of fetuses leads to stillbirth, mummification, embryonic death and infertility, the so-called SMEDI syndrome. The porcine reproductive and respiratory syndrome virus is now considered to be a more important cause of SMEDI syndrome than porcine parvovirus.

1.3.2 Etiologic Agent

1.3.2.1 Physical, Chemical and Antigenic Properties

Porcine parvovirus resembles feline and canine parvovirus. There is only one serotype, and porcine parvovirus is antigenically different with other parvoviruses.

1.3.2.2 Resistance to Physical and Chemical Agents

Porcine parvovirus is strongly resistant to heat, enzymes, and most commercial disinfectants. The virus is inactivated by heat at 73℃ for 30min or at 70℃ for 1h or by exposure to 0.5% sodium hypochlorite for 5min, to 0.06% potassium dichloroisocyanurate for 5min, or to 3% formaldehyde for 1h.

1.3.2.3 Infectivity for Other Species and Culture Systems

Porcine parvovirus apparently infects swine only. The virus can be propagated on primary or secondary cultures of fetal porcine kidney cells and swine testicle cells.

1.3.3 Host-Virus Relationship

1.3.3.1 Distribution, Reservoir and Transmission

Parvovirus infection is endemic in many herds, and infected swine serve as the reservoir of infection. Infected swine develop a viremia and shed virus in their oral secretions and feces. Since porcine parvovirus can persist in the environment for long periods of time, contaminated premises serve as a major reservoir of virus that is responsible for its transmission to susceptible animals. Carrier boars also can disseminate the virus in their semen. Although rats may be infected experimentally with porcine parvovirus, they are unlikely to serve as natural reservoirs of the virus.

1.3.3.2 Pathogenesis and Pathology

Swine infected with porcine parvovirus produce antibodies without developing clinical disease or obvious lesions. Reproductive disease occurs when sero-negative sows are exposed to the virus during gestation, and the consequences (fetal death with or without mummification, embryonic death or infertility) of transplacental transmission

of the virus are reflective of the gestational stage of the sow at infection. Lesions within stillborn fetuses are often nonspecific, but can include foci of necrosis and mononuclear cell infiltration in organs such as the liver, heart, kidney and cerebrum.

1.3.3.3 Host Responses to Infection

Piglets and nonpregnant adult swine infected with porcine parvovirus develop viremia without obvious clinical disease. These animals develop a strong humoral immune response and are resistant to subsequent reinfection with the virus. Piglets acquire high titers of virus-specific antibodies through the colostrum of immune sows.

1.3.4 Laboratory Diagnosis

Parvovirus antigen can be detected in the tissues of affected fetuses by immunofluorescent or immunohistochemical staining or by capture ELISA. A PCR assay has also been developed for the detection of porcine parvovirus.

1.3.5 Treatment and Control

There is no treatment for reproductive failure produced by porcine parvovirus, and vaccination of breeding gilts remains the best method to ensure that gilts develop active immunity prior to being bred. Both inactivated and modified live vaccines are available, but vaccination must be carefully limed to ensure that animals are immunized after passive antibodies are lost and before the animals are bred.

2 *Circoviridae*

The *Circoviridae* has two genera: *Circovirus* and *Cyclovirus*. Many circoviruses are important pathogens in animals. Like parvovirus, they are small non-enveloped icosahedral viruses (12-26nm in diameter) with a genome of single-stranded DNA. Important viruses within the *Circoviridae* include beak and feather disease virus, chicken infectious anemia virus (Fig. 24-2), and porcine circovirus. A pathogenic circovirus

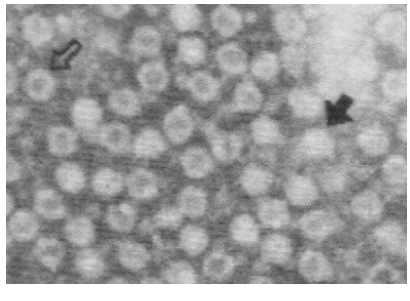


Fig. 24-2 Electron microscopy of chicken anemia virus (hollow core pointed by black arrow, solid core pointed by white arrow) (扈荣良, 2014)

also has been identified in pigeons, and similar viruses can infect humans as well as other bird species.

2.1 Porcine Circovirus

2.1.1 Disease

Porcine circovirus (PCV) have been divided into PCV1, PCV2 and PCV3 to distinguish the nonpathogenic and pathogenic viruses respectively. To date, PCV2 infection has been linked with a variety of syndromes, collectively termed porcine circovirus-associated disease (PCVAD). PCVAD describes a group of complex multifactorial syndromes that occur during all stages of pork production. Manifestations of PCVAD can range from non-overt clinical signs to acute death. Clinical signs typically present as wasting, diarrhea, respiratory distress, dermatitis or reproductive failure. The primary factor for the onset of PCVAD involves infection with PCV2. PCV2 isolates are divided into two main genotypes, known as PCV2a and PCV2b. Typical PCVAD syndromes include porcine multisystemic wasting syndromes (PMWS), PCV2-associated enteritis, PCV2-associated respiratory disease, proliferative and necrotizing pneumonia, PCV2-associated reproductive failure, porcine dermatitis and nephropathy syndrome, and acute pulmonary edema. PCV3 is a new member of PCV.

2.1.2 Etiologic Agent

Domestic and feral pigs are the primary hosts for PCV2. Porcine epithelial cells from kidney and testicle support PCV2 replication in cell culture systems. Experimental infections with PCV2 have been established in mice.

2.1.3 Host-Virus Relationship

2.1.3.1 Distribution, Reservoir and Transmission

Historically, PCV2 isolates were classified based on similarities with isolates from North America (PCV2a) or Europe (PCV2b). However, both genotypes are considered endemic within the pig population all over the world. PCV2 establishes a long-term infection and is shed in oronasal fluid, urine, blood and feces in pigs for up to 28 weeks of age on PCVAD affected farms. Transmission most often occurs from pig to pig through the horizontal route. In addition, PCV2 is very stable in the environment, which plays a role in virus transmission.

2.1.3.2 Pathogenesis and Pathology

The clinical manifestation and severity of PCVAD are linked to a variety of co-factors, such as the disease potential of the PCV2 isolate, the presence of pathogenic or opportunistic infections, host genetics, and use of immunostimulating agents such as

vaccines. In general, clinical and pathological signs are syndrome specific. Clinical signs of PMWS, the most common PCVAD syndrome, include lethargy, diarrhea, lymphadenopathy, discoloring of the skin, jaundice and wasting. Gross pathological signs include enlargement of the submandibular, inguinal and bronchial lymph nodes, non-collapsed mottled lungs, occasional spleen infarcts, atrophic and discolored liver, and enteritis. Histopathological characteristics of PMWS may include lymphocyte depletion with granulomatous inflammation of lymphoid tissues, which may include intracytoplasmic inclusion bodies, granulomatous to lymphohistiocytic interstitial pneumonia, lymphohistiocytic hepatitis, interstitial nephritis, and granulomatous enteritis.

2.1.3.3 Host Response to Infection

PCV2 infection results in a pronounced humoral and cell-mediated response that, in general, is incapable of clearing viral infection. Maternally derived antibodies resulting from natural infection typically provide minimal protection against PCV2 infection.

2.1.4 Laboratory Diagnosis

Clinical signs, history and histopathological examinations are useful in a presumptive diagnosis of PCVAD. Laboratory procedures used to confirm PCV2 infection include the followings: ①isolation of PCV2 from serum or tissues of infected animals on susceptible cell cultures, or by identification of PCV2 nucleic acid in infected tissues by PCR; ②detection of PCV2 antigens in histological sections of lung or lymphoid lesions by immunofluorescent or immunohistochemical staining with virus specific antibodies; ③detection of PCV2-specific antibodies in serum using serologic tests such as IFA, virus neutralization assay or ELISA.

2.1.5 Treatment and Control

Before the advent of vaccines, multiple measures were incorporated with varying effects. These included proper housing, stress reduction, practicing an “all-in-all-out” policy, and the prevention of age mixing. Further methods that were used with minimal success included antibiotics to control secondary infections, serum therapy and depopulation. The most effective method for controlling PCV2 today is vaccination. Field and experimental vaccine trials have demonstrated prevention of PCVAD, decreased viremia, and increased growth performance.

Currently, several commercial vaccines incorporate the PCV2a ORF2 antigen, which may be expressed in baculovirus, inactivated PCV2, or an inactivated PCV1/2 chimera. Of the five commercial vaccines currently available, four are recommended for piglets and only one for sows. Vaccine schedules for piglets are either one or two doses with the first dose administered at age of 3 weeks. In the case of the two-dose vaccine, the second dose is administered 3 weeks later. Sow vaccination is recommended at 2-5 weeks antepartum.

2.2 Beak and Feather Disease

2.2.1 Disease

While avian-specific medicine for poultry has significantly developed over the years, only minimal information is available for psittacine birds. Viral infectious diseases such as beak and feather disease, also termed psittacine beak and feather disease (PBFD), are some of the most common problems in psittacine birds. PBFD was first observed in Australia in the early 1970s, and since then it has emerged in many countries. The onset and severity of disease depend on the age of the host. PBFD typically presents as necrotic or otherwise abnormal feathers such as feathers that are bent, contain hemorrhages, or are prematurely shed. Chronic infection may result in beak and nail deformities. Neonatal birds are particularly susceptible to severe acute disease characterized by pneumonia, enteritis, rapid weight loss, and death.

2.2.2 Etiologic Agent

The primary host for the causative agent of PBFD, the BFDV, is psittacine birds. There is currently no cell culture system available for virus propagation.

2.2.3 Host-Virus Relationship

2.2.3.1 Distribution, Reservoir, and Transmission

PBFD has been described among birds in many countries. Movement of infected psittacine birds is likely to be the cause of dissemination. BFDV has been identified in over 60 species of captive and free ranging psittacine birds. Transmission of the virus occurs through both horizontal and vertical routes.

2.2.3.2 Pathogenesis and Pathology

Exposure to BFDV results in subclinical infection in the majority of free-ranging and captive birds. However, the outcome of infection depends on many factors, such as the age of the bird at initial exposure, the presence and level of protection provided by the maternal antibody, and the route and titer of the infecting virus. The incubation time for BFDV may be prolonged with clinical signs appearing long after the initial infection. Clinical signs in young birds may present as inappetence, lethargy, crop stasis, progressive feather abnormalities, and eventual death. In some cases, secondary infections stemming from immunosuppression are the primary cause of death. Histologic lesions depend upon many factors including the duration and severity of the disease. However, characteristic intranuclear and intracytoplasmic inclusion bodies may be present in macrophages in the thymus, bursa, and other lymphoid tissues, as well as in epithelial cells lining the shaft of the feather.

2.2.3.3 Host Responses to Infection

Exposure to BFDV results in resistance to reinfection. Maternal antibodies are likely to provide passive protection of chicks against BFDV infection for several weeks after birth.

2.2.4 Laboratory Diagnosis

Diagnosis of PBFD is based on identification of the clinical signs and characteristic gross and histological changes in the tissues of affected birds. Assays for the identification of BFDV infection include *in situ* hybridization, hemagglutination and HAI, electron microscopy, PCR and real-time PCR.

2.2.5 Treatment and Control

The most effective method to control PBFD is elimination or quarantine of carrier birds to prevent transmission of BFDV. Currently, there is no available commercial vaccine for BFDV, although experimental efforts involving recombinant BFDV capsid protein are underway.

2.3 Chickens Infectious Anemia

2.3.1 Disease

Chicken infectious anemia (CIA) is a highly contagious disease of young chickens (age of 2-4 weeks). Clinical disease resulting from infection with chicken anemia virus (CAV), the etiological agent of CIA, is rare largely due to the practice of widespread vaccination. However, the subclinical form of disease, which occurs at all stages of production, remains prevalent worldwide. Clinical signs in young birds include aplastic anemia, generalized lymphoid atrophy, and profound immune suppression which often results in secondary viral, bacterial and fungal infections. The subclinical form of disease, characterized by prolonged viremia and reduced growth performance, results in serious economic loss to the poultry industry. Within the *Circoviridae*, CAV represents the most distinct virus. Genetically distinct strains of CAV have been identified, although strains are similar in terms of antigenicity and virulence.

2.3.2 Etiologic Agent

The primary host for CAV is the chicken. CAV can be propagated in chicken embryos, chicken primary blood mononuclear cells, and the MDCC-MSB-1 cell line *in vitro*.

2.3.3 Host-Virus Relationship

2.3.3.1 Distribution, Reservoir and Transmission

CAV is ubiquitous in commercial and specific pathogen-free chicken stocks. While

chickens in all ages are susceptible to infection, older chickens are more resistant to clinical disease. Transmission of the virus occurs through both the horizontal and vertical routes.

2.3.3.2 Pathogenesis and Pathology

In unprotected chicks, CAV targets hemocytoblasts in the bone marrow and lymphocytes in the thymus, resulting in aplastic anemia, leukopenia, thrombocytopenia and thymic atrophy. Clinical signs generally appear 7-14 days after infection, presenting as gangrenous lesions on the wings. Furthermore, immunosuppression often results in opportunistic infections from other viruses, bacteria, or fungi. Early investigations indicated that maternal antibodies play a significant role in the prevention of disease. However, older chickens become susceptible to CAV infection once maternal antibodies decay, which may result in the subclinical form of disease. In addition to reduced growth performance, immunosuppression has recently been identified as a disease manifestation in older chickens.

2.3.4 Host Responses to Infection

Maternal antibodies provide passive protection to CAV infection and disease for several weeks after birth. Upon decay of maternal antibodies, CAV exposure may result in a persistent infection, resulting in reduced growth performance and immunosuppression.

2.3.5 Laboratory Diagnosis

A clinical diagnosis of CIA is based on flock history, clinical signs and pathological findings. Assays to confirm infection with CAV include virus isolation, PCR, real-time PCR, or immunohistochemical staining of tissues with CAV-specific antibodies. Serological assays for the detection of CAV infection include ELISA, indirect immunofluorescence (IFA) and virus neutralization assay.

2.3.6 Treatment and Control

The most effective practice for controlling CAV involves vaccination of breeding flocks with a modified live virus, which prevents vertical transmission of the virus and provides protective maternal antibodies. Specific treatment is not available for chickens infected with CAV.

Review Questions

1. What are the single-stranded DNA viruses that have important pathogenic effects?
2. Try to describe the pathogenesis of feline panleukopenia virus.
3. Try to describe the pathogenesis of canine parvovirus.

4. Try to describe the pathogenic mechanism of porcine parvovirus.
5. Try to describe the pathogenesis of porcine circovirus 2.
6. Try to describe the pathogenic mechanism of chicken anemia virus.
7. Try to describe the classification status of *Parvoviridae* and *Circoviridae*.

Chapter 25 Retroviruses

Synopsis

Retroviruses have a process of reverse transcription during replication. The genome of the retrovirus is a plus-stranded RNA in diploid, which has several important genes such as *gag*, *pol*, and *env*. Some retroviruses are replication-defective, which need helper virus for replication. *Retroviridae* has two subfamilies, *Orthoretrovirinae* and *Spumaretrovirinae*. The former has alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus and lentivirus. Avian leukosis/sarcoma virus, feline leukemia virus, bovine leukemia virus, Visna/Maedi virus, equine infectious anemia virus, feline immunodeficiency virus are important pathogenic agents to animals. *Hepadnaviridae* is a single-stranded circular DNA virus, which includes hepatitis B virus.

1 Summary about *Retroviridae*

Retroviruses of *Retroviridae* are enveloped, single-stranded RNA viruses which replicate through a DNA intermediate using an RNA-dependent DNA polymerase (reverse transcriptase). This family includes many members which are oncogenic, are related to immune system disorders, and cause degenerative and neurologic syndromes.

1.1 Classification

The *Retroviridae* has two subfamilies: *Orthoretrovirinae* and *Spumaretrovirinae.* Classification is based on genome structure and nucleic acid sequence, in addition to older classification criteria about morphology, serology, biochemical features, and the species of animals. The *Spumaretrovirinae* has only one genus *Spumavirus.* The *Orthoretrovirinae* has six genera, *Alpharetrovirus*, *Betaretrovirus*, *Deltaretrovirus*, *Epsilonretrovirus*, *Gammaretrovirus*, and *Lentivirus.*

Members of genus *Spumavirus* are nononcogenic viruses which can cause the formation of multinucleated and vacuolated (foamy) giant cells. No diseases have been

associated with spumaviruses in humans or animals.

The genus *Lentivirus* includes many important animal retroviruses, e.g, equine infectious anemia virus, Bovine immunodeficiency virus, Visna/Maedi virus, etc. Lentiviruses are often associated with chronic immune dysfunction and neurologic diseases.

The retrovirus of other 5 genera are oncoviruses or the RNA tumor viruses because they have ability to induce neoplasia, and they also can cause other kinds of diseases. These genera include *Alpharetrovirus* (e.g., avian leukosis virus), *Betaretrovirus* (e.g., mouse mammary tumor virus), *Gammaretrovirus* (e.g., murine leukemia virus), *Deltaretrovirus* (e.g., bovine leukemia virus) and *Epsilonretrovirus* (e.g., walleye dermal sarcoma virus).

Exogenous retroviruses spread horizontally (or vertically but non-genetically) in animals like other kinds of viruses, while endogenous retroviruses are transmitted genetically. Endogenous retroviruses persist as integrated DNA proviruses which are passed from generation to generation through the DNA in the gametes of the host. So, the endogenous proviral genome may exist in each cell of the host. Many vertebrates host possess endogenous retroviral DNA sequences. In general, endogenous retroviruses are not expressed and not pathogenic for their host. When endogenous viruses replicated in the original host cell, it is usually restricted. The endogenous transmissional mode occurs in many kinds of the oncoviruses, but is not known to occur in the lentiviruses or the spumaviruses.

Some oncoviruses are classified by their interaction with different cells. Ecotropic strains replicate only in cells from original animals and xenotropic strains replicate only in cells from other original animals. Amphotropic strains replicate in both. Most of endogenous retroviruses are also xenotropic.

Many features of retroviruses are known because of the extensive work on the oncoviruses in cancer research and the lentiviruses in the acquired immunodeficiency syndrome (AIDS) research. The members of the *Retroviridae* share many common features in their composition, organization, and life cycle, although the details of individual retroviruses vary (Fig. 25-1).

1.2 Components of Retroviruses

A typical retrovirus virion is composed of 2% nucleic acid (RNA), 60% protein, 35% lipid, and 3% (or more) carbohydrate. Its buoyant density is 1.16-1.18g/ml.

1.2.1 Lipids

Lipids of retroviruses are mainly phospholipid and exist in envelope. They form a bilayered structure similar to the outer cell membrane from which the retrovirus envelope is derived.

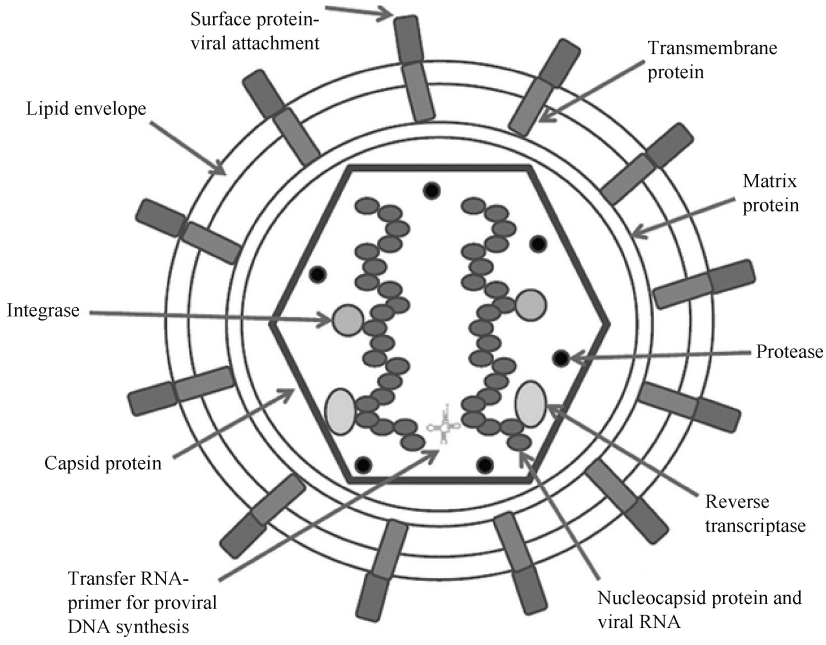


Fig. 25-1 Schematic diagram of a retrovirus particle (Scott McVey *et al.*, 2013)

1.2.2 Nucleic Acid

1.2.2.1 RNA

The genomic RNA of retroviruses exists in each virus particle as a dimer of two linear, single-stranded, positive-sense copies which are noncovalently joined near their 5′ ends. Hence, the genome of retroviruses virion is diploid. Upon denaturation, the sedimentation coefficient of each RNA copy is 38S. The monomer RNA is (7-11) $\times 10^3$ base pairs. Transfer RNA (tRNA) of the host cell is associated with genomic RNA near the 3′ terminus and serves as a primer for the synthesis of DNA by the reverse polymerase. The type of tRNA packaged in the virion is useful in the classification of retroviruses. The 3′ terminus of each RNA monomer has a poly (A) tract. The 5′ terminus has a methylated nucleotide cap.

1.2.2.2 Proviral DNA

In a cell, the retroviral RNA genome is reverse transcribed into a DNA copy which is the proviral DNA form serving as the intracellular retroviral genome. The retroviral DNA is several hundred bases, which are longer than the retroviral RNA genome due to duplication of repeated and unique terminal sequences presenting in the RNA genome during the reverse transcription process. These sequences form the long terminal repeats (LTR) which flank the genes in the retroviral DNA. The proviral DNA is covalently integrated in the DNA of the infected host cell. This integration is

facilitated by a viral enzyme, appropriately termed integrase which is encoded in the polymerase open reading frame.

1.2.2.3 Structure and Sequence of Nucleic Acid

From the 5′ end to the 3′ end of genomic RNA of retroviruses, the sequence of structural genes is *gag-pol-env*. Some retroviruses (e.g., lentiviruses, spumaviruses, and deltaretroviruses) have additional genes (e.g., *tax* and *rex*) which can regulate expression of the retroviral genome and have other accessory functions. Highly oncogenic retroviruses often have an oncogene in a place of the *pol* and/or *env* gene.

1.2.3 Proteins

1.2.3.1 Structural Proteins

Structural proteins of retrovirus are encoded by *gag* and *env* gene. Gag (group specific antigen) proteins form the core of the virion and consist of three major proteins. The nucleocapsid (NC) is a small protein (5-10kDa), which interacts with retroviral RNA. The capsid (CA) protein (about 25kDa) is the major structural element of the retroviral core. The matrix (MA) protein (about 15kDa) binds the retroviral core to the retroviral envelope. Some retroviruses have additional small core proteins.

Two glycoproteins are coded by *env* gene. The glycoprotein outside of the retrovirus (SU, surface) is a knob-like glycoprotein (about 100kDa) which binds the retrovirus to its cellular receptor. The other glycoprotein (TM, transmembrane) is a spike-like structure (about 50kDa) which binds the SU protein to the retroviral envelope.

1.2.3.2 Enzymes

Several proteins coded by *pol* gene are important for the replication of retroviruses because they have enzymatic activities. These enzymatic proteins exist in the retroviral particle, but its concentration is lower than structural proteins.

The reverse transcriptase (RT) is responsible for the production of the retroviral DNA from the retroviral RNA. Several catalytic functions of RT include an RNA-dependent DNA polymerase and an RNase H activity. RT requires a kind of divalent cation (magnesium or manganese) to function.

The retroviral protease (PR) participates the cleavage of Gag and Pol polyproteins during retroviral assembly and maturation. The retroviral integrase (IN) can covalently link the retroviral DNA into the host cell's DNA as an integrated provirus. Some retroviruses additionally encode a deoxyuridine triphosphatase enzyme (dUTP) is required for virus replication in nondividing cells.

1.2.3.3 Other Proteins

Most retroviruses only have the proteins encoded by the *gag*, *pol*, and *env* genes. Other retroviruses contain other proteins coded by additional genes which have some

functions, such as controlling the level of provirus transcription, facilitating transport of retroviral mRNA and enhancing retroviral replication in specific type of cells.

1.3 Retroviral Replication

A retroviral particle binds to a specific cell receptor via the SU protein. The retrovirus penetrates the cell and the structures of retroviral core changes. The retroviral RNA is reverse transcribed by RT using the related tRNA primer, first to a hybrid form of RNA/DNA, then to a linear double-stranded DNA form with long terminal repeats. The new retroviral DNA still connect with the integration complex of some core proteins and enzymes. Infection of retroviruses must occur within dividing cells in order that the integration complex can bind the host DNA. Additionally, in some retroviruses, the integration complex can actively transport into the nucleus of the host cell, which can allow such retroviruses to replicate in non-dividing or terminally differentiated cells (Fig. 25-2).

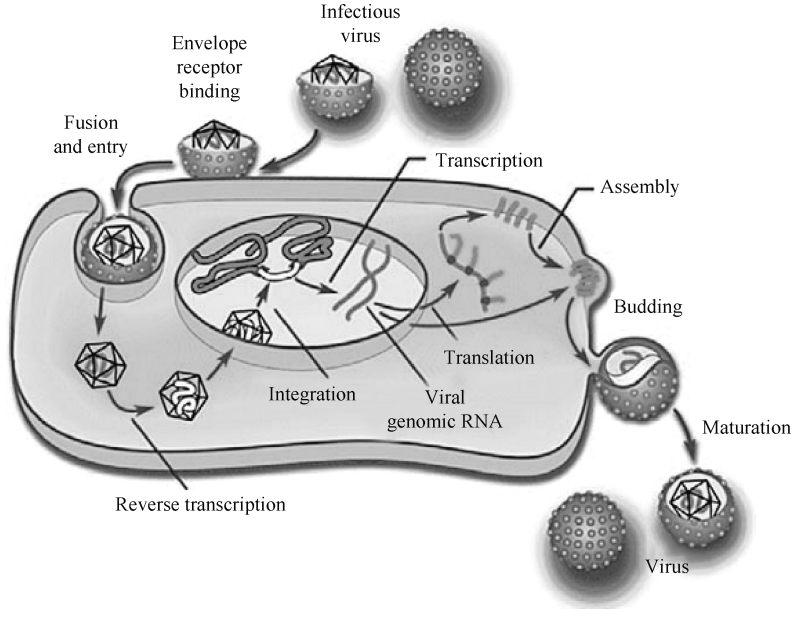


Fig. 25-2 An overview of the replication cycle of retroviruses (MacLachlan and Dubovi, 2011)

The IN enzyme mediates the integration of the retroviral DNA into the cell's DNA. The integration site in host cell's DNA is not specific. Namely, integration can occur at many sites. The integrated DNA may be transcribed into mRNA and genomic RNA using enzymes of the host cell to produce more viruses, or remain latent for a long time, and or replicate when the cell's DNA is replicated.

New retroviral particles are produced after budding from cell membranes. Immature

retroviral Gag polyprotein and genomic RNA are assembled, and retroviruses acquire envelopes when they leave infected cells by budding through the plasma membranes into which SU and TM proteins have been inserted.

In the last step, the retroviral protease (PR) cleaves the Gag polyprotein into the mature structural proteins of matrix, capsid, and nucleocapsid.

1.4 Immunologic Characteristics

Retroviral proteins possess a great many antigenic sites. Type-specific antigens that define the serologic subgroups are related to the envelope glycoproteins. Group-specific antigens are shared by related viruses and associated with the core proteins. Interspecies antigens are shared by other unrelated viruses from different host species. RT also contains type-, group-, and interspecies-specific determinants.

1.5 Oncogenic Viruses and Oncogenes

Oncogenic viruses can cause inappropriate cell growths in some tissues of susceptible hosts. Cancers are malignant tumors. The growth of normal cell in a tumor are out of controls, and these non-normal cells have ability to invade adjacent tissues and to metastasize to other parts of the body.

Oncogenic viruses cause cancer via genes they carry or activate. These genes are termed oncogenes.

1.6 Oncogenesis of Retroviruses

Different retroviruses have different mechanisms associated with cancer. Highly oncogenic or acutely transforming retroviruses cause cancer rapidly and efficiently within days or weeks after infection. Such retroviruses are rare in nature. Rous sarcoma virus (RSV) discovered in 1910 is the prototype highly oncogenic retrovirus.

The highly oncogenic retroviruses may have all or part of an oncogene which exists in the viral genome. More than 20 different retroviral oncogenes have been known, which can be found in normal cells. The normal gene equivalent to a viral oncogene is called a c-oncogene or proto-oncogene, the viral version is called a v-oncogene. In a normal cell without retroviral infection, the products of c-oncogenes have some function in growth, such as growth factors or their receptors, protein kinases, GTP binding proteins, or transcriptional activation factors. When a c-oncogene is captured by a retrovirus and became a part of a retroviral genome, it is controlled by the retroviral LTR rather than normal cell, and is expressed at high levels. The v-oncogene may be shortened, or contain point mutations, or be fused with other retroviral gene. These expressed proteins can lead to the abnormal growth of the infected cell and the

formation of neoplasia.

Most highly oncogenic retroviruses are lack their full complement of *gag-pol-env* genes, because the v-oncogene replace a portion of the retroviral genome. These defective retroviruses need the help of a closely related retrovirus which is not defective and contains the usual *gag-pol-env* complement of genes to supply the missing gene products for replication. Since the defective, highly oncogenic virus is packaged into a virion composed of the envelope proteins of the helper virus, the host range of the highly oncogenic retrovirus rely on the helper virus, because the defective retrovirus contains the envelope proteins of the helper retrovirus which is called a pseudotype.

Weakly oncogenic retroviruses cause neoplasia with slowly speed and low efficiency. These retroviruses are complement retrovirus and do not have a v-oncogene, So, they do not require a helper virus for replication. The examination of tumors caused by the weakly oncogenic retroviruses always shows abnormal proliferation of host cells. A retroviral genome in DNA of the host cell is always near a cellular oncogene. For example, avian leukosis virus is often integrated near or in the c-*myc* gene. The tumorigenic mechanism by weakly oncogenic retroviruses is considered as the insertional or *cis*-activation oncogenesis. During replication, proviral DNA is randomly inserted into many locations in the host genome. Occasionally, the integration occurs near a cellular c-oncogene. This may cause inadequate transcription of the oncogene either by read-through from the retroviral promoter or by enhancer activity of a retroviral LTR. The integration of the provirus DNA close to a c-oncogene is a very rare event. Therefore, the integration occurs with very low frequence and at very low efficiency. The tumors are considered as clonal products in origin because only one rare cell has occurred insertional oncogenesis and undergone the progresses to a tumor. However, abnormal activation of a c-oncogene is just one event in the course of tumorigenesis.

Another oncogenesis mechanism may occur in some retroviruses (e.g., bovine leukemia virus). Except of *gag*, *pol*, and *env*, these viruses have *tax* as a regulatory gene. The Tax protein functions can upregulate the transcription of the retroviruses by binding to specific DNA sequences within the LTR. Sometimes the Tax protein can bind to transcriptional activator sequences of cellular genes, resulting that the regulatory pathways of the infected cell are disrupted. The Tax protein can transactivate the c-oncogene rather than the proviral DNA itself, which is not like the insertional oncogenesis. The transactivation of a c-oncogene is just one of more incidents leading to cancer like insertional oncogenesis.

2 Avian Leukosis/Sarcoma Virus

2.1 Disease

Avian leukosis/sarcoma virus (ALSV) can cause many kinds of diseases in chickens,

including lymphoid leukosis, erythroblastosis, myeloblastosis, myelocytomatosis, sarcomas, osteopetrosis, hemangiomas, and nephroblastoma. These diseases cause great economic loss in poultry industry, but they are important tools in cancer research.

2.2 Etiologic Agent

2.2.1 Classification

Based on the differences in viral envelope glycoprotein antigens which decide the properties of virus-serum neutralization, and interference patterns between the same or different subgroups, ALSVs are classified into ten subgroups, A to J. The host of subgroup A-E and J is chicken, while subgroup F-I come from pheasants, quail, and partridges. ALV subgroup A-D and J are exogenous and have pathogenicity, while other ALSV subgroups are endogenous have no or low pathogenicity.

In some characteristics about size, shape, and ultrastructure, ALSVs are alpharetroviruses and are similar to one another. ALSVs of a definite subgroup have cross-neutralization to some extent. Viruses in different subgroups have no cross-neutralization exception of partial cross-neutralization between subgroup B and D.

2.2.2 Resistance

Lipid solvents (e.g., ether, sodium dodecyl sulfate) can remove the infectivity of ALSVs, and higher temperatures can inactivate rapidly ALSVs. However, ALVs can be saved for a long time at temperatures under 26℃. Stability of ALVs changes little at pH 5-9. However, outside the range of pH 5-9, inactivation rate of ALSVs is dramatically increasing.

2.2.3 Animal Hosts and Culture Systems

ALSVs can be isolated from chickens, pheasants, quails, and partridges and even turkeys in nature. Recently, some ALSV strains were isolated from wild birds. Experimentally, some ALSV strains especially RSV have a wide range of animal host. Some RSV strains can cause neoplasms in other species of birds and even mammals (e.g., monkeys).

Avian oncoviruses are not cytocidal for the cells in which they grow. However, RSV and other highly oncogenic ALSVs strains can induce rapid transformation of chicken embryo fibroblast cells (CEF) with alterations in cell growth and cell morphology. These cells multiplication lead to producing colonies or foci of transformed cells within several days. The number of transformed foci is negatively related to the viral dilution, which is useful for the measurement of viral concentration. Many sarcoma virus strains can cause cells transformation in cells from mouse, rat, hamster and chickens.

Weakly oncogenic ALSVs cannot produce markedly transformation or cytopathic effects (CPE) in CEF culture. But, their existence can be assessed with an immunofluorescence test using specific chicken antisera or with the induce-resistance testing to transformation by RSV.

2.3 Host-Virus Relationship

2.3.1 Distribution, Reservoir, and Transmission

ALSVs exist naturally in chickens and are distributed in most flocks of chicken worldwidely. In infected flocks, the incidence rate of lymphoid tumors is low and the mortality rate is usually 2% or less. However, losses caused by ALSVs can be much higher sometimes. The reservoir host of ALSVs is the infected chicken.

Transmission of exogenous ALSVs are vertical or horizontal. Vertically infected chicks from hens through eggs are immune tolerance resulting of failing to produce neutralizing antibodies, and have viremia for lifetime. Horizontally infected chickens through saliva or/and feces undergo transitory viremia and then develop antibodies. Tumors occur more frequently with vertical than horizontal ways in chickens.

Endogenous ALSVs such as ALV-E are transmitted genetically. Many endogenous ALSVs are defective, but some (e.g., RAV-O) are released in an infectious form and can be transmitted horizontally.

2.3.2 Pathogenesis and Pathology

ALSVs can cause many kinds of neoplasms. ALSV-infected chickens present bad growth and egg production. The pathogenesis of infected birds with ALSVs relies on whether a definite ALSVstrain carries oncogenes or not.

ALSV strains with a v-oncogene are highly oncogenic, and usually cause rapidly relatively repeatable kind of neoplastic diseases in infected chickens with a high incidence rate. While ALSVs strains without a v-oncogene are weakly oncogenic, and cause diseases that the kind of tumors rely on other factors (e.g., the number of virus, age and genotype of chicken, pathway of infection) by insertional oncogenesis near a proper c-oncogene.

Lymphoid leukosis caused by ALSV is most common under natural conditions. Transformation of lymphocytes presents in the bursa of *Fabricius* a few months after infection. These early lesions induced by ALSV sometimes fall back, while most lesions enlarge and eventually diffuse to other visceral organs. Visible neoplasms are different in size and distribution that always include the liver, spleen and bursa of Fabricius. Individual neoplasms are soft, smooth, lighting, and are usually miliary or diffuse, also may be nodular or a combination forms. These neoplastic masses are composed of large B lymphocytes which express surface immunoglobulin. Consistent

or significant hematologic changes in circulating blood and frank lymphoblastic leukemia are rare. Fully developed lymphoid leukosis present in birds at about 4 months-old or older.

Erythroblastosis occurs occasionally in chickens infected by ALSV. The liver and spleen of infected chickens are enlarged and the bone marrow is effaced because of the infiltration of proliferating erythroblasts. Sick chickens are anemic and thrombocytopenic. Blood smears present an erythroblastic leukemia. Naturally occurring erythroblastosis caused by slowly transforming ALSV may be the results of the activation of the c-oncogene c-*erbB* through insertional oncogenesis. Highly oncogenic ALSV strains carrying the v-oncogene v-*erbB* are called avian erythroblastosis virus (AEV). Erythroblastosis caused by some AEV strains can kill chickens within a week after infection.

Myeloblastosis often occur in adult chickens. The bone marrow is the target organ in myeloblastosis. The first neoplastic change is the formation of more foci composing of proliferating myeloblasts, and then the following signs are leukemia and invasion to other organs (e.g., liver, kidney and spleen). Microscopic examination uncovers massive accumulations of myeloblasts with promyelocytes in intravascular and extravascular sites. The highly oncogenic avian myeloblastosis virus (AMV) strains carry the v-*myb* gene, which can cause death several weeks after infection.

Myelocytomatosis present tumors which always occur on the surface of bones associated with periosteum, cartilage, costochondral junctions, posterior sternum, and cartilaginous bones of the mandible and nares. They are composed of compact more uniform myelocytes. Earliest changes occurring in bone marrow include the crowding of intersinusoidal spaces by myelocytes, the destruction of sinusoid walls and the overgrowth of the bone marrow. Tumors may crowd in the whole bone and penetrate through the periosteum. The highly oncogenic avian myelocytomatosis virus carries the oncogene v-*myc*.

Many benign and malignant connective tissue tumors occur sporadically in chickens infected by ALSV. Many avian sarcoma virus (ASV) strains [e.g., rous sarcom virus (RSV)] induce sarcomas, including fibrosarcoma and fibroma, histiocytic sarcoma, osteoma, myxosarcoma and myxoma, osteogenic sarcoma, and chondrosarcoma. Many highly oncogenic ASV strains contain oncogenes such as *src*, *fps*, *ros* and *yes*.

2.3.3 Host Response to Infection

Chickens infected by ALSV can be divided into four classes: no viremia and no antibodies (V–A–), no viremia and antibody (V–A+), existing viremia and antibody (V+A+), viremia and no antibody (V+A–). Chickens presenting V–A– are genetically resistant. Most infected chickens present V–A+, and in whole life persistently possess antibodies which can be passed to progeny chicks via the yolk. Passive immunity

supplied by maternal antibodies always sustains 3-4 weeks. The neutralizing antibodies are directly against the envelope proteins of virions, while antibodies against the internal group-specific antigens (Gag proteins) cannot provide protection because of its non-neutralizing roles. Neutralizing antibodies can restrict the number of virions, but they have no roles on the growth of the neoplasms induced by virus. Few chickens presenting V+A+ are in the course of an acute infection with ALSV. Most chickens presenting V+A– acquire ALSV through vertical pathway and are immune tolerant, which can transmit viruses to their progeny through the eggs.

2.4 Laboratory Diagnosis

ALSV can be isolated from serum, plasma, egg albumin, tumor tissue, embryo. Complement fixation, fluorescent antibody and radioimmunoassay (RIA) tests can be applied to detect and identify ALSVs in cell culture. An ELISA test can directly detect the virus in egg albumen or in vaginal swabs, which are used for the isolation of ALSVs. All tests need chicken embryos which are free of endogenous ALSV.

Phenotypic mixing of viruses was used for identifying the virus before. Recently, polymerase chain reaction (PCR) and PCR-based methods are widely applied for identification of the virus.

2.5 Treatment and Control

Attempts on effective vaccines are always failed, and there are no commercial effective anti-retroviral drugs for clinical application. That the establishment of breeder flocks free of exogenous ALSVs may be a practical way to eradicate ALSV from chickens.

3 Equine Infectious Anemia Virus

3.1 Disease

Equine infectious anemia virus (EIAV) can induce severe anemia in horse, but the clinical signs highly vary. Acute equine infectious anemia (EIA) occurs suddenly in 7-21 days post infection (DPI). Clinical signs include anorexia, fever, thrombocytopenia, severe anemia, profuse sweating and serous nasal discharge, which can last for 3-5 days. Then, the horse appears to recover. In the acute stage of EIA, horses are sero-negative against EIAV.

Subacute EIA often follow the acute infection after a recovery of 2-4 weeks. Signs in the acute stage are represented, while other new appear signs include weakness, petechiae, edema, lethargy, anemia, depression and ataxia. The horse again appears to health. But, the cycle of disease-health may recur.

Chronic EIA is similar to the subacute form, but it is milder and rarely leads to death. The cycle of clinical signs can recur more than five times. Each turn usually lasts 3-5 days, and the interval time is irregular. The frequency and severity may decrease after 6-8 cycles. Then, most horses have no clinical signs, but carry EIAV for the rest of their lives. However, EIA can be re-induced by stress or immunosuppressive drugs.

Subclinical or mild EIA is the common results of EIAV infection in horses with inapparent signs. But these horses have antibodies against EIAV and carry EIAV for lifelong, which can be observed more than 18 years.

3.2 Etiologic Agent

EIAV is a member of lentivirus. EIAV have two envelope glycoproteins (gp90=SU, gp45=TM) and four major non-glycosylated proteins (p26=CA, p15=MA, p11=NC, and p9). The p26 protein is the major core protein, which has group specificity. The envelope-associated glycoproteins have hemagglutination activity and type-specificity.

The genome of EIAV is highly variable. When EIAV is subjected to immune selective pressure of a horse. Mutations of nucleotide substitutions can produce new antigenic variants of envelope proteins, which cause the cycle of disease-health in the progress of EIA. When EIAV is in cell culture without immune selection, antigenic types are stable and neutralized by antibodies from the EIAV original horse. When an EIAV strain is introduced into a new horse, new antigenic variants are produced and are not any more neutralized by the original antibodies.

3.2.1 Resistance

EIAV can be inactivated by sodium hydroxide, sodium hypochlorite, most organic solvents, and so on. EIAV in horse serum at 58℃ for 30min has no infection to horses. However, EIAV at 25℃ holds infection for 96h.

3.2.2 Animal Hosts and Culture Systems

EIAV can infect horses, ponies, donkeys and mules. The try to propagate EIAV in lambs, mice, hamsters, pigs and rabbits have failed.

First EIAV isolates can be propagated only in equine cells of the monocyte/macrophage lineage. Laboratory EIAV strain can be propagated in many cell lines including human fetal lung fibroblasts. Signification of different sequence especially in the U3 region of the LTR exists between the primary isolates and laboratory strains.

3.3 Host-Virus Relationship

3.3.1 Distribution, Reservoir, and Transmission

EIAV is distributed worldwide and prevalent in warm climates. Horses, donkeys and

mules are only known reservoirs and natural hosts of EIAV.

EIAV can be transmitted by hematophagous insects especially deer and stable flies. Although EIAV cannot replicate in insect cells, but flies can transmit EIAV by mechanical transfer of blood containing EIAV. This transmission via blood can also occur through contaminated needles. Viral transmission can occur from a carrier mare to the nursing foal. EIAV can also be transmitted via the utero, but which is rare.

3.3.2 Pathogenesis and Pathology

Anemia of EIA is a consequence of hemolysis or/and erythrophagocytosis by activated macrophages. Complement levels are decreased, and complement-coated erythrocytes can be observed in EIAV-infected horses. That erythropoiesis level is decreased and pertubation in iron metabolism is disordered also contribute to anemia in chronic EIA.

Gross lesions of EIA include widespread hemorrhage and necrosis of lymphatic tissues, anemia, edema and emaciation. Microscopic lesions include the activation of the mononuclear phagocytic system in all lymphoid tissues, the activation of Kupffer cells, and hemosiderin deposition in many organs. Immune complex-mediated glomerulonephritis and hepatic centrolobular necrosis are common, the latter as a consequence of severe, acute-onset anemia. Granulomatous ependymitis, meningitis, choroiditis, subependymal encephalitis, and hydrocephalus are associated with ataxia.

3.3.3 Host Response to Infection

Horses infected by EIAV develop antibodies in 45 days. Most EIAV-infected animals become ELISA-positive in 12 days and agar diffusion test-positive in 24 days.

3.4 Laboratory Diagnosis

The detection of specific antibody using the Coggins test once are used widely. Recently, ELISA tests and ELISA-based methods, PCR and PCR-based methods are also available.

3.5 Treatment and Control

Supportive therapy is most important in recovery of EIAV-infected horse. Infected animals should be killed or isolated. Incidence of EIA can be decreased by the control of stable flies and mosquitoes. Reuse of hypodermic needles and transfusions from undetected donors must be avoided. Uninfected foals usually come from negative mares and negative stallions. A vaccine against EIAV is used in some countries, but it may not provide broad protection against all variants of EIAV.

Review Questions

1. Try to describe the classification status of retrovirus.
2. What are the retroviruses that have important pathogenic effects?
3. Try to draw the structure model of retrovirus.
4. Try to describe the function and structure of reverse transcriptase.
5. Try to describe the genome of the retrovirus.
6. Try to describe the the subgroup of avian leucosis virus and its pathogenicity.
7. Try to describe the pathogenesis of equine infectious anemia virus.
8. Try to illustrate the detection means and preventive measures of retrovirus with examples.

Chapter 26 Double-Stranded RNA Viruses

Synopsis

Reoviridae has two subfamilies, *Sedoreovirinae* and *Spinareovirinae*. The typical viruses of *Orbivirus* in *Sedoreovirinae* are arboviruses, of which blue tongue virus and african horse sickness virus have important pathogenic role in animals. Members of *Rotavirus* are distributed in humans and animals, and have multiple serotypes or genotypes, which can cause diarrhea in young animals. Avian orthoreovirus of *Orthoreovirus* in *Sedoreovirinae* is an important pathogenic agent in avian. The viral RNA of *Birnaviridae* is divided into two segments. Infectious bursal disease virus in *Birnaviridae* can damage the bursa and induce the immunosuppression of B cell.

Double-stranded (ds) RNA viruses are a multiple group of viruses that vary widely in host range (animals, humans, fungi, plants and bacteria), the number of genome segment (one to twelve), and virion organization (capsid layers, *T*-number, or turrets). Viruses with dsRNA are grouped into six families: *Birnaviridae*, *Reoviridae*, *Partitiviridae*, *Totiviridae*, *Hypoviridae* and *Cystoviridae*.

1 *Reoviridae*

1.1 Rotaviruses

Rotaviruses were firstly observed in faecal samples of mice and monkeys by electron microscopy. Spherical virion resembling the spokes of a wheel, with diameter about 75nm, were delineated (Fig. 26-1), so the Latin word rota (=wheel) were used as the name for the viruses. Similar viruses were observed 10 years later in faecal samples from children with diarrhoea by electron microscopy.

1.1.1 Morphology and Structure

The virion, with structures presenting icosahedral symmetry, is also referred to a triple-layered particle as the capsid contains three layers, each consisted of a distinct virus protein (VP). The middle and inner layers, consisted of VP2 and VP6 respectively, are perforated by channels. The middle layer has the “spokes” of the “wheel” and is the main composition of the virion. The outer layer is consisted of VP7, which is

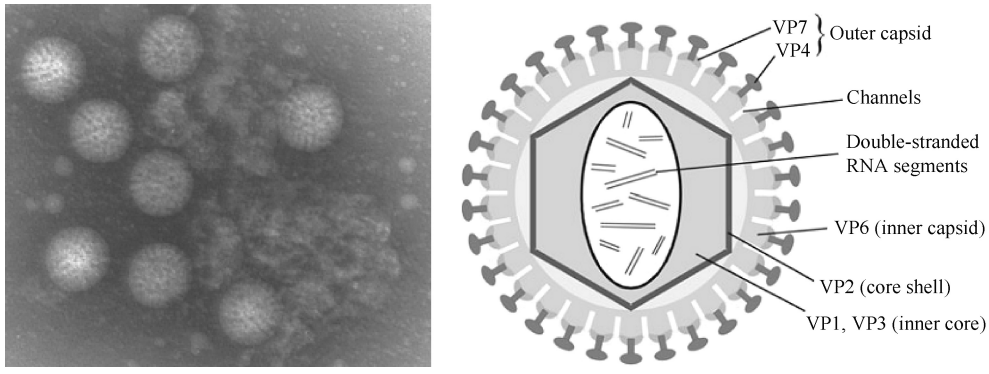


Fig. 26-1 Rotaviruses: electron micrograph (left) (Markey *et al.*, 2013), and structure (right) (Modrow *et al.*, 2013)

glycosylated. It is uncommon to find a glycoprotein in a naked virion, but VP7 is correlation with a membrane within the cell before it is included in the virion. Other protein species, including VP1 and VP3 in the core, and VP4, which forms 60 spikes at the surface, are found in the virion.

The proteins are limited in order of their sizes. The three largest proteins are found towards the centre of the virus particle; within the inner capsid layer (VP2) constructed from the genome are 12 copies of VP3 and VP1, which are enzymes. VP3 presents guanylyl transferase and methyl transferase activities while VP1 has the RNA-dependent RNA polymerase activities. There is only one copy each of VP3 and VP1 attached to the inner capsid layer at each of the 12 vertices of the icosahedron.

The 11 dsRNA segments that consist of the rotavirus genome can be isolated based on size by electrophoresis in a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Each segment of RNA encodes one protein, apart from one segment, which encodes two proteins. Hence after 12 proteins are encoded: six constitutive proteins (VP) and six non-structural proteins (NSP). In the diagram, the RNA segments are colour-coded to distinguish the plus strand (the coding strand) from the minus strand.

1.1.2 Rotavirus Replication

Rotaviruses infected cells are known as enterocytes at the ends of the villi (finger-like extensions) in the small bowel. The mechanisms by which rotaviruses adhere to and entry into their host cells are complicated and most details remain unclear. Pyrolysis of the spike protein VP4 to its products VP5 and VP8 by proteolytic enzymes such as trypsin leads to much faster enter the cell. Binding of a virus particle to a cell is originally via sites on the spike proteins (VP4, VP8, VP5) and then via sites on the capsid surface (VP7). It is probable that these proteins interact with amount of cell surface proteins; there is evidence that VP5 and VP7 bind to integrins.

There are two probable ways in which a virus particle can entry into the cell: direct

penetration of the virus particle across the endocytosis and plasma membrane, and indirect penetration which is mediated by a hydrophobic region of VP5. This region is hidden in the noncleaved VP4, so virion with spike proteins that have not been cleaved are unable to enter by this mechanism.

1.1.3 Rotavirus Disease

Enterocytes, line on the villi, are damaged as a result of rotavirus infection and this results in decreased absorption of sugars, salts and water from the gut. Evidence shows that non-structural protein NSP4 can destroy the tight junctions between cells, allowing leakage of fluid into the gut. These effects of virus infection, combined with the secreting water and solutes by secretory cells, lead to diarrhoea and this can result in dehydration. Treatment is a comparatively simple thing of rehydrating the patient with a solution of sugar and salts, but the methods for this are not currently available in some places of the world. It calculated that annually there are about half a million deaths of young children and infants as rotavirus infections. Although a majority of rotavirus infections are restricted to the gut, evidence shows that occasionally the virus may cross the gut and infect other tissues.

1.1.3.1 Animal Susceptibility

Rotaviruses have a wide range of host. A majority of isolates come from newborn animals with diarrhea. Cross-species infections can observe during experimental inoculations, but it is unclear if this occur in nature. Swine rotavirus infects both weanling piglets and newborn. Newborns often evincing signs of subclinical infection perhaps as the presence of maternal antibody, whereas obvious disease is more often occur in weanling animals.

1.1.3.2 Pathogenesis

Rotaviruses infected cells on the small intestine villi (colonic and gastric mucosa are spared). They proliferate in the enterocytes cytoplasm and destroy their transport systems. One of the non-structural protein, NSP4, is a viral intestinotoxin and induced secretion via activating a signal transduction pathway. Destroyed cells may shed into the intestine lumen and release a large number of viruses, which appear in the stool (up to 10^{10} virions per gram of faecal samples). Viral feces often continue 2-12 days in otherwise healthy patients but may be extended in those with lack of nutrients. Diarrhea driven by rotaviruses may be because of damaged sodium and glucose absorption due to impaired cells on villi are substituted by indigestible immature crypt cells. It may take 3-8 weeks for normal function to be restored.

1.1.3.3 Clinical Findings and Laboratory Diagnosis

Rotaviruses raise the main section of diarrheal diseases in children and infants

worldwide but not in adults. There is a latency period of 1-3 days. Hallmark behaves include fever, watery diarrhea, vomiting and abdominal pain, resulting in dehydration.

In children and infants, serious lack of fluids and electrolytes may be fatal unless treated. Patients with lighter cases have symptoms for 3-8 days and then full recover. However, viral feces in the stool may continue until 50 days after onset of diarrhea. Symptomless infections with seroconversion may occur. In children who have immunodeficiency, rotavirus can cause prolonged and severe disease.

Tight contacts may cause infection in adult, as the proof of seroconversion, but they hardly ever show symptoms, and virus is seldom tested in their stools. Pediatric cases are the common source for infection. However, severe diseases are common in adults, particularly in closed populations, as in a geriatric ward. Group B rotaviruses have been embroiled in massive outbreaks of serious gastroenteritis in adults in China.

Laboratory diagnostics remains on verification of virus in early collected stool in the illness and on an increase in level of antibody. Virus in stool is verified by ELISA, latex agglutination tests, or IEM. Genetic typing of rotavirus nucleic acid from stool samples by the PCR is the most sensitive detection method. Serologic tests can be applied to detect an increased level of antibody, especially ELISA.

1.1.4 Epidemiology and Immunity

The single and most important worldwide pathogen of gastroenteritis are rotaviruses in young children. There is a high incidence rate but a low mortality rate in developed countries. Representatively, rotaviruses caused up to 50% of cases of acute gastroenteritis in hospitalized children.

Rotavirus infections often predominant in winter. Symptoms of infections are extremely common in children between 6 months and 2 years, and transmission appears to be common by the fecal-oral route. Hospital-acquired infections are quite frequently.

Rotaviruses are everywhere. By age of 3 years, ninety percent of children have serum antibodies to one or more types. This high incidence of rotavirus antibodies is limited to adults, suggesting subclinical reinfection by the virus. Rotavirus reinfection are usual; it has been shown that small children could suffer up to five reinfections by age of 2 years. Non-symptomatic infections are very common with successive reinfection. Partial immune factors, such as excretive IgA or interferon, may be significant in protection against infection of rotavirus. Non-symptomatic infections are fairly common in infants before age of 6 months, the time during which protective maternal antibody acquired passively by newborns should be present. Such infection in newborns does not exhibit repeated infection, but it does protect against the development of serious disease during reinfection.

1.1.5 Treatment and Control

Treatment of gastroenteritis, correction of the loss of electrolytes and water that may result in shock, acidosis, dehydration, and death, is supportive. Management compose of replacement of restoration of electrolyte balance and fluids either orally or intravenously, as feasible. The rare mortality of developed countries infantile diarrhea is owing to routine use of effective replacement therapy.

In consideration of the fecal-oral mode of transmission, sanitation and wastewater treatment are important control measures.

An oral live attenuated rhesus-based rotavirus vaccine was approved in America in 1998 for vaccination of infants. It was cancelled a year later due to reports of intussusception (bowel blockages) as an unusual but severe side effect associated with the vaccine. However, an effective and safe vaccine remains the best hope for decreasing the worldwide onus of rotavirus infection.

1.2 Reoviruses

The viruses of this species, which have been studied drastically by molecular and cell biologists, are undefined to cause disease in human.

1.2.1 Classification and Antigenic Properties

Reoviruses are everywhere, with a wide range of host. Three specific but relevant types of reovirus have been recovered from many genera and are demonstrated by NT and HI tests. Reoviruses have a hemagglutinin for bovine erythrocytes or human O erythrocytes.

1.2.2 Epidemiology

Reoviruses cause numerous recessive infections, due to the majority of people have serum antibodies in early adulthood. Antibodies are also exhibit in other species. Three types have been reported can recover from healthy children and young children during outbreaks of mild febrile illness, from children with enteritis or diarrhea, and from epidemic rhinitis of chimpanzees.

Human volunteer studies have not demonstrated a distinct cause-and-effect relationship between reoviruses and human illness. In inoculated volunteers, reovirus is recovered far harder from nose or throat than from the feces. It has been suggested an association of biliary atresia with reovirus type 3 in infants.

1.2.3 Pathogenesis

Reoviruses have been become an important pattern system for the study of the molecular level of pathogenesis of viral infection. Defined recombinants consist of differing pathogenic phenotypes of two reoviruses are applied to infect mice.

Segregation analysis is then applied to associate specific features of pathogenesis with particular viral genes and gene products. The pathogenicity of reoviruses are originally demonstrated by the protein types based on the outer capsid of the virion.

2 *Birnaviridae*

Some members of *Birnaviridae* are very important in avian and marine industry.

2.1 Features of *Birnaviridae*

2.1.1 Description and Significance

Birnaviruses infect animals: insects, crustaceans, vertebrates and molluscs. They are dsRNA viruses and have double-stranded RNA genomes in two components.

2.1.2 Genome Structure

The genome of *Birnaviridae* is segmented and include linear double-stranded RNA. A few species of virion contain non-genomic nucleic acid. The total genome is 5,880-6,400 nucleotides long. Segment A has been fully sequenced and the complete sequence is 3,100-3,200 nucleotides long while segment B is 2,750-2,850 nucleotides long. The genome G+C content are 45.5-54.5%. The 5′-end of the genome has a cap on both segments genome-linked protein (VPg). The multipartite genome is found in one type of virion only.

2.1.3 Virion Structure

The viral particle in *Birnaviridae* have an ordinary construction. The particles are not enveloped and include a capsid. The capsid is round and presents icosahedral symmetry (T=13). The capsid shell of the virus particle is consist of a single layer. The capsid exhibit sexangular in outline. The arrangement of capsomer is clearly visible and there are about 132 capsomers in the capsid. The capsid all have the same outline have no projections on surface.

2.1.4 Reproduction Cycle in Host Cells

The replication and proliferation of birnaviruses occurs in the cytoplasm. The dsRNA uses as a template for the formation of mRNA (+) and progeny genomes.

2.1.5 Viral Ecology and Pathology

The *Avibirnavirus* is the pathogene of infectious bursal disease. The road of infection is primarily oral, but the respiratory tract and conjunctiva can also take part in infection. The virus is identified in liver Kuppfer cells shortly after onset of infection. The virus can also be identified in lymphoid cells and macrophages of the duodenum, jejunum

and cecum. The cells of the bursa of Fabricius are infected less than 12h. The Harderian gland, the thymus and spleen are infected after viremia. The immune response is damaged due to the depletion of the bursa.

2.2 Infectious Bursal Disease Virus (IBDV)

Infectious bursal disease (IBD) is a highly infectious disease of young chickens caused by infectious bursal disease virus (IBDV), presented by immunosuppression and mortality usually at age of 3-6 weeks. It is financially significant to the poultry industry worldwide because of increased susceptibility to other diseases and no interference with efficient vaccination. Now, very virulent strains of IBDV (vvIBDV) which can cause serious mortality in chicken have emerged in world.

2.2.1 Description of IBDV

IBDV with bi-segmented genome, is a double-stranded RNA virus, belongs to the genus *Avibirnavirus* of *Birnaviridae*. IBDV has two different serotypes, but only serotype 1 IBDV cause disease in poultry. There are six antigenic subtypes of IBDV serotype 1 have been found by cross-neutralization assay *in vitro*. Viruses pertain to one of these subtypes are usually known as variants, which were demonstrated to break through high levels of maternal antibodies in commercial chickens, causing up to 60-100% mortality rates in flocks (Fig. 26-2).

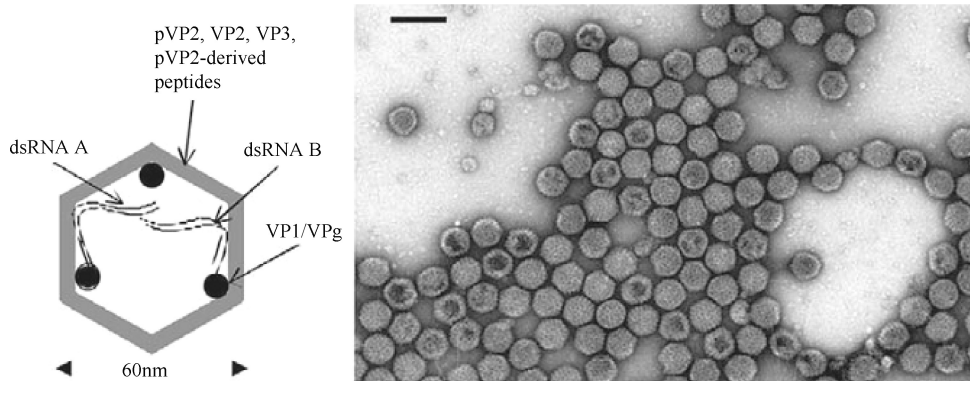


Fig. 26-2 IBDV: diagram of a particle (left), electron micrograph (right, the bar represents 100nm) (MacLachlan and Dubovi, 2011)

2.2.2 Genome of IBDV

IBDV genome include two segments, A and B, which are wrapped within a non-enveloped icosahedral capsid. The smaller segment B (2.9kb) encodes VP1, the presumptive viral RNA polymerase. The genome segment A (3.2kb) encodes viral proteins VP5, VP4,

VP3 and VP2. Among them, VP2 protein has significant neutralizing antigenic sites and triggers protective immune response and most of the amino acid (AA) changes between antigenically distinct IBDV are clustered in the VP2 hypervariable region. Thus, the hypervariable region of VP2 is the distinct target for the molecular techniques used to IBDV detection and strain variation studies.

2.2.3 Viral Structure

The IBDV capsid protein presents structural domains that exhibit homology to some positive-sense single-stranded RNA viruses capsid proteins, such as the tetravirusesand nodaviruses, as well as the *T*=13 capsid shell protein of the Reoviridae. The *T*=13 shell capsid of the IBDV is constructed by trimers of VP2, a protein produced by its precursor without the C-terminal domain, pVP2. The pruning of pVP2 is executed on immature particles as part of the ripening process. The other main structural protein, VP3, is a multipurpose element lying under the *T*=13 shell that affects the innate structure of polymorphism of pVP2. The virus-encoded RNA-dependent RNA polymerase, VP1, is become a part of the capsid through its related to VP3. VP3 also interacts widely with the viral dsRNA genome.

2.2.4 Pathogenesis

The virus attack lymphoid cells and particularly those of B-lyphocyte origins. Young birds at age of 2-8 weeks that contain highly active bursa of Fabricius are more susceptible to disease. Birds over 8 weeks are resistant to infection and will not exhibit clinical symptoms unless challenged by highly virulent strains.

After ingestion, the virus damages the lymphoid follicles in the Fabricius bursa as well as the circulating B-cells in the secondary lymphoid tissues such as BALT (bronchial) caecal tonsils, CALT (conjunctiva), GALT (gut-associated lymphoid tissue), Harderian gland, etc. Acute disease and death are because of the necrotizing effect of these viruses on the tissue of host. If the bird survives and recovers from the phase of the disease, they still maintaining immunocompromised which means they are more susceptible to other diseases and vaccination in the face of outbreak will not be valid.

Passive immunity can protect against disease, as does previous infection with avirulent strains. In broiler farms, breeder flocks are immunized against IBD so that they would confer protective antibodies to their offspring which would be slaughtered for eat before their passive immunity depletion.

2.2.5 Clinical Signs

In the acute form birds are depressed, dehydrated and debilitated. They exhibit watery diarrhea, swollen, and blood-stained vent. Mortality rates vary with involved strain with different virulence, the challenge dose as well as the flock's ability to product an effective immune response. Less virulent strains infection may not exhibit overt

clinical symptoms but the birds may have cystic or fibrotic bursa of Fabricus that has atrophied untimely (before age of 6 months) and may die of infections by reagents that would not generally cause disease in immunocompetent birds.

2.2.6 Clinical Detection and Diagnosis

With the advent of hypersensitivity molecular techniques, such as reverse transcription polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP), it became practicable to detect the vvIBDV, to distinguish IBDV strains, and to apply such information in exploring the molecular epidemiology of the virus.

Review Questions

1. What are the double-stranded RNA viruses that have important pathogenic effects?
2. Try to describe the classification status of *Reoviridae*.
3. Try to describe the pathogenesis of blue tongue virus.
4. Try to describe the pathogenesis of rotavirus.
5. Try to describe the classification status of *Birnaviridae*.
6. Try to describe the pathogenesis of infectious bursal disease virus.
7. Try to describe the microbiological diagnosis of bluetongue disease.
8. Try to describe the diagnosis, prevention and control measures of infectious bursal disease.

Chapter 27 *Mononegavirales*

Synopsis

Mononegavirales has four families, *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae* and *Bornaviridae*. Members of *Mononegavirales* have a similar gene structure and replication mode. Many members of *Paramyxoviridae* have important pathogenic significance in humans and animals, such as Newcastle disease virus, canine distemper virus and rinderpest virus. Rabies virus is a typical representative of *Rhabdoviridae*, which has great pathogenic role to humans and animals. Ebola virus and marburg virus in *Filoviridae* can cause virulent diseases in humans. Vaccines can be used to prevent and control the infection of paramyxovirus and rhabdovirus.

RNA viruses can be further classified according to the sense or polarity of their RNA into negative-sense and positive-sense, or ambisense RNA viruses. Positive-sense viral RNA is similar to mRNA and thus can be immediately translated in the host cell. Negative-sense viral RNA is complementary to mRNA and thus must be converted to positive-sense RNA by an RNA polymerase before translation. As such, purified RNA of a positive-sense virus can directly cause infection though it may be less infectious than the whole virus particle. Purified RNA of a negative-sense virus is not infectious by itself as it needs to be transcribed into positive-sense RNA; however, each virion can be transcribed to several positive-sense RNAs. Ambisense RNA viruses resemble negative-sense RNA viruses, except they also translate genes from the positive strand.

1 *Paramyxoviridae*

Viruses in *Paramyxoviridae* are enveloped, pleomorphic particles (150nm in diameter) containing a linear single-stranded RNA genome. Its nucleocapsid has helical symmetry (Fig. 27-1). Based mainly on size of nucleocapsid, presence or absence of neuraminidase, and the antigenic relationship, members of *Paramyxoviridae* are divided into seven genera, *Aquaparamyxovirus*, *Ferlavirus*, *Respirovirus*, *Rubulavirus*, *Morbillivirus*, *Avulavirus* and *Henipavirus*. Some members cause serious respiratory or systemic diseases in mammalian and avian species.

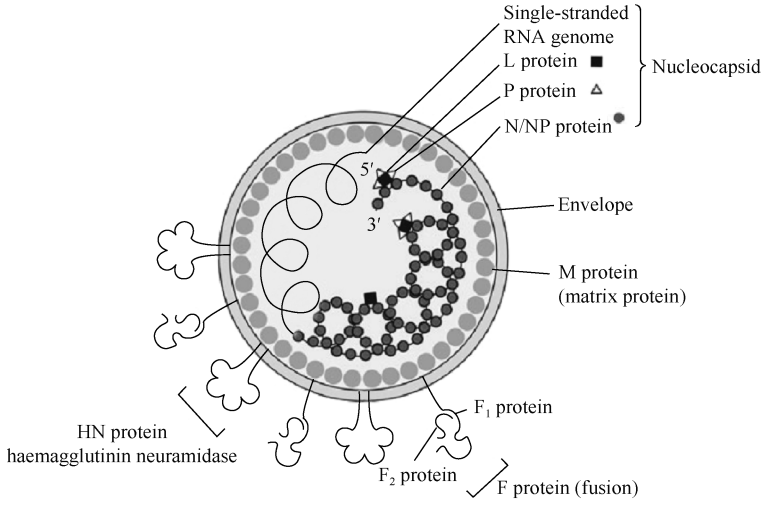


Fig. 27-1 Structure of a paramyxovirus particle (Modrow *et al.*, 2013)

1.1 Canine Distemper

Canine distemper (CD) is the most serious viral disease of dogs. The classic textbook description of the disease is of an acute viral infection characterized by diphasic fever, ocular and nasal discharges anorexia, depression, vomiting diarrhea, dehydration, leukopenia, thrombocytopenia, respiratory distress, skin rash, hyperkeratosis of the foot pads and nose, muscle spasms, and signs of central nervous system (CNS) involvement. The most frequent symptoms are diarrhea, respiratory signs, fever, catarrhal ocular or nasal discharges, hyperkeratosis of foot pads, and nervous signs. It is important to recognize that canine distemper infection in dogs can produce a variety of clinical signs that range from no visible disease to several symptoms in the infected animals. The disease has an incubation period of 3 to 5 days. The mortality rate of the disease depends largely on the immune status of the infected dogs. Mortality is highest among puppies and in cases where there are complications such as pneumonia and encephalitis.

1.1.1 Physical, Chemical and Antigenic Properties

Canine distemper virus (CDV) is variable in shape and size. Virus particles are spherical and vary in diameter from 150 to 300nm. The viral nucleocapsid is helical in symmetry and is enclosed by a lipoprotein envelope (5-8nm in thickness) that contains spikes 9-13nm in length. The virus has a buoyant density of 1.23g/cm^3 in cesium chloride. The viral nucleic acid is a linear single, stranded RNA and the viral capsid is composed of six major polypeptides. The large glycosylated H polypeptide is responsible for the adsorption of CDV to receptor sites of susceptible cells. Other glycosylated polypeptide (F) causes fusion of cells infected by CDV. All members of

the *Morbillivirus* are closely related antigenically since they all share the six major polypeptides. However, differences in the major polypeptides of different members of *Morbillivirus* could be distinguished by peptide mapping. Canine distemper virus has only one antigenic type.

1.1.2 Resistance to Physical and Chemical Agents

CDV is sensitive to environmental factors. Such as extreme of pH, temperature, and several kinds of disinfectants. It can be inactivated by visible light, ultraviolet light, and heating at 60℃ for 30min. Therefore, the virus can survive in tissues for 48h at 25℃ or for 14 days at 5℃. Optimal pH stability for CDV is 7.0. Infectious virus is lost above pH 10.4 or below pH 4.4. CDV is readily inactivated by disinfectants, such as 0.2% roccal quaternary ammonium compound or 0.75% phenol solution. As we know that most viral inactivation studies are carried out in laboratory conditions, labile viruses, such as the CDV, can exist longer in cool, shady environments or in serum or tissue debris.

1.1.3 Infectivity for Other Species and Culture Systems

CDV can infect a number of animals, in addition to dogs, other members of the Canidae (e.g., fox, wolf, coyote), the Mustelidae (e.g., badger, ferret, skunk, mink) and the Procyonidae (e.g., raccoon, panda), are all susceptible to CDV. Canine distemper in some members of the Felidae (lion, tiger) has been reported. Ferrets are especially susceptible to CDV and are frequently applied as laboratory animals in the study of this disease. There is no evidence that CDV can infect humans.

CDV can be isolated and propagated in primary canine and ferret kidney cell cultures. The virus has been successfully adapted to embryonated chicken eggs and various cell cultures, such as bovine kidney, Vero monkey kidney, and human amnion or fibroblast continuous cultures. The virus can also be adapted to newborn Swiss mice and weanling hamsters.

1.1.4 Distribution, Reservoir, Transmission

Canine distemper is a worldwide spread disease. The dog is the main host for the disease. Infected dogs can produce CDV in their nasal and ocular secretions during the course of the disease. Virus can be detected in the urine of experimentally infected dogs 6-22 days after exposure to CDV. In addition, feces of infected dogs also contain the virus. It is possible that the disease is transmitted by direct contact or droplet infection.

1.1.5 Pathogenesis and Pathology

To dogs experimentally infected with CDV by the aerosol route, virus can be detected in bronchial lymph nodes and in tonsils 2 days after viral exposure. Leukocyte-

associated viremia appears on the second or third day post-infection, and on the fourth day, the virus may be detected in mononuclear cells of the lymphatic system in the digestive tract and the respiratory tract. From about the seventh to the ninth day, CDV spread to the epithelial cells of the conjunctiva, the lymphatic system, the alimentary tract, the respiratory tract, the urogenital tract, the endocrine system, and the foot pad occurs, resulting in characteristic clinical symptoms of the disease. Viral antigen first appears in the meningeal macrophages on the ninth day and later in ependymal cells and glial cells. And neurons damage to these cells in the brain gives rise to the central nervous disorders seen in canine distemper. A subacute, delayed, demyelinating encephalomyelitis is found in dogs with persistent infection by CDV, available evidence, such as isolation of infectious virus from brain tissue, high levels of antibody to CDV polypeptides, and interferon in cerebrospinal fluid of infected dogs, indicates that CDV undergoes complete replication in the brain, causing chronic neurologic symptoms.

Pathologic lesions in canine distemper are a reflection of organ systems in which viral replication has occurred. A depletion of lymphocytes in the tissues of the lymphatic system is frequently found, followed by regenerative hyperplasia during the recovery stage. Degenerative changes and intracytoplasmic, and to a lesser extent, intranuclear eosinophilic inclusion bodies are observed in the epithelial cells of the urogenital tract, the alimentary tract, and the endocrine system. There is often a diffuse interstitial pneumonia with desquamation of alveolar epithelial cells and macrophages along with thickening of alveolar walls. Hyperkeratosis of footpads and nose may be found. Central nervous system lesions are those observed with diffuse demyelinating meningoencephalomyelitis comprised of infiltration of mononuclear cells; swelling and desquamation of meningeal cells, glial cells, and neurons; perivascular cuffing; dilation of blood vessels; and extensive breakdown of myelin and axis cylinders.

1.1.6 Host Response to Infection

Canine distemper can produce a long-lasting immunity in dogs who recover from the viral infection. Neutralizing antibodies first appear in the serum of infected dogs 8-9 days after viral exposure, reaching the peak in 4-5 weeks. Neutralizing antibody can keep at a significant level in most animals for at least 1 year after infection. To dogs experimentally infected with CDV, specific IgA can't be detected in serum. The antiviral IgM levels are equivalent in both persistently infected dogs and recovered ones, while high levels of IgG can be found only in animals recovered from this disease. A lymphocyte-mediated immune response is also produced by dogs infected with CDV and is partially responsible for the recovery from the disease.

Acute canine distemper infection can cause immune depression. Fatal infection of

CDV in dogs is associated with systemic depletion of T and B lymphocyte-dependent areas in lymphoid tissues, whereas in persistently infected or convalescent dogs, repopulation of lymphoid tissues with associated germinal center formation occurs 2-3 weeks after a viral infection.

1.1.7 Diagnosis

Because the clinical signs that accompany canine distemper infection are nonspecific and variable in dogs, definitive diagnosis of the disease must depend on laboratory tests, which include: ①isolation and identification of CDV by inoculating susceptible dog or ferret cell cultures with secretions or tissue suspensions from dogs; ②demonstration of canine distemper viral antigen in mononuclear cells in the blood or conjunctival smears by the immunofluorescent technique or the peroxidase technique; and ③demonstration of rising IgG titers in paired sera by ELISA.

1.1.8 Control

Treatment for canine distemper is supportive in nature. Antibiotics are only used to prevent secondary bacterial infections and electrolyte solutions are vital in restoring the fluid balance. Anticonvulsants can be used for the symptomatic relief of nervous disorders. Despite intensive supportive care, some dogs infected with CDV are failure to make a satisfactory recovery. Animals who recover from CDV infection can develop long lasting immunity.

Vaccination against canine distemper is the best means of preventing and controlling the disease. Both modified live and inactivated CDV vaccines have been commercialized. Modified live viral vaccines can induce to produce a higher antibody titer, a longer duration of immunity, and in general afford better protection against the disease. The use of these biological products by veterinarians around the world has greatly reduced the incidence of this disease in dogs, and in many countries. Vaccination for canine distemper has become an important medicine of any canine health program. Recent commercial development of CDV in canarypox viral vector has been proven to be able to protect ferrets against canine distemper viral challenge.

Maternally derived antibody to CDV can interfere with the successful vaccination of puppies against canine distemper. Ideally, puppies should be vaccinated for canine distemper at one age when maternally derived antibody has declined to a level that allows the replication of vaccine virus to induce an active immunity. Since it is impossible to detect the serum of every puppy for neutralizing antibody prior to vaccination, one of the following two alternatives has been recommended for canine distemper vaccination in puppies. If early vaccination of puppies is required, a second booster canine distemper vaccine should be administered at 12-14 weeks of age regardless of the time the first canine distemper vaccine is given. The second

alternative is to give the puppy a measles viral vaccine followed by a canine distemper viral vaccine one week later. Measles virus is antigenically related to canine distemper virus and confers protection to CDV infection in puppies. Measles virus is not neutralized by antibodies to canine distemper virus, and this heterotypic measles virus is able to sensitize antibody-producing cells to produce a rapid and pronounced canine distemper neutralizing antibody titer following exposure to canine distemper virus. The precise mechanisms of heterotypic vaccination are not fully comprehended at present. Caution should be exercised, when vaccination for wild and exotic animals against canine distemper is required. Inactivated canine distemper viral vaccine has been shown to be effective in immunizing ferrets, foxes, bush dogs, mink, and maned wolves. Though modified live CDV vaccine is proven to be safe and immunogenic in bush dogs, fennec foxes, and maned wolves, there have been reports of canine distemper in kinkiest and lesser pandas following vaccination with a modified live CDV vaccine. It is always advisable to vaccinate exotic species with an inactivated viral vaccine, especially when information regarding live viral vaccines on those species is never found.

1.2 Newcastle Disease

The majority of avian paramyxovirus isolates from domestic and wild birds can be divided into nine serologic groups. Newcastle disease virus (NDV) is the prototype virus of the avian paramyxovirus serotype 1 and is the most serious disease causing avian paramyxovirus. Newcastle disease is a highly infectious disease in chickens and is characterized by respiratory distress, diarrhea, and neural signs. The severity of the disease is dependent upon the virulence of the strain that is responsible for the infection. The most virulent strains are referred to as velogenic and produce mortality rates in affected birds as high as 90% or more. The disease caused by mesogenic strains is less severe and the mortality rate is often less than 25%. The lentogenic strains are almost avirulent and are frequently employed as vaccines. The incubation period of Newcastle disease varies from 4 to 11 days.

1.2.1 Physical, Chemical and Antigenic Properties

Newcastle disease virus is pleomorphic. Its diameter varies from 100 to 300nm and consists of an envelope and internal structures (Fig. 27-2). The envelope contains spikes (8nm in length) that are antigenic components involving in hemagglutination and neuraminidase activities. The internal component or nucleocapsid is 17-18nm in diameter. The molecular weight of the RNA of NDV is about 5.5×10^6Da, which codes for six major polypeptides of which two are glycosylated. The glycoprotein HN is related to hemagglutination and neuraminidase activities and glycoprotein F causes fusion among infected cells.

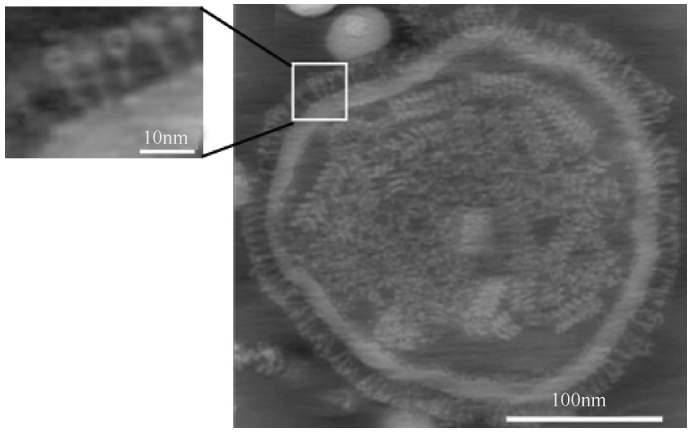


Fig. 27-2 Electron tomographic analysis of newcastle disease virus (Swayne, 2013)

1.2.2 Resistance to Physical and Chemical Agents

NDV can be inactivated in varying degrees by such physical and chemical agents as heat, pH, ultraviolet light, oxidation processes, and chemical compounds (lysol, detergents, phenol, and butylated hydroxytoluene). It is important to remember that the rate of viral inactivation varies with the strain of NDV, quantity of virus initially exposed, time of exposure, and presence of organic matter in the environment. Infectious virus has been recovered from contaminated areas and eggshells several weeks following an outbreak of Newcastle disease. It can survive in fresh eggs for many months and in frozen carcasses for a few years.

1.2.3 Infectivity for Other Species and Culture Systems

NDV can infect chickens, turkeys, guinea fowls, and a large number of species of domestic and wild birds. Sea birds are less susceptible, but may act as carriers. Humans accidentally infected with NDV when exposed to infected birds or live viral vaccines may develop a self-limiting disease.

NDV can be propagated in the chorioallantoic cavities of 10-12 day-old embryonated chicken eggs. Primary chick embryo kidney, primary chick fibroblast, and baby hamster kidney (BHK) cells are the most commonly used cell to culture for the cultivation of NDV.

1.2.4 Distribution Reservoir and Transmission

Newcastle disease is worldwide in distribution and domestic birds are the major reservoir for the disease. Although NDV has been isolated from a large number of wild birds, such as ducks, geese, crows and sparrows. They play a minimal role in transmitting this disease.

Aerosol via the respiratory tract is the most common route for transmission of NDV

infected birds begins to shed virus 2-3 days after exposure from their respiratory tracts. Investigations following NDV outbreaks suggest that flies and wind-borne forces may be the responsible factors in some instances.

1.2.5 Pathogenesis and Pathology

In natural or experimental infection of chickens with NDV viral replication occurs at the site of initial exposure, followed by primary viremia. Widespread multiplication of virus in cells of parenchymal organs leads to a secondary viremia, which in some instances causes to the infection of the cells of the CNS. Disease presents several different forms in chickens, depending on the virulence of the strains involved. The very virulent velogenic strains cause very rapidly fatal infections involving the visceral organs (Doyle's form) or the CNS (Beach's form). Mesogenic strains of NDV caused a disease represented by respiratory and, occasionally, nervous symptoms in infected chickens with low mortality (Beaudette's form), while lentogenic strains can produce a mild or often recessive disease (Hitchner's form).

Lesions in ND vary greatly. Lesions are rarely observed in chickens within apparent infections. In more severe forms of the disease, hemorrhagic necrosis can be found in the respiratory tract, intestinal tract, and visceral organs. In chickens with CNS involvement, necrosis of the glial cells, neuronal degeneration, perivascular cuffing, and hypertrophy of endothelial cells are often observed.

1.2.6 Host Response to Infection

Chickens infected with NDV can produce antibodies after viral exposure for 6-10 days, reaching a peak in 3-4 weeks. Antibodies to the envelope glycoprotein, HN, exhibit viral neutralizing and hemagglutination inhibition activities and are responsible for host immunity to the disease. Humoral antibodies (IgM and IgG), secretory antibody (IgA), and cell-mediated immunity all appear to play a role in immunity to ND.

1.2.7 Diagnosis

As the clinical symptoms and pathologic lesions of ND are variable and nonspecific, definitive diagnosis of the disease must depend on laboratory methods. These include the followings: ①isolating and identifying the virus by inoculating embryonated eggs or cell cultures with respiratory exudate or tissue suspensions (lung, spleen, or brain), due to the widespread use of live vaccine strains of NDV in the field, it is necessary to reproduce the disease in chickens with the viral isolate; ②demonstrating NDV antigen in affected tissues or cell cultures by the immune-fluorescence technique; ③demonstrating rising NDV antibody titers by the hemagglutination inhibition test, neutralization test, or ELISA method.

1.2.8 Treatment and Control

Sanitary management to prevent exposure of susceptible chickens to NDV is one of the most important aspects to control against the disease.

Since there is only one serotype of NDV vaccination, which is another major step in preventing ND. Minor antigenic differences among strains of NDV are not sufficient to prevent gross immunity. The majority of live vaccines used are lentogenic strains of NDV administered in drinking water or applied as aerosols. Occasionally, mesogenic strains are used as vaccines and are inoculated by wing, feather, follicle, or intramuscularly in chickens 4 weeks old or older. Inactivated oil-emulsion NDV vaccines by formalin and betapropiolactone are available and are administered by the parenteral route. Live NDV vaccine is effective in protecting market turkeys when it is administered 2-3 times by spray.

2 *Rhabdoviridae*

Members of *Rhabdoviridae* are very widely distributed in nature, one of which can infect vertebrates, invertebrates, and plants. *Rhabdoviridae* has 12 named genera and one unnamed genus. Rabies virus (new name in ICTV net is rabies lyssavirus) belongs to *Lyssavirus*, and can cause a zoonosis disease termed as rabies.

2.1 Structure of Rabies Virus

Rabies virus is a rhabdovirus with morphologic and biochemical properties in common with vesicular stomatitis virus of cattle and several animal, plant, and insect viruses. The rhabdoviruses are rod- or bullet-shaped particles about 75nm in width and 180nm in length (Fig. 27-3). The particles are surrounded by a membranous envelope with protruding spikes which is 10nm long. The peplomers (spikes) are composed of trimers of the viral glycoprotein (G). Inside the envelope is a ribonucleocapsid. The genome is single-stranded, negative-sense RNA (12kb; MW 4.6×10^6Da). Virions contain an RNA-dependent RNA polymerase. The particles have a buoyant density in CsCl of about 1.19g/cm^3 and a molecular weight of (300-1,000) $\times10^6$Da.

2.2 Classification

Viruses are classified in *Lyssavirus* of *Rhabdoviridae*. Rabies virus is the only medically important rhabdovirus.

2.3 Reactions to Physical and Chemical Agents

Rabies virus survives storage at 4℃ for weeks and at –70℃ for years. It is inactivated

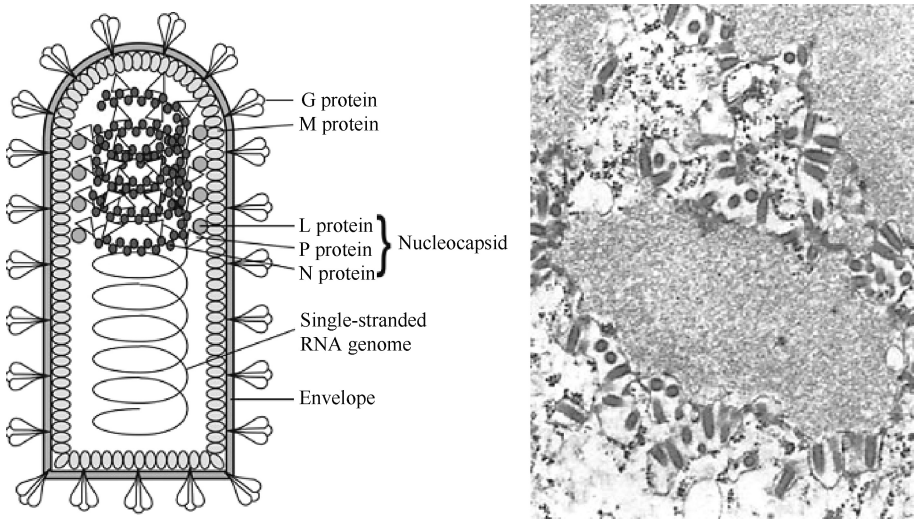


Fig. 27-3 Structure of a rhabdovirus particle (left) (Modrow *et al.*, 2013) and rabies virus in the brain (right) (MacLachlan and Dubovi, 2011)

by CO_2, so on dry ice it must be stored in glass-sealed vials. Rabies virus can be killed rapidly by exposure to ultraviolet radiation or sunlight, by heat for 1h at 50℃, lipid solvents (ether, 0.1% sodium deoxycholate), trypsin, detergents or by extremes of pH.

2.4 Virus Replication

Rabies virus can attach to cells via glycoprotein spikes; the nicotinic acetylcholine receptor may serve as a cellular receptor for rabies virus. The single-stranded RNA genome is transcribed by the virion-associated RNA polymerase to five mRNA species. The template for transcription is the genome RNA in the form of ribonucleoprotein (RNP) (encased in N protein and containing the viral transcriptase). The monocistronic mRNAs code for the five virion proteins: glycoprotein (G), matrix (M), nucleocapsid (N), and polymerase proteins (L, P). The genome RNP is a template for complementary positive-sense RNA, which is responsible for the generation of negative-sense progeny RNA. The same viral proteins serve as polymerase for viral RNA replication as well as for transcription. Ongoing translation is required for replication, particularly of viral N and P proteins. New replicated genomic RNA associates with the viral transcriptase and nucleoprotein to form RNP cores in the cytoplasm. The particles acquire an envelope by budding through the plasma membrane. The viral matrix protein forms a layer on the inner side of the envelope, whereas the viral glycoprotein is on the outer layer and forms the spikes.

2.5 Animal Susceptibility and Growth of Virus

Rabies virus has a wide host range. All warm-blooded animals, including humans, can

be infected. Susceptibility varies among mammalian species, ranging from very high (wolves, foxes, coyotes) to low (opossums); those with intermediate susceptibility include raccoons, skunks, and bats (Table 27-1). The virus is widely detected in different organs or tissues of infected animals, especially in the nervous system, urine, saliva, lymph, blood and milk. Recovery from infection is rare except in certain bats, where the virus has become peculiarly adapted to the salivary glands. Vampire bats may transmit the virus for months without themselves ever showing any signs of disease.

Table 27-1 Animal susceptibility to rabies virus (胡建和等, 2011)

Very High	High	Moderate	Low
Foxes	Hamsters	Dogs	Opossums
Coyotes	Skunks	Sheep	
Jackals	Raccoons	Goats	
Wolves	Cats	Horses	
Cotton rats	Bats, Rabbits, Cattle	Nonhuman primates	

When freshly isolated in the laboratory, the strains are referred to as street virus. Such strains have long and variable incubation periods (usually 21-60 days in dogs) and regularly produce intracytoplasmic inclusion bodies. Serial brain-to-brain passage in rabbits yields a "fixed" virus that no longer multiplies in extraneural tissues. This fixed (or mutant) virus multiplies rapidly, and the incubation period is shortened to 4-6 days. But inclusion bodies are difficult to be found.

2.6 Antigenic Properties

Though there is a single serotype of rabies virus, the viruses isolated from different species (canines, foxes, raccoons, skunks, bats) in different geographic areas are usually different. These viral strains can be distinguished by epitopes in the nucleoprotein and glycoprotein recognized by monoclonal antibodies as well as by specific nucleotide sequences. Up to now, there are at least seven antigenic variants found in terrestrial animals and bats.

The G glycoprotein is a major factor in rabies virus neuroinvasiveness and pathogenicity. Avirulent mutants of rabies virus have been selected using certain monoclonal antibodies against the viral glycoprotein. A substitution at amino acid position 333 of the glycoprotein results in loss of virulence, indicating some essential role for that site of the protein in disease pathogenesis. Purified spikes containing the viral glycoprotein elicit neutralizing antibody in animals. Antiserum prepared against the purified nucleocapsid is used in diagnostic immunofluorescence for rabies.

2.7 Pathogenesis and Pathology

Rabies virus multiplies in muscle or connective tissue at the site of inoculation and then enters peripheral nerves at neuromuscular junctions and spreads up to the central nervous system. However, it is also possible for rabies virus to enter the nervous system directly without local replication. It multiplies in the central nervous system and progressive encephalitis develops. Then the virus spreads through peripheral nerves to the salivary glands and other tissues. The organ with the highest titers of virus is the submaxillary salivary gland. Other organs where rabies virus has also been found in kidney, pancreas, retina, heart, and cornea. However, rabies virus has not been isolated from the blood of infected persons.

Susceptibility to infection and the incubation period may depend on the age of host, immune status, and genetic background, the viral strain involved, the amount of inoculum, the severity of lacerations, and the distance the virus has to travel from its point of entry to the central nervous system. There is a higher infection rate and a shorter incubation period in persons bitten on the face or head; the lowest mortality occurs in those bitten on the legs.

Rabies virus produces a specific eosinophilic cytoplasmic inclusion, the Negri body, in infected nerve cells. Negri bodies are composed with viral nucleocapsids. The presence of such inclusions is pathognomonic of rabies, but is not observed in at least 20% of cases. Therefore, the absence of Negri bodies does not rule out rabies as a diagnosis. The importance of Negri bodies in rabies diagnosis has been lessened by the development of the more sensitive fluorescent antibody diagnostic test.

2.8 Clinical Findings

Rabies is primarily a disease of lower animals and is spread to humans by bites of rabid animals or by contact with saliva from rabid animals. The disease is acute, fulminant, fatal encephalitis. The incubation period in humans is typically 1-2 months but may be as short as one week or as long as many years (up to 19 years). It is usually shorter in children than in adults. The clinical spectrum can be divided into three phases: a short prodromal phase, an acute neurologic phase, and coma. The prodrome, lasting 2-10 days, may show any of the following nonspecific symptoms: anorexia, malaise, photophobia, headache, nausea and vomiting, sore throat, and fever. Usually there is an abnormal sensation around the wound site. During the acute neurologic phase, which lasts 2-7 days, patients show signs of nervous system dysfunction, such as nervousness, hallucinations, apprehension, and bizarre behavior. General sympathetic over activity is observed, including lacrimation, pupillary dilatation, and increased salivation and perspiration. A large fraction of patients will exhibit hydrophobia (fear of water). The act of swallowing

precipitates a painful spasm of the throat muscles. This phase is followed by convulsive seizures or coma and death. The major cause of death is respiratory paralysis. Paralytic rabies occurs in about 20% of patients, most frequently in those infected with bat rabies virus. The disease course is slower, with some patients surviving 30 days. Recovery and survival are extremely rare.

Rabies should be considered in any case of encephalitis or myelitis of unknown cause even in the absence of an exposure history, and particularly in a person who has lived or traveled outside country. Most cases of rabies are in individuals with no known exposure. Because of the long incubation period, people may forget a possible exposure incident. People who contract bat rabies often can't remember being bitten by a bat.

In general, the incubation period in dogs is 3 to 8 weeks, but sometimes, it may be as short as 10 days. Clinically, the disease in dogs is also usually divided into the same three phases as human rabies.

2.9 Diagnosis

2.9.1 Rabies Antigens or Nucleic Acids

Tissues infected with rabies virus are currently identified most rapidly and accurately by means of immunofluorescence or immunoperoxidase staining using anti-rabies monoclonal antibodies. A biopsy specimen is usually taken from the skin of the neck at the hairline. Impression preparations of brain or cornea tissue may be used. A definitive pathologic diagnosis of rabies can be based on the finding of Negri bodies in the brain or the spinal cord (Fig. 27-4). They are sharply demarcated, more or less spherical, and 2-10nm in diameter, and they have a distinctive internal structure with basophilic granules in an eosinophilic matrix. Negri bodies contain rabies virus antigens and can be demonstrated by immunofluorescence. Both Negri bodies and rabies antigen can usually be found in animals or humans infected with rabies, but they are rarely found in bats.

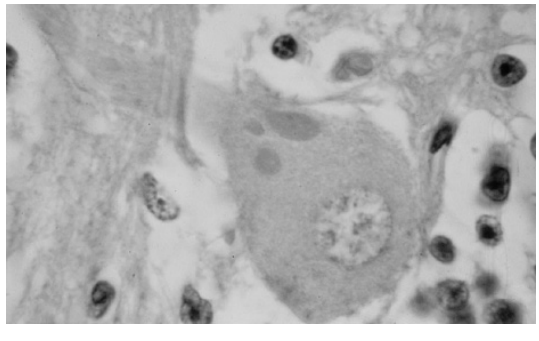


Fig. 27-4 Negri bodies in a Purkinje cell from a dog with rabies (HE×1,000) (Markey *et al.*, 2013)

RT-PCR can be used to amplify parts of a rabies virus genome from fixed or unfixed brain tissue. Although unusual as a diagnostic test, it can be identified that belong to the infecting virus strain by analyzing the sequence amplified.

2.9.2 Viral Isolation

Available tissue is inoculated intracerebrally into suckling mice. Infection in mice can cause encephalitis and death. The central nervous system of the inoculated animal is examined for Negri bodies and rabies antigen. In specialized laboratories, hamster and mouse cell lines can be used to inoculate for rapid (2-4 days) growth of rabies virus; rabies virus can proliferate much faster in above cell lines than virus isolation in mice. An isolated virus can be easily identified by fluorescent antibody tests with specific antiserum. Virus isolation takes too long time to be useful in making a decision about whether to give the vaccine.

2.9.3 Serology

Serum antibodies to rabies can be detected by immunofluorescence or Nt tests. Such antibodies develop slowly in infected persons or animals during progression of the disease but promptly after vaccination with cell-derived vaccines. Antibodies in cerebrospinal fluid are produced in rabies-infected individuals but not in response to vaccination.

2.9.4 Animal Observation

All animals considered “rabid or suspected rabid” should be sacrificed immediately for laboratory examination of neural tissues. Other animals should be quarantine observation for about 10 days. If they show any signs of encephalitis, rabies, or unusual behavior, they should be killed humanely immediately and the tissues should be examined in the laboratory. If they appear normal after 10 days, decisions must be made on an individual basis in consultation with public health officials.

2.10 Immunity and Prevention

All the data show that rabies virus has only one antigenic type. More than 99% of infections in humans and other mammals which develop symptoms end fatally. Survival infected rabies virus with the typical symptom is extremely rare. It is therefore essential that individuals at high risk receive preventive immunization, that the nature and risk of any exposure be evaluated, and that individuals be given post-exposure prophylaxis if their exposure is believed to have been dangerous. Because treatment is of no significance after the onset of clinical disease, it is essential that post-exposure treatment should be initiated promptly. Post-exposure rabies prophylaxis consists of the immediate and thorough cleansing of all wounds with soap and water, administration of rabies immune globulin, and a vaccination regimen.

2.10.1 Pathophysiology of Rabies Prevention by Vaccine

The virus must proliferate in muscle near the site of inoculation until the concentration of virus is sufficient to accomplish infection of the central nervous system. If immunogenic vaccine or specific antibody can be administered promptly, virus replication can be depressed and virus can be prevented from invading the central nervous system. The action of passively administered antibody is to neutralize some of the inoculated virus and lower the concentration of virus in the body, providing additional time for a vaccine to stimulate active antibody production to prevent entry into the central nervous system.

2.10.2 Vaccines

All vaccines for human use contain only inactivated rabies virus. Now, many kinds of commercial vaccines against rabies virus can be available easily.

2.10.3 Pre-exposure Prophylaxis

The person who contact with rabies virus (research and diagnostic laboratory workers, spelunkers) or with rabid animals (veterinarians, animal control and wildlife workers) will be at high risk to infect rabies. The goal is to attain an antibody level presumed to be protective by means of vaccine administration prior to any exposure. It is recommended that antibody titers of vaccinated individuals be monitored periodically and that boosters be given when required.

2.10.4 Post-exposure Prophylaxis

The decision to administer rabies antibody, rabies vaccine or both depends on several factors followed: ①the nature of the biting animal (species, state of health, domestic or wild) and its vaccination status; ②the availability of the animal for laboratory examination (all bites by wild animals and bats require rabies immune globulin and vaccine); ③the existence of rabies in the area; ④the manner of attack (provoked or unprovoked); ⑤the severity of the bite and contamination by saliva of the animal; ⑥advice from local public health officials.

2.11 Treatment and Control

There is no successful treatment case for clinical rabies. Ribavirin, interferons, and other drugs have shown no beneficial effects. Symptomatic treatment may prolong life, but the outcome is almost always fatal. Pre-exposure vaccination is desirable for all persons who are at high risk of contact with rabid animals, such as veterinarians, certain laboratory workers, animal care personnel, and spelunkers. Persons traveling to developing countries where rabies control programs for domestic animals are not

optimal should be offered pre-exposure prophylaxis if they plan to stay for more than 30 days. However, pre-exposure prophylaxis does not eliminate the need for prompt post-exposure prophylaxis if an exposure to rabies occurs. Isolated countries that have no indigenous rabies in wild animals can establish quarantine procedures for dogs and other pets to be imported. In countries where dog rabies exists, stray animals should be destroyed and vaccination of pet dogs and cats should be mandatory. In countries where wildlife rabies exists and where contact between pets, domestic animals, and wildlife is inevitable, so in order to ensure the safe, all domestic animals and pets should be vaccinated.

An oral vaccinia-rabies glycoprotein recombinant virus vaccine (V-RG) proved effective at controlling rabies in foxes. Added to baits, the oral vaccine is being used to eliminate rabies epizootics in wildlife.

Review Questions

1. Try to describe the classification status of *Mononegavirales*.
2. What are the viruses in *Paramyxoviridae*, which have important pathogenic roles?
3. Try to describe the morphological structure and characteristics of paramyxovirus.
4. Try to describe the pathogenic mechanism, detection method, prevention and control measures of Newcastle disease virus.
5. Try to describe the pathogenic mechanism, detection method, prevention and control measures of canine distemper virus.
6. What are the viruses in *Rhabdoviridae*, which have important pathogenic roles?
7. Try to describe the characteristics of the morphological structure and the genome of rhabdovirus.
8. Try to describe the pathogenic mechanism, detection methods, prevention and control measures of rabies virus.

Chapter 28 Minus Strand RNA Viruses of Segmentation

Synopsis

The viruses with single negative strand segmented RNA mainly include *Orthomyxoviridae*, *Bunyaviridae* and *Arenaviridae*. Influenza A virus is a typical representative of *Orthomyxoviridae*, and whose genome is divided into 8 segments which are prone to genetic variation. HA and NA of influenza virus often occur with antigenic drift or antigenic drift. Highly pathogenic influenza virus has an important pathogenic effect on humans and animals, which often cause epidemics. Bunyavirus as arbovirus have many species, whose genome has 3 ring segments which can cause gene reassortment. Rift Valley fever virus, a typical representative of bunyavirus, is an important zoonoses pathogens.

1 Overview of *Orthomyxoviridae*

Influenza viruses are incorporated into the *Orthomyxoviridae*, and are grouped into three types (A, B, and C) according to the antigenic differences between their matrix (M) proteins and nucleoprotein (NP). Type A influenza viruses generally occur in birds, swine, horses, and humans. Type A is the most common. Type A strains taken responsibility for many panzootic (worldwide epidemics). The most flagrant is the great flu epidemic of 1918 that led to more than the World War Ⅰ. Types B and C infect humans, and type C also influences pigs. They don't give rise to pandemics. Outbreaks of type B occur once every two or three years. Type C causes mild colds or subclinical infections illnesses. All three types of flu viruses are resembled in structure and mode of repication, but there are some differences in capsid proteins.

Influenza A viruses are further divided into kinds of subtypes. The standard nomenclature system of influenza virus isolates consists of the following information: host of origin, type, strain number, geographic origin, and year of isolation. Antigenic accounts of the NA and the HA are given in brackets for type A. The host of origin is not demonstrated for human isolates, e.g., A/Hong Kong/03/68 (H3N2). The nomenclature

system in animals consists of the geographic origin, host of origin, year of isolation, and strain number. A detailed characteristic of the two major surface antigens, the neuraminidase (NA) and the hemagglutinin (HA), is given in brackets, such as A/swine/Iowa/15/30(H1N1) and A/swine/New Jersey/8/76(H1N1). By convention, the host of origin for human strains is now removed.

Up to now, nine subtypes of NA (N1-N9) and sixteen subtypes of HA (H1-H16), in many various combinations, have been recovered from animals, birds, or humans. Four HA (H1-H3, H5) and two NA (N1, N2) subtypes have been recovered from humans.

2 Influenza Viruses

2.1 Morphology

Influenza virions are anomalously shaped circular particles of 80-120nm in diameter (Fig. 28-1). The surface of the particle has three kinds of spike proteins NA, and HA and matrix (M2) protein- embedded in a lipid bilayer based on the host cell and covers the matrix (M1) protein which encircles the viral core (Fig. 28-2). The ribonucleoprotein complexus assembling the core composes of at least one of each of the eight single-stranded RNA segments related to the nucleoprotein (NP)and the three polymerase proteins (PB2, PB1, PA). RNA segments contain base pairing between their 3′ and 5′ ends developing a panhandle. Their organization and the function of NS2 in the virus particle remain undetermined. The virus particle envelope is based on the membranes of host cells. There are two different types of surface spikes (peplomers); one is rod-shaped and accordance with the HA, and the other is mushroom-shaped and exhibits NA activity. Both the NA and the HA are viral glycoproteins that adhere to the viral envelope by short sequences of hydrophobic amino acids. The viral envelope encircles a matrix protein (M) shell, which successively encircles the genome of eight (seven in type C influenza viruses) individual molecules of single-stranded RNA,

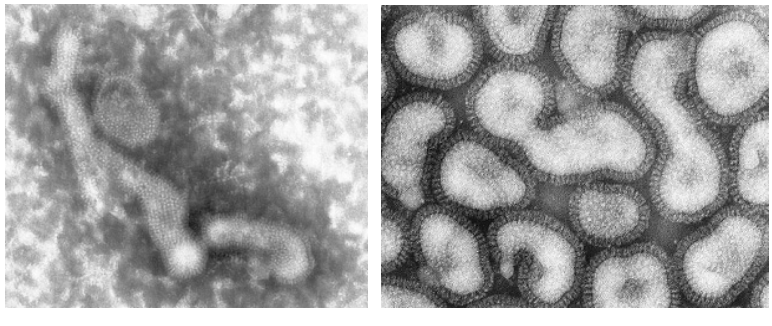


Fig. 28-1 Electron micrograph of influenza A virus (left) (Markey *et al.*, 2013) and AIV (right) (Swayne, 2013)

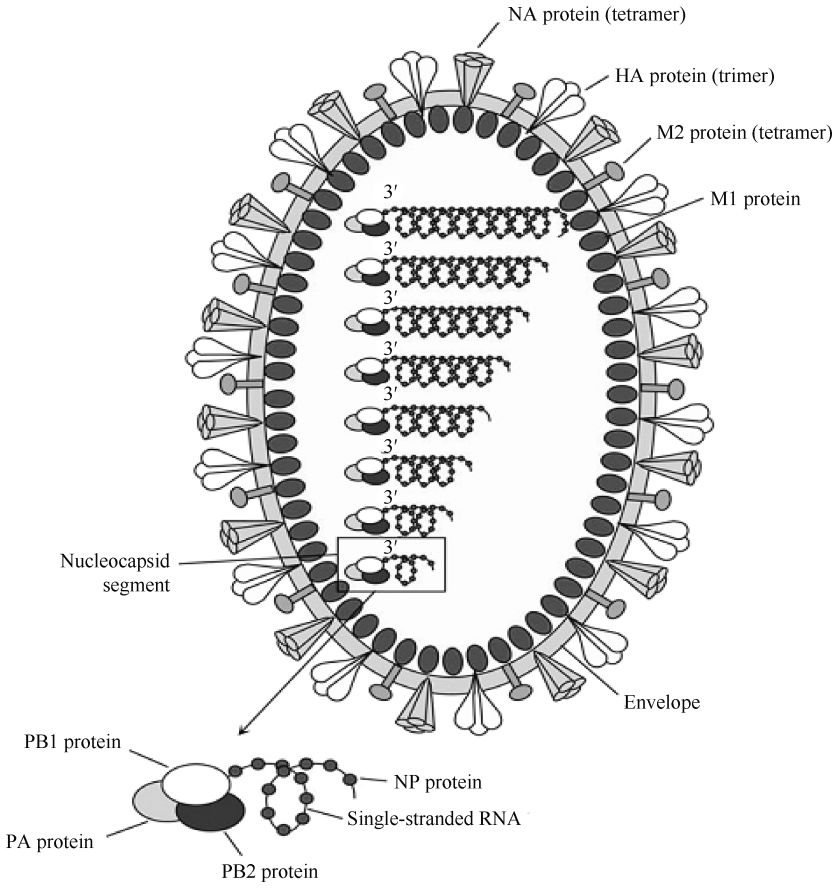


Fig. 28-2 Structure of an influenza A virus particle (Modrow *et al.*, 2013)

accompanying by the NP and three large proteins (PB1, PB2 and PA) that are in charge of RNA replication and transcription (Fig. 28-2). Each of the eight genomic RNA species encodes for one, or sometimes two, polypeptides. This independent nature of the individual viral gene segments leads to the phenomena of high frequency recombination (reassortment of gene segments from two parental viruses to generate offspring with new genotypes) during mixed infections, and accounts for the origin of some new pandemic strains of influenza virus.

The worthiest attention was given the circulation of three isolates which exhibited declined sensitivity to the NA inhibitor oseltamivir in an enzyme inhibition assay, each with different NA mutations: I222L, V116A and K150N, and a preceding unreported N246S mutation. Additionally, six isolates had an S31N mutation in the M2 protein, which confers to stand up to amantadine not preceding reported. Two H5N1 reassortants were isolated whose polymerase genes, PB1 and PB2, are homologous to those of Eurasian viruses producing a novel H5N1

genotype, genotype P.

2.2 Viral Proteins

2.2.1 Hemagglutinin

All strains of influenza are able to agglutinate erythrocytes from chickens, and humans as well as many other species. Antibodies to the HA prevent infection of host cells. The NA and HA are main strain-particular surface antigens and are significant to host immunity. As a matter of fact, it is the variation of these molecules that is originally in charge of emergence of new strains of the virus resulting in new outburst of influenza, and the failing to control them by vaccination. The functions of HA in original virus adhere to its cellular reception, and subsequent HA cleavage allows fusion of the viral envelope with an intercellular membrane allowing transfer of the nucleocapsid into the cytoplasm. There are now 16 identified influenza HA proteins.

HA substitution-containing viruses replicated more efficiently than those with NA stalk deletions. NA deletion mutants were not recovered from contact chickens, meaning inefficient transmission. Amino acid substitutions in HA proteins exhibited in pairs in chickens, but were not dependent in ducks, indicating adaptation in chickens.

2.2.2 Neuraminidase

The NA is in charge of the cleavage of the sialic acid-containing receptor and the elution of the virion from the host cell. This phenomenon hampers self-aggregation and furthers release of the virus from the infected cell. Antibody against the NA does not protect against infection but does confer protection against disease and decreases transmissibility. There are nine identified NA subtypes.

2.2.3 Nucleoprotein

The nucleoprotein (NP) was initially designated the soluble, or S, antigen and is the innermost ingredient of the innermost ingredient of the influenza virus particle. It is coiled into a double helix of 50-60nm in diameter and is closely related to each RNA segment and the three distinct polymerases (Fig. 28-2).

The NP is one of the type-specific antigens used to distinct genera of influenza virus, and can be confirmed by double immunodiffusion, ELISA, single radial diffusion, complement fixation, agar-gel precipitation, and the hemagglutination inhibition (HI) tests.

2.2.4 Matrix Protein

The nonglycosylated matrix is not only protein but also a type-particular antigen of influenza viruses. However, antibodies against M provide little, even if, avoid from infection. This structural protein encircles the nucleoprotein to form the inner part of

the viral envelope (Fig. 28-2).

2.2.5 Nonstructural Proteins

There are at least two non-structural proteins, NS1 and NS2. Their role is at this time unclear.

2.2.6 Polymerase Proteins

The polymerase proteins (PB1, PB2, and PA) localize with viral RNA and the NP. They are the biggest of the viral proteins and are in charge of RNA polymerization of the viral genome (Fig. 28-2). PB1 and PB_2 are required for complementary RNA synthesis; whole PA and NP are necessary for viral RNA synthesis.

2.3 Viral Genome

The influenza A genome includes at least 10 different open reading frames (genes) in its eight segments of negative-sense RNA. The segmented genome of influenza viruses promotes reassortment, leading to in the yield of new strains. Influenza viral variation is continual and taken place in two ways, drift and shift. Owning to point mutations, antigenic drift causing amino acid substitutions mainly in the HA protein. Antigenic shift taken place with reassortment of individual RNA segments when a cell is infected with two distinct influenza viruses so that to generating new viruses caused pandemics of human influenza.

2.4 The Pathogenesis Mechanism of Avian Influenza Viruses

Avian influenza viruses are enveloped minus-strand RNA viruses with protein spikes in their membranes. There are two kinds of spikes, HA and NA. HA is the protein by which the virus adheres to its host cell. As its name suggests, it can also adhere to red blood cells, causing them to agglutinate or aggregate. NA is an enzyme that contributes to release the virus particles from their host cell.

2.4.1 Minus-Strand RNA Virus

A majority of viruses have a single characteristic shape, but the shape of the influenza virus is highly variable. Within a single strain of the virus, some virions are almost round, others are elongated and sometimes bent, and others are still long filaments. Like virion of other minus-strand RNA viruses, influenza virion includes molecules of RNA-dependent RNA polymerases.

Many of the particular properties of the influenza virus arise from its having a segment genome. That is, its RNA exists in the virus particle as eight separate pieces, each of which is enclosed in a helical capsid. Each piece, except for the smallest two,

encodes a single protein. The smallest two encode overlapping genes for two proteins. All eight helical capsids are packaged in a single larger capsid and enveloped.

2.4.2 Antigenic Drift and Shift

In case of influenza virus infection, humans become immune, but only to that specific strain. Immunity relies on the ability to devitalize the HA and NA on the surface of virus. However, a majority of humans get influenza several times in their lives, because influenza endlessly changes the HA and NA to present. These changes take place suddenly and dramatically by antigenic shift or gradually and incrementally by antigenic drift.

The segmented genome of the influenza virus bases on its ability to change by antigenic shift. This occurs when two distinct influenza viruses infect the same cell. The progeny virion then obtains some RNA molecules from each of the infecting virion. In other words, the RNA molecules of the two infecting virions recombinate in various ways among the progeny virion. The product is a virus that is a deal of difference from either of the initial infecting strains. Small mutational changes cause antigenic drift.

Influenza viruses contain the uncommon property of having a highly segmented genome. And type A, which sometimes causes destroying pandemics, has the additional uncommon property of infecting many animal species that act as reservoirs and places in which antigenic shift occurs. Type A accidentally alters dramatically by antigenic shift; it endlessly changes slowly by an antigenic drift.

2.4.3 Effect

Avian influenza influences the respiratory, enteric, or nervous systems of many species of birds. Viruses with relatively low virulence may create few signs whereas others cause high mortality. A majority of outbreaks yield respiratory signs such as coughing, sneezing, sinusitis, rales, and lacrimation. Other signs include diarrhea, depression, and a decline in egg production or fertility. The disease caused by a highly pathogenic virus was once called fowl plague. Viruses that cause virulent diseases should belong to highly pathogenic virus were once called fowl plague. Viruses that cause virulent diseases should be incorporated into highly pathogenic avian influenza virus. Domestic turkeys, especially, have usual been influenced by influenza, which has caused substantial losses over the years. There is marked genetic diversity among avian viruses and at least 16 HA and 9 NA proteins which have been confirmed among avian influenza viruses. Therefore, a great number of subtypes of avian influenza viruses are possible, and birds act as the natural reservoir of influenza. There is evidence exists that all HA subtypes are maintained in aquatic bird populations, including gulls, ducks, and shorebirds. A majority of infections in aquatic birds yield no clinical signs. In wild ducks, influenza virus replicates in intestinal mucosal cells and is excreted in high concentrations. Virus has been isolated from pond and lake water, and surveys have

exhibited that as many as 60% of juvenile birds may be infected as they congregate prior to migration.

2.4.4 Host-Virus Relationship

Highly virulent avian influenza is a main menace to the world's poultry industry. Up to date, it has been discovered in many countries, and has been contacted with HA subtypes H7and H5. As H5N2 virus, early isolations yielded virus with low pathogenicity. It appears which the HA gene is important to pathogenicity due to highly pathogenic viruses possess that are fast cleaved. Early Pennsylvania isolates contain a glycosylation site in the cleavage region that may have blocked cleavage. It appears that a single mutation removed that glycosylation site, which led to a highly pathogenic virus. Similarly, the differences were observed in low and high-pathogenicity H5N2 viruses involving the cleavage site. In the end, it is suggested that the HA cleavage site sequence also be determined in evaluating pathogenicity of isolates.

2.4.5 Transmission

The disease is transmitted through poultry flocks principally through ingestion of virus, but it may also be transmitted by mechanical and inhalation means involving movement of personnel throughout flocks or between premises. What's more, some believe that for waterfowl, a fecal-water-cloacal route of transmission may be significant apart from the fecal-water-oral route. Outbreaks of highly virulent avian influenza may be self-limiting as few birds survive the disease to act as carriers.

Waterfowl have been entangled as the main natural reservoir for influenza. Infected ducks can shed virus for extended periods without showing clinical signs or yielding a detectable antibody response. Evidences show that influenza can stick to some birds for some months after infection. In the past, although avian influenza has been frequently isolated from imported exotic birds, prompting strict quarantine measures on newly imported birds, there is no sign for spreading in this manner just as has been elucidated with newcastle disease virus. What's more, avian influenza may be transmitted on such objects as clothing, shoes, and crates that come in contact with infected birds or premises. While the possibility of vertical transmission of avian influenza through infected eggs exits, there are no sign exists for its spread by this means.

Avian influenza viruses have been discovered to be able to infect a wide rang of mammalian species except for birds. Actually, avian strains have been implicated in the deaths mink and in the disease of seals as well as the emergence of new strains in northern China affecting horses. Avian influenza virus can be propagated in cell cultures of human, chicken, canine, bovine, and rabbit origin.

H5N1, highly pathogenic avian influenza virus, was firstly detected in a goose in Guangdong Province of China in 1996. Multiple genotypes of H5N1 viruses have been demonstrated from apparently healthy waterfowl since 1999. In the years of 2004-2008,

over 100 outbreaks in domestic poultry taken place in 23 provinces and led to severely economic damage to the poultry industry in China. Beginning from 2004, a culling plus vaccination strategy has been enforced for the control of epidemics. Since then, over 35,420,000 poultry have been depopulated, and over 55 billion doses of the distinct vaccines have been applied to control the outbreaks. Although it is logistically impossible to vaccinate every single bird in China owing to the large poultry population and the complicated rearing styles, there is no doubt that the increased vaccination coverage has led to reduced disease epidemic and environmental virus loading. The experience in China suggests that vaccination has played very important role in the protection of poultry from H5N1 virus infection, the decrease of virus load in the environment, and the prevention of H5N1 virus transmission from poultry to humans.

The pathogenesis of avian influenza viruses widely relying on age, strain of virus and species, concurrent infections, and husbandry. At necropsy, lesions include foci of necrosis of different sites including the liver, wattles, skin, spleen, comb, lung, intestine, kidney, and pancreas. There may be fibrous exudates in the air sacs, pericardial sac, oviduct, or peritoneum. Other lesions include petechiation of the abdominal fat, heart muscle, and the mucosa of the nonsuppurative encephalitis, proventriculus, and a serofibrinous pericarditis.

2.5 Laboratory Diagnosis

A definitive diagnosis requires viral isolation and identification or the demonstration of rising antibody titer by soluble antigen fluorescent antibody test, viral neutralization tests, HI, agar gel precipitation, or ELISA test. Avian influenza can be improved in chick embryos; calf, duckling, and monkey kidney cell cultures; and from samples of trachea, sinus exudate, air sac, lung, or cloacal swabs and so on. Antigen-capture ELISA tests have been applied for rapid viral detection.

Clinical features of viral respiratory infection can be produced by various viruses. Therefore, diagnosis of influenza dependents on isolation of the virus, identification of viral nucleic acid or viral antigens in the animal's cells, or demonstration of a specific immunologic response.

2.5.1 Isolation and Identification of Virus

Diachorema, nasal washings, and throat swabs are the best specimens for viral isolation and should be obtained within 3 days of the onset of symptoms. The sample should be held at 4℃ until inoculation into cell culture, as freezing and thawing decrease the ability to recover virus. However, if storage time will exceed 5 days, the sample should be frozen at –70℃.

Typically, primary monkey kidney cells and embryonated eggs have been used to establish the isolation methods with choice for influenza viruses, although some

continuous cell lines may be applied. Inoculated are incubated the absence of serum, which may contain nonspecific viral inhibitory, and in the presence of trypsin, which cleaves and activates the HA in order to replicate virus will spread throughout the culture.

HI was used to examine virus in the culture fluid after 7 days. If the results are negative, a passage is made into fresh cultures. This passage may be needful, because primary viral isolates are often fastidious and grow slowly.

Viral isolates can be detected and identified by HI, a procedure that permits rapid determination the influenza type and subtype. To do this reference sera to currently popular strains must be used. HI by the new isolate will be inhibited by antiserum to the homologous subtype. For rapid diagnosis, cell cultures on coverslips in shell viral may be inoculated and stained 1 or 2 days later with pools of monoclonal antibodies to respiratory agents. Positives are confirmed by use of single fluorescent antibodies.

It is possible to demonstrate viral antigen directly in exfoliated cells in nasal aspirates using fluorescent antibodies. Although, this test is fast, it is as insensitive as viral isolation, does not provide full details about the viral strain, and does not produce an isolate that can be characterized. Rapid tests based on detection of influenza RNA in clinical specimens using polymerase chain reaction are also possible.

2.5.2 Serology

Antibodies to NP, NA, HA and M are yielded during infection with influenza virus. The immune responses against the HA and NA glycoproteins are relative with resistance to infection. Routine serodiagnostic tests in use are the basis of ELISA and HI. Paired acute and convalescent sera are imperative, because normal individuals often have influenza antibodies. A fourfold or greater increase in titer must occur to indicate influenza infection. The sera often have nonspecific mucoprotein inhibitors that must be damaged by treatment with RDE (receptor-destroying enzyme of *Vibrio cholerae* cultures), trypsin, or period date before testing.

The HI test uncovers the strain of virus in charge of infection only if the correct antigen is helpful for use. The tests are the most sensitive and the best predictor of susceptibility to infection but are more time-consuming and unwieldy to perform than the other tests.

2.6 Treatment and Control

Recovered birds remain immune to succulent challenge by a homologous strain for at least several months. Birds immunized parent rally with inactivated by a heterologous strain with the same HA and are partially protected against heterologous strains possessing the same neuraminidase. Following immunization, chickens remain immune for at least 84 days, about twice as long as turkeys remain immune. It has been proved that anti-

HA antibody is significant for protection against infection while anti-neuraminidase antibody protects against disease and decreases virus shedding but does not prevent infection.

In practice, appropriate vaccines are available to the poultry industry though great genetic and antigenic diversity among avian viruses. There is hope that a polyvalent vaccine with a broader protective range may be developed.

In most cases, control of avian influenza occurs through the prevention of exposure. Careful husbandry to hold back the introduction of the virus into the flock is important. New birds should not be introduced into a started flock, and careful precautions should be taken to prevent either direct or indirect contact with migratory, wild, or exotic birds. Since turkeys have been also found to be susceptible to a strain of influenza which typically associated with pigs, it is a good management practice but not have pigs on the same farm as turkeys. Eggs for hatching should come from flocks demonstrated to be free of the virus. Virus has been demonstrated to persist for 105 days in liquid manure following depopulation. Strict measures should be employed to eliminate movement of personnel and equipment, potentially contaminated by manure, between flocks and premises. During an outbreak, isolation of a flock accompanied with orderly marketing of the flock should be considered.

Drugs such as amantadine, used for human influenza prevention, have not been cleared for use in birds for consumption and to date there is no satisfied treatment for avian influenza. Treatment of infected flocks with broad-spectrum antibiotics is useful in controlling secondary bacterial infections, and proper nutrition and husbandry may help decrease mortality.

2.7 Zoonotic Significance of Animal Influenza

A growing body of evidence indicates that pandemic strains of human influenza occur because of recombination between human and animal strains of influenza virus. Waterfowl appear to be particularly significant in the origin of new human isolates. Ducks appear to act as a “melting pot” which in various strains of influenza can come together and undergo genetic reassortment, leading to the generation of new strains of influenza. Swine have also been implicated as intermediate host of “avian-like” and “human-like” influenza viruses. Since the internal genes are crucial for host range, and the HA and NA are of importance to host immunity, reassortment events can result in the formation of new virus strains that contain the same or similar internal genes but possess very different HA and NA proteins. The new viruses generated in this manner might still be infective to humans but possess surface antigens that are highly different than those to which the human population previously was exposed (and immune). The result could be a substantial influenza pandemic as the new strain fast diffuses through a susceptible population.

In the past, major antigenic shifts had place among human influenza viruses leading to prevail in the mankind. Each of the last three shifts had its origin dated from China. The role of China in the emergence of these popular strains has not been completely settled; What's more, China is know to contain a large reservoir of different influenza A viruses among wild and domestic species, particularly ducks. Furthermore, the large number of production of ducks and the proximity of human habitations to the farms, also in tight association with swine, have been implicated by some as an ideal situation for establishing new antigenic strains and bringing in these viruses to the human population.

Review Questions

1. What are the negative strand segmented RNA viruses that have important pathogenic effects?
2. Try to describe the classification of *Orthomyxoviridae*.
3. Try to describe the morphological structure and genomic characteristics of orthomyxovirus.
4. Try to describe the naming characteristics of influenza viruses with examples.
5. Try to describe the pathogenesis and the trend of genetic variation of highly pathogenic avian influenza virus.
6. Try to describe the detection and control measures of influenza virus.
7. Try to describe the classification status of *Bunyaviridae*.
8. Try to describe the pathogenesis of Rift Valley fever virus.

Chapter 29 *Nidovirales*

Synopsis

Members of *Nidovirales* with a nested transcription include *Coronaviridae*, *Arteriviridae*, *Roniviridae* and *Mesoniviridae*. The genome of coronavirus is easy to mutate resulting the changing of antigenicity and pathogenicity. Transmissible gastroenteritis virus of swine and avian infectious bronchitis virus are typical representative of *Coronaviridae*. SARS coronavirus is a new virus that is highly pathogenic to humans. Porcine reproductive and respiratory syndrome virus and equine arteritis virus are typical representatives of *Arteriviridae*, which have important pathogenic significance to pigs and horses. It has great significance for animal industry to strengthen the detection of coronavirus and arteritis virus.

Despite differences in virion structure and genome size, but members of the order *Nidovirales* exhibit a similarities genome organization and replication strategy. In infected cells, all these viruses carry out a nested set transcription strategy, i.e., the expression of their genes is mediated by set of several 3′-coterminal subgenomic mRNAs. This unique strategy has been recognized by the establishment of the order *Nidovirales* (from the Latin nidus, nest). The order *Nidovirales* includes four families: *Coronaviridae*, *Mesoniviridae*, *Roniviridae* and *Arteriviridae*.

1 *Coronaviridae*

The *Coronaviridae* comprises two subfamilies: *Coronavirinae* and *Torovirinae*. The *Coronavirinae* contains many pathogens of animals and humans.

1.1 Properties of Viruses in *Coronavirinae*

1.1.1 Classification

Coronavirinae includes four genera: *Alphacoronavirus* including porcine epidemic diarrhea virus, *Betacoronavirus* including Middle East respiratory syndrome-related coronavirus and severe acute respiratory syndrome-related coronavirus, *Deltacoronavirus* including common moorhen coronavirus HKU21 and *Gammacoronavirus* including

avian coronavirus.

1.1.2 Virion Properties

The *Coronaviridae* viruses are enveloped, 80-220nm in size, and roughly spherical in shape or 120-140nm in size and disc, kidney (Fig. 29-1). Coronaviruses have large club-shaped (about 20nm) peplomers enclosing that appears to be an icosahedral and with a helical nucleocapsid within internal core. Some coronaviruses also have a second fringe of shorter (about 5nm) peplomers.

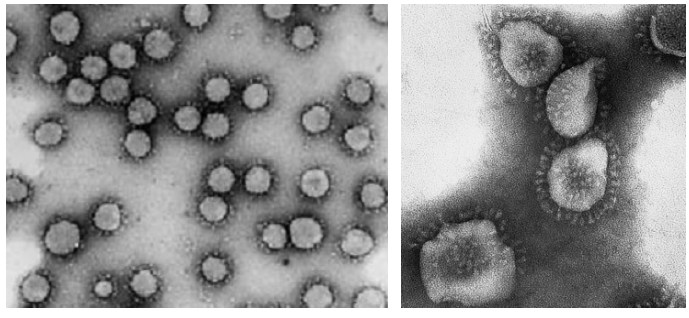


Fig. 29-1 Electron micrograph: TGEV (left) (MacLachlan and Dubovi, 2011), IBV (right) (Swayne, 2013)

The genome consists of a linear single-stranded and positive-sense RNA, 27-32kb in size, the latter being the largest RNA virus genomes known. The genomic RNA is infectious and with a 5′-capped and 3′-polyadenylated.

The major proteins of coronaviruses include a nucleocapsid protein (N) and several envelope/peplomer proteins: ① peplomer glycoprotein (S); ② a triple-spanning transmembrane protein (M); ③a minor transmembrane protein (E), which together with M is essential for virion assembly. The secondary, smaller peplomers consist of a dimer of class Ⅰ membrane protein, a hemagglutinin-esterase (HE) that shares 30% nucleotide identity with the N-terminal subunit of influenza C virus hemagglutinin-esterase fusion (HEF) protein.

1.1.3 Viral Replication

The coronavirus genome expression strategy is complex. First, the viral RNA serves as mRNA for synthesis procedure of the RNA-dependent RNA polymerase. The two large open reading frames (totaling 20kb in size) encoding the units of the polymerase are translated, the much larger via ribosomal frame shifting, as a single polyprotein that is then cleaved. These proteins then assemble to form the active RNA polymerase.

This enzyme is then employed to transcribe a full length complementary (negative-sense) RNA, from which is transcribed not only full-length genomic RNA, but also a

3′-coterminal nested set of subgenomic mRNAs in turn. The nested set comprises five to seven (different in the various viruses) overlapping mRNAs that extend for different lengths from common 3′ ends and share a common 5′-leader sequence. They are generated by a leader-primed discontinuous transcrip-tion mechanism: the polymerase first transcribes the non-coding leader sequence from 3′ end of the complementary (negative-sense) RNA. Second, the capped leader RNA dissociates from the template and then reassociates with a complementary sequence at the start of any genes to continue copying the template right through to its 5′ end. Only a unique sequence which is not shared with next smallest mRNA in the nested set is translated; this strategy yields the various regulated amounts of viral proteins. Inter-genic sequences serve as promoters and attenuators during transcription.

The synthesis, processing, oligomerization, and transport of the several envelope glycoproteins of coronaviruses exhibit some unusual features. For example, the envelope protein M, which in some coronaviruses contains O-linked rather than N-linked glycan is pointed to cisternae of the endoplasmic reticulum. And as a result of, virions budded only there and not from the plasma membrane. Virions are then transported by vesicles to the plasma membrane and are released in exocytosis. Following their release, many of the mature enveloped virions still adherent to the outside of the cell at last. The whole replication cycle is confined in the cytoplasm, which indeed it can occur in the enucleated cells. Genetic recombination occurs at high frequency between in the genomes of different but related coronaviruses. This may be a critical mechanism for the generation of the genetic diversity seen with these viruses in nature.

1.2 Transmissible Gastroenteritis of Swine

There are four distinct coronavirus disease patterns in swine, which referred to vomiting and wasting disease, porcine epidemic diarrhea, transmissible gastroenteritis and respiratory disease. Transmissible gastroenteritis of swine is an important infectious disease in swine industry.

1.2.1 Clinical Features

Clinical signs are most severe in the little young piglets. Most, if not all, neonates succumb, however, few deaths occur in animals that more than 3 weeks. Piglets demonstrate with vomiting followed by yellowish diarrhea, weight loss, and dehydration. Time death is in 2-7 days after the onset of clinical signs in piglets less than one week of age. In older animals, the duration of diarrhea is shorter and vomiting is seen rarely. Exceptions to these disease patterns pose important clinical diagnostic problems: in some outbreaks, there is high mortality in adult swine, or in others sows that may show anorexia, fever, vomiting, diarrhea and agalactia, and in yet others

infection may be inapparent or not obvious.

1.2.2 Pathogenesis, Pathology and Immunity

The virus enters the body by ingestion, then after an incubation period of 18-72h it causes clinical signs that vary according to the age of the infected animals. There are several reasons for the susceptibility of very young piglets: ①their gastric secretions are not yet as acid as those of older animals that the virus proper labile in it; ②their diet of milk buffers the gastric acid which protecting the virus. Virus is therefore free to reach and infect of the small intestinal villous enterocytes. These cells are destroyed under this infection, resulting in shortening and blunting of villi. Villous enterocytes are the source of lactase and other disaccharidases that needed in the digestion of milk-absence of enzymes induce an increased osmolarity of the intestinal contents, resulting in water transport from tissues into the intestinal lumen. The consequence is diarrhea. The crypt epithelial cells are remaining uninfected, and as the recovery of the integrity and function of villi is rather fast.

Gross pathology (except for the dehydration) exhibit that pathology is restricted to the gastrointestinal tract and consists of a distended stomach and small intestine, often containing yellowish undigested milk. When the gut is submerged in isotonic buffer, the destruction of villi can be markedly seen, results in a thinning of the intestinal wall.

Maternal IgA antibodies, passed to piglets via colostrum and milk, provide protection against infection while systemic IgG antibody does not protect. IgA antibodies are also more resistant to proteolytic degradation in the intestine and adhere more firmly to the gut epithelium. The gut-mammary linked of lymphocyte trafficking results in local mammary gland IgA production. Immunity cannot be stimulated by parenteral immunization, only by the mucosal immunization.

1.2.3 Laboratory Diagnosis

At early stages of the disease, mucosal impression smears or cryostat sections of intestinal tissue prepared from piglets in may be examined by immune-fluorescence or immune-peroxidase staining, and these methods can provide a rapid result. Virus isolation is carried out in porcine thyroid or testicle cells; and cytopathology or isolates are identified by serology such as using an enzyme immunoassay. Using paired serum samples and either neutralization serum or enzyme immunoassay, serology allows retrospective diagnosis and is also quite valuable in epidemiological investigations.

1.2.4 Epidemiology, Prevention and Control

Between epidemics, transmissible gastroenteritis virus probably persists in some swine and that as a reservoir in another host species seems unlikely. When the virus is introduced into a non-immune herd there is spread epidemic among animals of all ages and with 100% mortality in newborn piglets, usually less severe disease in older

animals. When no susceptible swine are left and at this time no new animals are reintroduced, the epidemic terminates, usually within a few weeks.

Another epidemiologic pattern occurs in intense production facilities where the farrowing system makes susceptible piglets available continuously. Endemic infection and background immunity usually lead to a low mortality that is most obvious after weaning when maternally acquired IgA-based immunity has waned.

Protection of swine by attenuated virus vaccines has not been very effective while the best protection has been obtained when virulent virus has been administered to pregnant sows, thereby boosting lactogenic immunity in the born piglets. Control also involves good sanitation and management practices. Control was maintained through extensive serological monitoring.

1.3 Avian Infectious Bronchitis

Infectious bronchitis has been found in almost every country of the world and is one of the most important viral diseases in chickens. Infectious bronchitis virus has many antigenic variants as a consequence of mutations in its large genome.

1.3.1 Clinical Features

The clinical presentation of infectious bronchitis depends on the age of the bird infected, and the route of exposure, or the bird's immune status, and the strain of virus. And outbreaks may be explosive, with the virus spreading rapidly and then to affect the entire flock within a few days. In chicks 1-4 weeks of age infected with virulent virus strains exhibit gasping, coughing, rales, nasal exudate, and respiratory distress.

Infected chicks demonstrate plaintive, high-pitched cheeps, become lethargic, lose appetite, and may die suddenly. Mortality in young chicks is usually 25-30%, but it can reach up to 75% in some outbreaks. Less virulent strains may induce few respiratory signs; whether clinical signs are seen or not, infection usually results in retarded growth (stunting).

When the infection through an opportunistic bacterial is uncomplicated, respiratory signs usually last for 5-7 days and then disappear from the flock in 10-14 days. However, cessation of growth may sustain for several days at this age. High mortality can appear in broilers due to secondary infection with *E. coli* or pathogenic mycoplasmas. There appears have no influence on the reproductive system of birds that nearly sexual mature stage when provided egg production has not started. Birds in egg production stage show respiratory rales and coughing with prominent involvement of the reproductive tract with a decline or cessation of egg production. When infected bird is recovered, many eggs show abnormalities such as no shells, thin shells, shells with deposits, distortions, dimples, depressions, or ridging; eggs that should be colored are often pale or white. There is loss of albumen quality, sometimes the thick white almost

completely disappeared. An additional clinical feature (caused especially by strains of low virulence) is "pasting", i.e., the accumulation of a white, sticky exudate around with the vent that can plug the cloaca; after a few days, the plug may slough, leaving a bare patch.

1.3.2 Pathogenesis, Pathology and Immunity

The virus replicates to a high titer in the respiratory tract (ciliated epithelial cells) firstly which is accompany with a viremia (within 1-2 days of infection), then distributes the virus to many organs. At last, the virus causes major injury to the reproductive system and the kidneys. The intestinal tract is another site of primary infection. Infection originating in the upper respiratory tract may display a less severe disease than the primary infection occurs in the lower respiratory tract. Infectivity declines rapidly and isolation of virus beyond 10 days post-infection is unusual (except from chicks). Rarely, virus may sustain for up to 50 days after primary infection. Virus can be isolated from many organs, such as kidneys and bursa of Fabricius (which may be the reason for the immunosuppressive effects noted). Some strains may produce permanent anatomical damage to the immature oviduct.

The most frequent gross pathological manifestation is mucosal thickening with serious or catarrhal exudate distribute in the nasal passages, trachea, bronchi, and air sacs. In the very young chicks, the main bronchi may be also distributed with caseous yellow casts, which may be the main reason for the death at last. Pneumonia, conjunctivitis, and swollen sinuses are sometimes seen. In laying birds, ova can be congested and sometimes ruptured then caused the free yolk in the abdominal cavity. The desquamation of respiratory epithelium, edema, epithelial hyperplasia, while mononuclear cell infiltration in the submucosa, and regeneration are view in various combinations. Repair processes begin after 6-10 days, and a return to normal is fulfilled in 14-21 days. Virus strains affecting the kidney mostly occur in Australia, but some degree of nephro-tropism (interstitial nephritis) is exhibit worldwide.

Infection induces IgM, IgG, and IgA antibodies. In immune laying hens, it begins to acquire IgG antibody (some of it virus specific) from the mother' blood about 5 days before the egg is actually layed. As it becomes surrounded with albumen during passage down the oviduct, the ovum acquires both IgM and IgA antibodies, and then flow into the amniotic fluid during about pass through halfway of its development. During the last third of embryonation, IgG enters the circulation from the yolk; and at this exact time, antibody can inhibit virus replication. The chick hatches with a circulating IgG level similar to that of the mother hen. IgG antibody is metabolized with a half-life of approximately 3 days and may sustain for 3-4 weeks. The vaccine or virus may survive until passive immunity declines to a level then it can replicate again, at this indicate time the chicken mounts an active immune response, scribed in

pheasants in England, racing pigeons in Australia, and guinea fowl in South America.

1.3.3 Laboratory Diagnosis

Direct smears an immunofluorescence staining of tracheal tissue is useful for the diagnosis of early cases before secondary bacterial infection has occurred. For the virus isolation, embryonated eggs are inoculated via the intraallantoic route. Changes remind us the presence of a coronavirus include congestion of the main blood vessels in the chorioallantoic membrane and embryo stunting. To characterize this virus in the chorioallantoic membrane is usually done by immunofluorescence, gel diffusion, or electron microscopy. In some laboratories, the PCR (with appropriate identification of amplified products) or DNA probes are being widely used. Isolates are usually typed and subtyped by serologic methods.

1.3.4 Epidemiology, Prevention and Control

Infectious bronchitis virus (IBV) spreads between birds through aerosol and ingestion of food contaminated with feces. In the environment, virus can usual survive on fomites for several days and possibly for weeks, especially at a low environmental temperature. Recently, outbreaks of infectious bronchitis have declined for years due to the wide use of vaccines; however, the disease may still occur even in vaccinated flocks following the introduction of infected replacement chicks. To minimize this risk, most poultry producers obtain 1-day-old chicks from the strict certified virus-free sources and rear them in isolation.

Attenuated virus vaccines are widely used to protect chicks from IBV infection. These vaccines are derived either from avirulent isolates or by serial passage in embryonated eggs. They are administered through drinking water, or by coarse spray, or by deposition on the conjunctiva. The vaccine is usually given between 7 and 10 days of age and then again at 4 weeks. The vaccination when earlier than 7 days old may be unsuccessful because of passively acquired maternal antibody. Vaccination failure is common because of the variable presence of new antigenic variants. Such variants will continue to emerge and spread, posing continuing problems for poultry producers.

Control of infectious bronchitis is difficult for the existence of persistently infected chickens in many flocks. Eggs have been found to contain the virus for approximately 50 days after infection. The domestic chicken is the major ground but not the only host for infectious bronchitis virus.

2 *Arteriviridae*

The *Arteriviridae* has *Arterivirus* and an unassigned genus. Arteriviruses can infect horse, swine, mice and monkey. All arteriviruses have the capacity to develop asymptomatic persistent infections and then to cause severe disease in certain

circumstances in their natural hosts.

2.1 Properties of Arteriviruses

2.1.1 Classification

Arterivirus has 13 species. Important arteriviruses in animals include equine arteritis virus, lactate dehydrogenase-elevating virus, simian hemorrhagic fever virus, porcine reproductive and respiratory syndrome virus 1-2.

2.1.2 Virion Properties

Arteriviruses are consist of an isometric (probably icosahedral) nucleocapsid and surrounded by a closely adherent envelope with 35nm honeycomb-like surface structures, that the virion is properly 50-70nm in diameter. The genome consists of a single molecule of linear positive-sense, nearly 13-15kb single-stranded RNA. Virion RNA has a capacity of infection and includes a 5′-type terminal cap and a 3′-terminal poly (A) tract. The RNA polymerase gene takes up about 75% of the 5′ end of the genome; genes that encode the viral structural proteins are located in the 3′ end of the genome. The nucleocapsid protein (N), a nonglycosylated triple-membrane spanning integral membrane protein (M), and at least two *N*-glycosylated peplomer proteins (Gs and GL) composed the virion (Fig. 29-2).

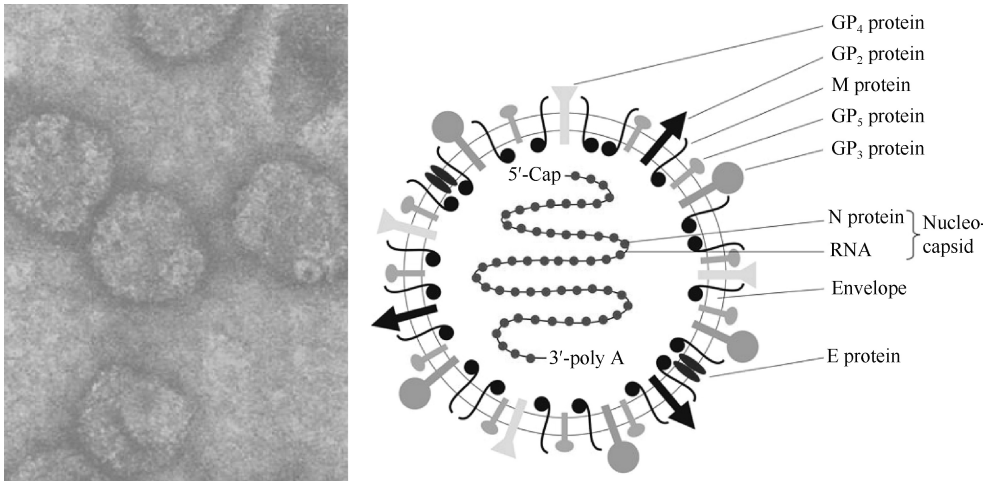


Fig. 29-2 Electron microscopy of equine arteritis virus virions (left) (MacLachlan and Dubovi, 2011) and structure of PRRSV (right) (Modrow *et al.*, 2013)

2.1.3 Viral Replication

Arteriviruses replicate in the perinuclear cytoplasm of host cells, usually as macrophages.

Equine arteritis virus exhibits a high replicates titer in equine cells. The lactate dehydrogenase-elevating virus replicates to very high titer in mice, reaching a titer of 10^{11} ID_{50}/ml in plasma within the early few days of infection.

Full-length genomic RNA is synthesized which was mediate via a full length, negative-sense replicative. Transcription involves of a nested set of seven or eight 3′-coterminal mRNAs synthesis, each according a single open reading flame. These subgenomic RNAs are composed of a common leader sequence (shared by all mRNAs) linked to the unique sequence for each individual mRNA. Thus, each protein is not encoded contiguously on the viral genome. The leader sequence of each mRNA is identical and derived from the according extreme 5′ end of the genome. Conserved sequence motifs define its junctions where the leader sequence joins a unique part of each subgenomic mRNA. The unique part of each mRNA is derived from a subgenomic negative-strand template or is transcribed individually from a genome-sized negative-stranded template is not total understand. Some adjacent genes which encoded in each subgenomic mRNA are using a different reading frames; ribosomal frame shifting is applied to assure that only the 5′-terminal encoded protein is translated from each of its subgenomic mRNAs.

The genome of equine arteritis virus contains eight open reading frames. The 5′-three fourths of the viral genome are occupied by two large open reading frames, which together encode the viral replicase. The products of both open reading frames are multidomain polyproteins, that are cleaved by viral and host proteases to form a subunit of the mature replicase. Open reading frames 2, 5, and 6 expressed from the subgenomic mRNAs; encode not only the large and small glycoproteins and but also the transmembrane protein, respectively. While open reading frame 7 encodes the phosphorylated nucleocapsid protein. Virions bud through membranes of the endoplasmic reticulum into intracellular vesicles; from there they move to the surface of cell in vesicles and are released by exocytosis.

2.2 Porcine Reproductive and Respiratory Syndrome

Among the arteriviruses, porcine reproductive and respiratory syndrome virus (PRRSV) and lactate dehydrogenase-elevating virus of mice are more closely related to each other. From such data, it has been thought that the swine virus may represent the consequence of a species-jumping event of a rodent virus. Today porcine reproductive and respiratory syndrome (PRRS) is recognized as an important economically disease in domesticated and wild pigs.

2.2.1 Clinical Features

The characterization of this disease is anorexia, fever, a blue discoloration of the ears, snout, and vulva, agalactia, and abortion late in gestation (around 110 days). Premature

births, stillbirths, and mummified fetuses are visualized and, if born alive, piglets are weak, half of them dying during the first week, often with respiratory distress. Many of these clinical manifestations during infection can also be in older swine.

2.2.2 Epidemiology

The virus spreads quickly in naive swine populations, with up to 95% of swine becoming seropositive in this herd within 2-3 months after an introduction. The virus has reached high levels of endemicity in many swine-producing countries. At low ambient temperatures, the virus survives particularly well in these environment, and spread quickly especially in winter months. Contact transmission, airborne, and sexual transmission via semen have been reported. The virus is sustained persistently by infected healthy swine, and it has been isolated from the oropharynx more than 5 months after infection.

2.2.3 Pathogenesis, Pathology and Immunity

In infected pigs, viremia may persist for many weeks despite in the presence of antibody. Antibody-dependent enhancement (ADE) of infection has been demonstrated, and subneutralizing concentrations of IgG antibody may as well contribute to the pathogenesis of the disease in other ways. Transplacental infection of piglets leads to gross lesions in the umbilical cord, usually segmental hemorrhagic lesions. Histologically, a necrotizing umbilical arteritis with periarterial hemorrhage is visualized. Endometritis and myometritis have been observed in sows.

2.2.4 Laboratory Diagnosis

The virus is inactivated rapidly in aborted fetuses, so moribund piglets should be used for virus isolation assay. The virus is quite fastidious, that only a few cell types can sustain it replication. As demonstrated that the virus grows well in swine lung macrophages and in an African green monkey kidney cell line (MA-104) and its descendant clones. As to diagnose virus in equine arteritis, the sensitivity of neutralization tests can be increased by the addition of guinea pig complement.

2.2.5 Control and Prevention

Control of PRRSV in free herds is important, because the virus is spread between herds by the movement of infected swine or infective semen, or spread mechanically by fomites, and perhaps by long-distance aerosol. Once introduced, the virus spreads quickly in naïve swine populations. So, separation of pens markedly reduces the rate of transmission.

Control in herds with enzootic infection is difficult, and usually achieved through a combination of vaccination and management strategies. Live-attenuated and inactivated vaccines are commercially available, but vaccines are not infallible because of the

remarkable genetic variation amongst strains and the uncertain nature of a protective immune response.

Review Questions

1. Try to describe the classification status of *Nidovirales*.
2. What are the coronaviruses which have important pathogenecity?
3. Try to describe the pathogenesis and preventive measures of transmissible gastroenteritis virus of swine and avian infectious bronchitis virus.
4. Describe the variability of coronavirus with examples.
5. Describe the pathogenesis of porcine reproductive and respiratory syndrome virus.
6. Describe the enhancement mechanism of porcine reproductive and respiratory syndrome virus.
7. Describe the microbiological diagnosis of porcine reproductive and respiratory syndrome virus.

Chapter 30 *Picornavirales*

Synopsis

Picornavirales is a new order that has important relations with veterinary medicine. *Aphthovirus* and *Enterovirus* is the typical representative of *Picornaviridae*. Foot-and-mouth disease virus has an important pathogenic effect on humans and animals, and has seven serotypes. Avian encephalomyelitis virus, a member of *Tremovirus*, can cause high fatality rate in chickens. Duck hepatitis A virus, a member of *Avihepatovirus*, can cause the hepatitis of duck. The disease caused by foot-and-mouth disease virus and duck hepatitis A virus are notable diseases with OIE rules. The vaccines can effectively prevent foot-and-mouth disease, avian encephalomyelitis and duck virus hepatitis.

Picornavirales has five named families (*Dicistroviridae*, *Iflaviridae*, *Marnaviridae*, *Picornaviridae*, *Secoviridae*) and one unassigned family.

1 General Characteristics of *Picornaviridae*

Picornaviruses contain a large family of small RNA viruses. The *Picornaviridae* recently was confirmed to include 31 genera. Many of them contain members that are important in veterinary medicine, e.g., foot-and-mouth disease virus (FMDV).

Viruses of the *Picornaviridae* have similar general properties. Viruses are short of an envelope and exhibit a cubic symmetry with diameters about 30nm and densities in cesium chloride of 1.33-1.45g/cm^3. The capsid, which is icosahedral, is made of 6 subunits, every one consisting of three surface proteins (VP1, VP2 and VP3 or, respectively, 1D, 1B, and 1C) and (generally) an internal protein (VP4 or 1A) which is closely associated with the RNA. Each of these proteins is obtained by systematic cleavage with a single precursor protein, from which 11 or 12 viral proteins finally produced by post-translational cleavage.

The viral genome is made of a single piece of single-stranded RNA of 7-8.5kb. The genome of viral is positive-sense RNA, so its function as messenger RNA and it is also infectious. Small viral-specified protein, VPg, is connect of the 5′ terminus of the genome and it could have a role in initiating RNA synthesis and in viral maturation

(RNA packaging).

2 *Aphthovirus*

Foot-and-mouth disease virus and equine rhinitis A virus are the important members of genus *Aphthovirus*. These are typical picornaviruses with the notable exception which are labile under pH 7 (acid-labile) and they have a leader protein which is encoded immediately prior to the capsid proteins. Foot-and-mouth disease virus is the type species. The VP1 protein of aphthoviruses is responsible for cellular attachment and for viral neutralization.

2.1 Foot-And-Mouth Disease

Although foot-and-mouth disease (FMD) is not as widely distributed as it once was, particularly in industrialized countries, but it still an enormously important disease of food animals. The economic harm of FMD reflects not only direct losses associated with disease in affected animals, but also limits international and regional movement of animals. All wild and domestic cloven-hoofed animals can be infected, but the disease is easy to infect cattle and swine; sheep and goats are difficult affected. The economic losses of FMD, because it can be vast.

2.1.1 Disease

The characterize of FMD in cattle and swine is fever, depression, excessive salivation, lameness, and formation of vesicles on the mucous membranes of the oral cavity (tongue, dental pad, and gums) and muzzle, epidermis of the coronary band and interdigital spaces, udder, and teats. The epithelium of the pharynx, larynx, trachea, esophagus, and rumen may make Vesicles growth. Some young animals may occur heart muscle necrosis. The vesicles rapidly erode to leave ulcers which result in reduced food consumption, weight loss, and emaciation. Secondary bacterial infections of some affected animals may occur ulcers. While mortality is generally low not exceed 3%, morbidity is very high and economic losses reflect decreased productivity and protracted convalescence of affected animals. Mortality is notably increased in young pigs and sometimes calves.

2.1.2 Etiologic Agent

2.1.2.1 Physical, Chemical and Antigenic Properties

FMDV (Fig. 30-1) has seven different serotypes, named as O, A, C, SAT l, SAT 2, SAT 3 and Asia 1. The individual serotypes are extensive genetic heterogeneity, and each serotype may have distinct virus subtypes. The function of VP1 protein is virus neutralization

and compose of a conserved RGD integrin-binding motif that is responsible for the binding of virus to integrin cellular receptors.

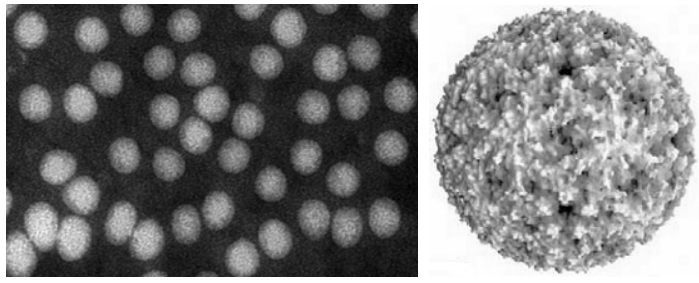


Fig. 30-1 FMDV: electron micrograph (×300,000) (left) (Scott McVey *et al.*, 2013), reconstruction structure (right) (MacLachlan and Dubovi, 2011)

2.1.2.2 Resistance to Physical and Chemical Agents

The FMDV animal secretions and products can survive for extended periods. The virus is dead by heating above 50℃ and is sensitive to both add (pH<6.5) and alkaline (pH>11.0) treatments. The lipid solvents of FMDV are used to resist inactivation. Sodium hydroxide (1%) is used for disinfecting premises after outbreaks.

2.1.2.3 Infectivity of Other Species and Culture Systems

Different kinds of animals are susceptible to infection and disease caused by FMDV, including cattle, domestic and wild pigs, sheep, goats, certain wild ruminants, buffalo, camels, and humans (rare). Cattle and swine of domestic livestock are easy affected. It was reported that dogs, cats, rabbits, and chinchillas by experimental infections. Guinea pigs, suckling mice, rats, rabbits, and hamsters are susceptible laboratory animals. Chick embryos, day-old chicks, and several other avian species can propagate some viral strains. FMDV can replicates in a variety of cell cultures include bovine and ovine origin, baby hamster kidney (BHK) cells, and rabbit and mouse cells.

2.1.3 Host-Virus Relationship

2.1.3.1 Distribution, Reservoir, and Transmission

FMDV is scattered in worldwide largely mirrors which the global economic situation, so the disease occurs principally in developing countries; most industrialized countries are free. The epidemiology of FMD differs somewhat based on whether or not FMDV is endemic in a country/region. Outbreaks of FMD in previously free regions spread very rapidly, the results of the highly infectious nature of the virus and its short incubation period, the respiratory tract of affected animals can excrete large quantities virus, and the relative stability of the virus in the environment. Direct contact, aerosol, and fomites and possibly arthropod vectors may transmit the virus. Virus may be

recovered by all body secretions/discharges (tears, nasal, saliva, urine, feces, milk, vaginal, semen, and the placenta of aborted fetuses). The survival of the virus in such excretions relies on temperature, pH, and humidity. FMD can be transported over long distances by infected animals or their products and is viable for up to 3 months in frozen meat and up to 2 months in ham, bacon, and certain sausages. When discarded as garbage, contaminated pork products form a source of FMDV infection especially swine. A source of virus can be served for extended periods of time in animal hides. Infected humans may disseminate virus by mechanically (fomites) or biologically. The source of some outbreaks because of long-distance airborne spread. It is to be emphasized that epidemics or FMD can occur in endemic areas while new serotypes or strains of the virus are introduced and the severity of an outbreak reflects the inherent biological properties of the infecting virus strain.

2.1.3.2 Pathogenesis and Pathology

Respiratory is the most important route of FMDV infection, although ingestion of contaminated food or direct inoculation also are both highly effective in transmitting infection. Viremia precedes the development of lesions and overt disease, and body fluids and secretions can isolate a virus at this time. Virus rapidly moves by the blood during viremia to infect the epithelium of the oral cavity and feet, where lesions develop. The oral cavity of infected animals can make the virus persist for long periods after the acute infection.

The vesicle is the characteristic epithelial lesion of FMD, which forms as a result of both intracellular (spongiosis) and intercellular edema. These vesicles rapidly rupture to leave erosions and ulcers in the affected epithelium, thus these vesicles rapidly rupture. The heart musculature, causing degeneration and necrosis of young animals can be infected by FMD; the characteristic yellow-white streaks of myocardial necrosis are referred to as tiger heart.

2.1.3.3 Host Response to Infection

Develops of serum IgG approximately 2 weeks post-infection and is type-specific. It is reported that colostral antibody in newborn calves can interfere with vaccination. It is not well defined that the relative importance of local secretory, systemic, and cell-mediated immunity. Duration of immunity is shorter in swine than in cattle but apparently persists for only about a year. Serum-neutralizing antibody, actively or passively acquired, appears to correlate with protection in pigs.

2.1.4 Laboratory Diagnosis

Because of the economic and political significance of FMD and its similarity to other vesicular diseases-vesicular stomatitis (VS), swine vesicular disease (SVD), and vesicular exanthema play important roles in finally diagnosis for swine. FMDV is

usually detected by PCR techniques, and sequence analysis of any PCR-positive material then can be used to type the infecting strain of FMDV and to undertake molecular epidemiologic studies. Clinical samples can isolate FMDV (vesicular fluid and others) by propagation in cell cultures or laboratory animal s followed by physicochemical characterization and serology using viral neutralization, ELISA, fluorescent antibody (FA), or agar gel immunodiffusion (AGID). The rapid diagnosis of FMD can use electron microscopy (EM) and immuno-EM.

ELISA, AGID and viral neutralization can be used to confirm the serologic diagnosis of FMDV infection (either in cell cultures or suckling mice). Antibody to group-reactive antigen can be identified by AGID test (viral infection-associated antigen); such antibody is typically found only in animals which have experienced an active infection and not in animals vaccinated with inactivated virus, recombinant FMDV nonstructural proteins (2C and 3AB1) can also be used in serology to distinguish vaccinated from naturally infected animals as vaccinates do not make antibody to these proteins.

2.1.5 Treatment and Control

The specific treatment of FMD has not existed. But the secondary bacterial infections should be reduced through proper animal husbandry practices and treatment.

It is difficult to control FMD because of its highly contagious nature, multiple hosts, viral stability, multiple antigenic types and subtypes, and transient immunity. Countries could be free of FMD by imposing strict import regulations on animals, animal products, and potentially contaminated materials. Previously unaffected countries control outbreaks by resorted to massive slaughter and quarantine programs.

To control outbreaks and to prevent spread of the disease, we often use quarantine and vaccination programs. Control depends heavily on vaccines (e.g., inactivated vaccines of tissue) in FMD endemic countries. It is a question that the safety of live attenuated vaccines. Thus, they are not widespread. When immunity is short-term, vaccination is conducted one to three times a year. It is essential that vaccines must have appropriate type/subtype. The developing subunit, synthetic peptide, and recombinant-type vaccines are directed in current research.

2.2 Swine Vesicular Disease

2.2.1 Disease

Swine vesicular disease (SVD) is similar to FMD, vesicular stomatitis (VS), and vesicular exanthema of swine (VES). Affected swine exhibit fever, lameness, and vesicles in and around their feet (coronary bands, soles, and interdigital areas) and, less commonly, the oral cavity and nares.

2.2.2 Host-Virus Relationship

Swine vesicular disease virus (SVDV) is a zoonoses pathogen and closely related to human coxsackie virus B5. SVDV is very stable in sausage of pork products. SVDV can be isolated from a variety of tissues and feces when following oral infection. Some infected pigs may occur encephalitis. The mortality of uncomplicated infections is low.

2.2.3 Laboratory Diagnosis

It is critical for SVD rapid diagnosis because it must be distinguished from FMD and other vesicular diseases such as VS and VES. PCR is the best method to diagnosis SVD and other enteroviruses can distinguish SVD virus by sequence analysis. Immunohistochemical staining or electron microscopy, or by virus isolation on primary porcine cell cultures or in suckling mice can identify the virus or viral antigens in vesicles. Serological diagnosis of convalescent animals can be accomplished by ELISA or viral neutralization.

2.2.4 Treatment and Control

It has no available treatment for SVD, and only experimental vaccines have been described. The economic importance of SVD is similar to FMD. So, control requires strict quarantine and import restrictions in countries free of the disease. The previously unaffected regions should control incursions of the virus by strict quarantine and animal depopulation.

Review Questions

1. Try to describe the classification status of *Picornavirales*.
2. Try to describe some common features of the picornavirus.
3. Why are foot-and-mouth disease antigens susceptible to change?
4. Describe the pathogenic mechanism of foot-and-mouth disease virus.
5. Describe the diagnosis, prevention and control measures of foot-and-mouth disease.
6. How to detect foot-and-mouth disease virus and swine vesicular disease virus?

Chapter 31 Other Viruses of Positive- Sense RNA

Synopsis

Other positive-sense RNA viruses mainly include *Caliciviridae*, *Hepeviridae*, *Astroviridae*, *Togaviridae*, *Flaviviridae* and *Nodaviridae*. The surface of viruses in *Caliciviridae* present hollow like a cup. Rabbit hemorrhagic disease virus, a member of *Caliciviridae*, are wide distributed, and has highly pathogenic to rabbits. Hepatitis E virus can cause hepatitis in humans and pigs. Bovine viral diarrheal virus and classical swine fever virus, members of *Pestivirus* in *Flaviviridae*, can cause persistent infections. Rabbit haemorrhagic disease, bovine viral diarrhoea and swine fever are notable diseases in OIE rules.

1 *Caliciviridae*

1.1 General Properties

The *Caliciviridae* has five genera: *Lagovirus*, *Norovirus*, *Sapovirus*, *Nebovirus* and *Vesivirus*. Caliciviruses are small (27-40nm in diameter), nonenveloped, icosahedral viruses, and its genome is single-stranded, positive-sense RNA. The viral RNA serves as mRNA and has a capacity of infection. The name calicivirus is defined as the chalice-shaped spheres on the surface of negatively stained virus particles.

The genome of caliciviruses has only two or three open reading frames (ORF). Virions are comprised of a single major capsid protein while the nonstructural proteins share common features with those of picornaviruses. Replication of caliciviruses occurs in the cytoplasm, although both cytoplasmic and intranuclear inclusions developed in infected cells.

1.2 Vesiviruses

Based on genus, vesicular exanthema virus and feline caliclvirus are together classified as in the *Vesivirus*. Viruses within this genus are readily propagated in cell culture, and distinct from caliciviruses in other genera. Here, we mainly introduce vesicular

exanthema of swine virus.

1.2.1 Disease

Vesicular exanthema of swine (VES) is an acute viral disease, and the characteristic is formation of vesicles in the oral cavity, interdigital spaces, and coronary band of the foot. VES is indistinguishable from FMD, swine vesicular disease, and vesicular stomatitis in clinical manifestation. The incubation period of the disease is 24-72h and within 1-2 weeks' course. The disease demonstrates a high morbidity but a low mortality. It is of some economic importance as a disease in pigs; however, its main influence is that it mimics FMD, from which it must be distinguished. VESV can infect marine mammals (e.g., seals, sea lions, walrus, and dolphins), and cause vesicular diseases and reproductive failure. Outbreaks of VES occur in these marine mammals, VESV could spread to swine, likely induced feeding of dead marine mammals to swine.

1.2.2 Etiologic Agent

1.2.2.1 Physical, Chemical, and Antigenic Properties

Vesicular exanthema of swine virus (VESV) as a typical calicivirus, its virus particle is associated with cytoplasmic cisternae in infected swine cells and crystalline arrays in the cytoplasm. VESV is stable at low a pH (e.g., pH 5). A large number of antigenically distinct types of VESV have been charactered, and a flock of antigenically distinct viruses that originally were isolated from other species instead of swine are capable of causing VES that classified as VESV, including bovine calicivirus, cetacean calicivirus, primate catictvirus, and a number of so-called San Miguel sea lion viruses. Similar viruses have been isolated from fish, birds, reptiles, and other mammals, including skunks. These viruses are distinguished according serological tests, usually serum neutralization, and the virulence of these viruses to pigs varies significantly.

1.2.2.2 Resistance to Physical and Chemical Agents

VESV could remain in the environment and in contaminated meat products for very long time. The virus is completely inactivated by 2% NaOH or 0.1% NaClO.

1.2.2.3 Infectivity for Other Species or Culture Systems

Natural occurred VES disease is confined only to swine at all ages and breeds. Experimentally, VESV also causes vesicles at inoculated sites in seals. Vesicles are also appeared at the sites of inoculation in horses and hamsters. The viral titers are low that from some sites of inoculation and draining lymph nodes. VESV can be propagated in cell lines of swine kidney or Vero monkey kidney.

1.2.3 Host-Virus Relationship

1.2.3.1 Reservoir and Transmission

Marine mammals have served as a reservoir for VESV. This calicivirus was named as San Miguel sea lion virus (SMSV), which was indistinguishable from VESV according to morphologic, biophysical, and biochemical criteria. Experimental SMSV infection of swine produces a different a disease compared with VES. SMSV also has been collected from asymptomatic domestic swine. Serum-neutralizing antibodies to several serotypes of SMSV and VESV have been demonstrated in marine mammals and both wild and domestic swine in California. Earlier epidemiologic studies confirmed the relationship between feeding of raw garbage during the outbreaks of VES, and the dead sea lions are known to have been utilized as a food source for swine.

Although outbreaks of VES properly originated from feeding SMSV-infected marine animal parts to swine, the infection then subsequently spread rapidly within affected herds by direct contact.

1.2.3.2 Pathogenesis and Pathology

VES is properly characterized as the appearance of fluid-filled vesicles on the snout, coronary band, and tongue of infected swine. These same lesions occurred in swine which are inoculated intradermally with either VESV or SMSV. Infected animals demonstrate febrile, and virus is appeared in blood and nasaloral secretions for several days after infection. Vesicles present on the coronary band and interdigital space of the fingers at 3-4 days after infection. The vesicles rapidly rupture and healing unless complicated by secondary bacterial infection. High titers of virus are present within the fluid in vesicles, which may also contaminate the surrounding environment. Some swine infected with VESV exhibit a mild encephalitis, and virus also may be isolated from brain tissue of SMSV infected swine.

1.2.4 Host Response to Infection

Neutralizing antibodies to VESV and SMSV appear in the sera of animals soon after viral infection, and then viral titers peak within 7-10 days.

1.2.5 Laboratory Diagnosis

VES must quickly distinguish from other vesicular diseases of swine, such as FMD, swine vesicular disease, and vesicular stomatitis. Laboratory diagnosis is established by virus isolation in cell cultures, direct electron microscopic examination of the vesicle fluid, or polymerase chain reaction (PCR). Although these vesicular diseases produce similar signs in swine, there are major differences: whereas VES and swine vesicular disease are almost exclusively diseases of swine; while vesicular stomatitis frequently affects horses as well as ruminants, and FMD aim affects ruminants.

1.2.6 Treatment and Control

There is no treatment or useful vaccine to therapy and control this disease. It considered to be eradicated in the United States nowadays. Enforcement of laws requiring cooking of corresponding garbage before feeding it to swine was the most important factor in eliminating the disease.

1.3 Lagdviruses

Lagdvirus mainly has two members, rabbit hemorrhagic disease virus and european brown hare syndrome virus. Here, we mainly introduce rabbit hemorrhagic disease virus.

1.3.1 Disease

Rabbit hemorrhagic disease (RHD) and European brown hare syndrome (EBHS) are similar diseases that are induced by related but antigenically different caliciviruses. RHD is an acute infectious disease to European rabbit and *Oryctolagus cuniculus*, and frequently develop a very high mortality in susceptible rabbit swarm. A novel feature of RHD is that the disease is just only fatal to rabbits over 2 months of age. The disease is characterized by a short incubation period, followed by fever, disseminated hemorrhage in all body tissues, and death rapidly. The disease was first described in China in 1984 and then rapidly spread throughout much of the rest of the world. EBHS occurs in European hare, *Lepus europacus*.

1.3.2 Etiologic Agent

1.3.2.1 Physical, Chemical and Antigenic Properties

Based on serological, various strains of RHD and EBHS viruses are recognized and distinguished.

1.3.2.2 Infectivity for Other Species and Culture Systems

Neither RHD nor EBHS viruses are readily propagated in cell culture, and through to characterize these virus by using homogenates of the livers of affected animals. The viruses appear to be highly species specific.

1.3.3 Host-Virus Relationship

1.3.3.1 Distribution, Reservoir, and Transmission

RHD was first reported in China, while EBHS had been recognized earlier in Europe. It is likely that a mutation of the EBHS calicivirus led to the emergence of RHDV, then causing the lethal pandemic in rabbits. The disease is transmitted by the oral-fecal route.

1.3.3.2 Pathogenesis and Pathology

Rabbits with RHD display an enlarged spleen, swollen liver, and disseminated hemorrhages. There is an abroad necrosis distributed in liver and it potentially explains the disseminated intravascular coagulation (DIC) that occurs in affected animals. The DIC induced by RHDV is not a characteristic of other calicivirus infections, but does occur in such flavivirus-induced diseases as yellow fever and dengue in humans.

1.3.4 Laboratory Diagnosis

Immunofluorescence and ELISA tests have been established for the rapid diagnosis of RHD. The genome of RHDV has been completely sequenced, so PCR readily can be applied and used for rapid diagnosis of the infection.

1.3.5 Treatment and Control

There is no useful treatment for the acute disease. A formalin-inactivated vaccine that incorporates with infected rabbit tissue could provide effective immunization against the disease. Control can also be achieved through strict quarantine and isolation to prevent transportation of RHOV contaminated materials into commercial rabbitry. It is interesting to notice that, although most countries have focused on the control and prevention of RHD, RHDV has been used as a biologic weapon to control rabbit numbers in other countries.

2 *Flaviviridae*

The *Flaviviridae* has four genera: *Flavivirus*, *Pestivirus*, *Pegivirus* and *Hepacivirus*. Many members of the *Flaviviridae* are very important pathogens in human and animals.

2.1 *Pestivirus*

The genus *Pestivirus* are important pathogens to livestock, and includes bovine viral diarrhea virus (BVDV), border disease virus (BDV) and classical swine fever virus (CSFV). Compared to the genus *Flavivirus*, members of the genus *Pestivirus* are not arthropod-borne. They are inactivated by low pH, heat, organic solvents and detergents easily. Virions are spherical to pleomorphic (40-60nm in diameter) and are enveloped with small surface projections (spikes). The virions consist of an envelope and nucleocapsid and include four structural proteins: a nucleocapsid protein (C) and three envelope glycoproteins (E^{rns}, El and E2). In the infected cells, some seven or eight viral nonstructural proteins are also produced. The genome is a positive-sense, single-stranded RNA that contains a large open reading frame which encodes a large polyprotein that is co-translationally and post-translationally cleaved into the various structural and

nonstructural viral proteins.

The pestiviruses are all antigenically related including the cross-reactive epitopes. Several other pestiviruses have already been collected that are genetically distinct from BVDV, BDV and CSFV. Based on this, it is proposed that pestiviruses isolated from a giraffe or reindeer have to be identified as new species. Neutralizing antibodies are directed against the E^{rns} and E2 envelope glycoproteins. Infected animals also proposed a strong immune response against the NS3 protein, whereas the antibody response to other viral proteins is rather weak.

All these three viruses are important food-animal pathogens and will described sequentially. They are rather related that can only be distinguished by employed the monoclonal antibodies and/or molecular biology techniques; however, they tend to be host-specific.

2.2 Classical Swine Fever Virus

2.2.1 Disease

Classical swine fever is an important disease of swine worldwide; although it has been eliminated in many intensive swine-producing countries, however, it reemerges to induce serious, economically devastating outbreaks. Disease is characterized by fever, leukopenia, and loss of appetite. Infected animals may appear dull and drowsy and crowd together as if chilled. While vomiting and diarrhea are common, as is conjunctivitis, erythema of the skin, and neurologic signs such as paralysis and locomotory disturbances. Pregnant sow infection can result in small litters, fetal death, premature births, stillbirths, and the birth of piglets with cerebellar ataxia or congenital tremors. Morbidity and mortality are both high during epidemics caused by virulent strains in fully susceptible swine, whereas disease is less apparent when the virus is endemic which making detection and eradication more difficult.

2.2.2 Etiologic Agent

2.2.2.1 Physical, Chemical, and Antigenic Properties

CSFV is one member of the genus *Pestivirus*, and there is considerable genetic and antigenic variation compared with other stains.

2.2.2.2 Resistance to Physical and Chemical Agents

CSFV is chloroform-labile and ether-labile while relatively stable to pH. Virions are quickly inactivated in drying but can persist for long time in uncooked pork or garbage. The virus is completely inactivated in canned hams with an internal temperature of 65℃ maintained for 90min. The virus could survive for 3 days at 50℃ in the defibrinated blood.

2.2.2.3 Infectivity for Other Species and Culture Systems

Domestic swine and wild hogs are the only natural susceptible species. CSFV replicates in the porcine cells cultures such as spleen, kidney, testicle, and peripheral blood leukocytes. Most strains are noncytopathogenic and may persist in cell culture for many passages. The presence of CSFV in infected cell cultures is readily marked by immunofluorescent or immunohistochemical staining techniques. Some cytopathogenic strains have been reported.

2.2.3 Host-Virus Relationship

2.2.3.1 Distribution, Reservoir, and Transmission

CSFV occurs worldwide, however it has been eliminated in many countries. Domestic swine and wild hogs are serve as reservoir hosts, often as inapparent carriers. Pigs infected in utero may become persistently infectious carriers, which similar to the reservoirs of other pestiviruses like BVDV and BDV. Transmission is occurred through droplet, fomites, and ingestion of infected materials, especially uncooked garbage.

2.2.3.2 Pathogenesis and Pathology

Hog cholera is an acute, highly contagious disease and is characterized by disseminated intravascular coagulation or hemorrhage and infarction in many tissues. The incubation period of this disease is short (3-8 days) with virus first replicates in lymphoid tissues of the upper respiratory tract or tonsils. The virus then spreads widely and replicates in endothelial cells and mononuclear inflammatory cells throughout the whole body. The obvious lesions are appeared in petechial hemorrhages on all serous surfaces, lymph nodes (hemorrhagic lymphadenitis), and kidney, and the presence of infarcts in the spleen.

More chronic forms of this disease occur toward some endemic areas. Affected pigs may exhibit growth retardation (stunting), chronic diarrhea, and secondary bacterial pneumonia.

2.2.3.3 Host Response to Infection

Animals which recover from hog cholera sustain a long-lasting immunity. Neutralizing antibody titers correlate with resistance to CSFV infection. Suckling pigs acquire antibodies in colostral from the immune dam. The half-life of this colostral antibody is almost two weeks. Pigs that have maternal antibody titers of 1∶1,000 or above still have some antibody at 4 months.

Pigs infected in utero are often persistently infectious carriers, whether or not they are still healthy at birth.

2.2.4 Laboratory Diagnosis

Diagnosis of hog cholera have to do in free areas where outbreaks of severe disease in

pigs, but the diagnosis always requires laboratory confirmation that distinguish it from another septicemia. The virus is identified in the tissues collected from affected pigs by immunohistochemical staining or by virus isolation from the spleen, tonsils, lymph nodes, and blood. Since many strains are noncytopathogenic in cell culture, the fluorescent antibody method is required for the strain infection. PCR detection now also is available.

The diagnosis of chronic form of CSF is much harder and requires careful laboratory investigation.

2.2.5 Treatment and Control

Control of CSF depends on whether the virus is endemic toward particular country or region. In free areas, elimination is accomplished by regulating the movement (importation) of swine from endemic areas and strict prohibiting feeding of garbage and/or food scraps containing pork products to swine. In endemic areas, vaccination and/or eradication are applied. Vaccines for CSF are attenuated and although effective in control disease. They complicate efforts to eradicate CSFV from a region or country.

Review Questions

1. Try to describe the pathogenesis, prevention and control measures of rabbit hemorrhagic disease virus.
2. Try to describe the classification status of *Flaviviridae*.
3. Try to describe the pathogenesis, prevention and control measures of classical swine fever virus.
4. Try to describe the structural characteristics and genomic characteristics of calicivirus.
5. Try to describe the microbiology diagnosis method of swine fever.
6. Try to describe the main characteristics of *Flaviviridae*.

Chapter 32 Prion

Synopsis

Prion is the pathogen of the transmissible spongiform encephalopathy of humans and multiple animals. The composition of prion is only proteins. The amino acids of the prions are same as those of normal cellular PrP^c, but they have different structures. The prions form SAF after coagulation, which leads to the plaques of neurons in the brain. Prions are very resistant to conventional physical and chemical factors. The protein of the prion is as the template, then PrP^c form a dimer, and subsequently form the basic unit of the prion, namely PrP^{sc}. Bovine spongiform encephalopathy and sheep scrape are important prion diseases caused by prions.

Prions are infectious proteins that differ from all other known infectious pathogens, and are believed to be devoid of nucleic acid. The prion concept was proposed by Stanley Prusiner in 1982, and the term "prion" derived from protein aceous infectious particles (abbreviated as PrPRes) to emphasize its proteinaceous and infectious nature. Prusiner received the Nobel Prize for this work in 1997. Prion is thought to be the pathogens of transmissible spongiform encephalopathies (TSE), which are a unique group of progressive, uniformly fatal, neurodegenerative disorders occurring in humans and animals. The concept of the prion as an infectious agent is now widely accepted.

1 Biological Characteristic

Prion protein (PrP) exists in two different conformational forms, i.e., cellular prion protein (PrP^c) and scrapie prion protein (PrP^{sc}). PrP^c is the normal cellular protein found on the surface of neurons. PrP^{sc} is thought to be the pathogens of transmissible spongiform encephalopathies (TSE). PrP^c is rich in α-helical content and has little β-sheet structure, whereas PrP^{sc} has less α-helical content and abundant β-sheet structure (Fig. 32-1). The gene encoding for the human PrP is located at the short arm of human chromosome 20. PrP^c is converted into PrP^{sc} through a posttranslational process. The conversion of PrP^c into PrP^{sc} involves a conformation change whereby the α-helical content diminishes and the amount of β-sheet increases. This structural transition is accompanied by profound change in the properties of the protein. PrP^c is

soluble in nondenaturing detergents, whereas PrP^{sc} is not; and PrP^{c} can be digested by proteases wherea PrP^{sc} is partially resistant. Since the PrP^{sc} cannot be broken down through the body's normal process, it aggregates mostly in the central nervous system causing neurologic disease.

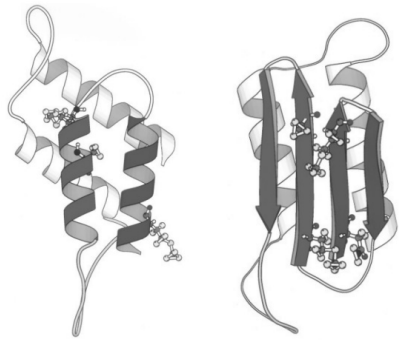


Fig. 32-1 Structure models of PrP^{c} (left) and PrP^{sc} (right) (Huang *et al*., 1996)

The mechanism of prion replication is not well understood. Propagation of this protein is a posttranslational event, not requiring nucleic acid or genetic material. PrP^{sc} is formed and must involve a template process. PrP^{sc} binds to the normal PrP^{c} on the cell surface, causing it to refold and acquire the structure of PrP^{sc}. The PrP^{sc} is released from the cell and aggregates as amyloid-like plaques in the brain. The cell then replenishes the PrP^{c}, and the cycle continues. PrP^{sc} protein is very resistant to many environmental insults, chemicals, and physical conditions that would destroy any virus or microorganism. PrP^{sc} is also resistant to endogenous proteases, which is the key to its accumulation into aggregates, called scrapie-associated fibrils (SAF, a term derived from scrapie but in general use for all prion diseases), that form neuronal plaques and are associated with spongiform damage and neuronal dysfunction.

Prions are highly resistance to conventional inactivation procedures, including irradiation, boiling; dry heat, proteases, DNases, RNases, and chemicals (formaldehyde, alcohol, and glutaraldehyde). However, they are inactivated by 2.0mol/L NaOH, 4.0mol/L guanidinium hydrochloride or isocyanate, sodium hypochlorite, and steam autoclaving at 1h, 121℃. Guanidine thiocyanate is highly effective in decontaminating medical supplies and instruments.

To date, only a few cell culture models permissive to prion replication are available. Mouse neuroblastoma cells (N2a) were found to propagate prions derived from sheep with scrapie and human with Creutzfeldt-Jakob disease (CJD) if they were first passage through mice. Rat pheochromocytoma cells (PC12) were reported to propagate mouse prions. Mice and hamsters are commonly used in experimental studies of prion disease.

2 Pathogenesis and Immunity

Prion disease is neurodegenerative disease, which affects human and a variety of domestic and wild animal species. The human prion diseases include Kuru, Creutzfeldt-Jakob disease (CJD), variant Creutzfeldt-Jakob disease (vCJD) which is the exception because of its relationship with bovine spongiform encephalopathy (BSE), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). Some of these human prion diseases (familial CJD, GSS, and FFI) are inherited, while sporadic CJD occurs spontaneously in a small percentage of humans. The human prion disease has a limited host range which is limited to humans. The most common prion diseases in animals are scrapie of sheep and goats, chronic wasting disease (CWD), and bovine spongiform encephalopathy (BSE) or "mad cow disease" (Table 32-1). The animal prion diseases have a narrow host range with the notable exception of BSE. Prion diseases have similar pathologic features. They are characterized in all species by spongiform change and amyloid plaque formation in the central nervous system. This is accompanied by neuronal death and a reactive proliferation of astrocytes and microglia in the absence of inflammation. Long incubation periods (months to decades) precede the onset of clinical illness and are followed by chronic progressive disease (weeks to years). Prion diseases are always fatal, with no known case of remission or recovery.

Table 32-1 Human and animal prion diseases (胡建和等, 2011)

Human prion disease	Animal prion disease
Kuru disease	Scrapie of sheep and goats
Creutzfeldt-Jakob disease (CJD)	Bovine spongiform encephalopathy (BSE)
Variant CJD (vCJD)	Transmissible mink encephalopathy (TME)
Gerstmann-Sträussler-Scheinker syndrome (GSS)	Chronic wasting disease of deer or elks (CWD)
Fatal familial insomnia (FFI)	Feline spongiform encephalopathy (FSE, one subgroup of vCJD)

The exact mechanism of spread of prion disease is unknown. The diseases scrapie, TME, BSE, FSE and EUE are all thought to occur after the consumption of prion-infected foods. The diseases are transmissible by inoculation or ingestion of infected tissues or homogenates, and infectivity is present at high levels in brain or other central nervous system tissues, and at slightly lower levels in lymphoid tissues including spleen, lymph nodes, gut, bone marrow, and blood. Ingested prions may be absorbed across the gut wall at Peyer's patches, lymphoid cells then phagocytose prion protein and travel to lymphoid to other lymphoid sites such as nodes, spleen and tonsils. Many of these sites are innervated and eventually the prion gains access to nerve and then

propagate back up the axon to the spinal cord and eventually the brain.

Familial CJD, GSS, and FFI are all dominantly inherited prion diseases. Many different mutations of the PrP gene have been shown to be genetically linked to the development of inherited prion disease.

There is no immune response against to both of PrP^{c} and PrP^{sc} since the prion protein is the product of a normal cellular gene. There is also no inflammatory response in the infected brain tissue.

3 Prion Diseases

3.1 Scrapie

Scrapie, the prototypical TSE, has been studied most extensively. Scrapie is a chronic, progressive, and uniformly fatal degenerative CNS disease. Although recognized as a distinct disease of sheep and goats in many countries for centuries, scrapie was not understood to be transmissible until an episode in Scotland in 1935. More than 1,500 cases of iatrogenic scrapie followed the use of a formalin-inactivated louping ill vaccine prepared from sheep brain. Scrapie is distributed widely in Europe and North America and occurs sporadically in some countries in Africa and Asia. Typically, only a few sheep in a flock are diseased at any given time, but infected flocks suffer losses continuously over many years. In the United Kingdom and the United States, most cases occur in the Suffolk and Hampshire breeds, although most other breeds are affected if genetically susceptible sheep are exposed. Goats seem to be incidental hosts, with infection following commingling with scrapie-infected sheep or exposure to contaminated pastures. Sheep, goat, and mouflon are susceptible to natural scrapie. The disease is characterized by the development of amyloid plaques in the central nervous system of infected animals. Typical clinical signs of scrapie in sheep are with unusual restlessness, signs of nervousness, continuous rubbing against the fence and hair loss. There is no evidence for transmission of scrapie to humans.

3.2 Bovine Spongiform Encephalopathy (BSE)

Bovine spongiform encephalopathy (BSE) or “mad cow disease” was first recognized in United Kingdom in 1986. The disease has a long incubation period of four to five years, but ultimately is fatal for cattle within weeks to months of its onset. It is an emerging prion disease of cattle and spreads rapidly throughout the cattle population. The epidemic of BSE peaked in Great Britain in 1993. Over 180,000 cases of BSE had been confirmed in cattle in the United Kingdom between 1986 and October 2004. BSE had also been found in other European countries. This outbreak was traced to the use of cattle feed that contained contaminated meat and bone meal (MBM) from scrapie-

infected sheep and BSE-infected cattle carcasses. The use of such cattle feed was prohibited in 1988, the BSE epidemic has now been under control.

4 Laboratory Diagnoses

The diagnosis of prion disease includes detection of the spongiform changes in the brain biopsy specimen and immunoassay. Immunoassays with monoclone antibodies and polyclonal antibodies are currently employed include western blot, ELISA and immune fluorescence. Confirmation of the diagnosis can be made by detection of a proteinase K-resistant form of PrP in a Western blotting using antibody to PrP in a biopsy specimen. At autopsy, the characteristic amyloid plaques, spongiform vacuoles, and immunohistologically detected PrP can be observed. The presence of PrP^{sc} in any of these target tissues is considered diagnostic. Antemortem testing is based on biopsy sampling of lymphoid tissue from the nictitating membrane, palatine tonsil, or rectal mucosa. The antemortem tests are generally useful only in sheep older than 14 months.

5 Prevention and Treatment

There is no drug or vaccine available for the prevention and treatment of prion diseases. The causative agents are also impervious to the disinfection procedures used for other viruses. Autoclaving at 15psi for 1h or treatment with 5% hypochlorite solution or 1.0mol/L sodium hydroxide can be used for decontamination. Because these agents can be transmitted on instruments and brain electrodes, such items should be carefully disinfected before being reused.

Countries with enzootic scrapie may seek to eradicate the disease. For example, an eradication scheme was established in the United States following the introduction of scrapie into the United States in 1947 and again in 1952. The program has been modified to reflect scientific advances and has involved an integrated program of large-scale active and passive surveillance, animal identification to allow trace-back to the farm of origin of diseased sheep, recognition and identification of a prion gene variant associated with nearly absolute resistance to classical scrapie, financial indemnity for removal of genetically susceptible sheep exposed to scrapie, and certification of scrapie-free flocks. This program has resulted in a steady decrease in the prevalence of scrapie. Given the adverse impact of scrapie and the expenses associated with its eradication, Australia and New Zealand, with their large and scrapie-free sheep populations, have instituted stringent quarantine programs to protect their industries.

Review Questions

1. Term explanation: prion, scrapie associated fibrils.
2. What are the differences between prions and traditional viruses?
3. Try to describe the classification status of prion.
4. Try to describe the replication of prions.
5. Try to describe the public health significance of prions.
6. Try to describe prevention and control measures for diseases caused by prions with example.
7. Try to describe the pathogenesis of mad cow disease.
8. What are the implications of the emergence of prions?

Chapter 33 Bacteriophage

Synopsis

Bacteriophage is a type of virus that infects bacteria, and have host specificity. The most common phages present as tadpoles, such as *E. coli* lambda phage and T4 phage. According to the differences of host relationship, phages were divided into virulent phage and temperate phage. The phenotype, pathogenicity or serotype of the host bacterium can be changed when the phage genome is inserted into the host bacterium. Bacteriophage immunity is that the presence of prophage in the lysogenic bacteria hinders the second infection of homologous phage. Phages can be used for bacterial identification and classification, as a tool for genetic engineering research, and as a therapeutic agent for bacterial infection.

Bacteriophage, generally called phage, are known as viruses that infect bacteria. They obligate intracellular endoparasites that are able to exist as phage particles outside the bacterial cell but can only duplicate inside the cell. They compose of a nucleic acid genome surrounded by a protein coat called a capsid. Phage demoniac the ability to infect a bacterium and redirect the host cell to synthesize phage components.

1 Classification

Based upon the current findings, International Committee on Taxonomy of Viruses classifies the bacteriophage into 12 species. The species are listed in Table 33-1.

Table 33-1 Classification of bacteriophage (胡建和等, 2011)

Family	Group	Type species	Host
Tectiviridae	*Tectivirus*	Enterobacteria bacteriophage PRD1	Bacteria
Siphoviridae	λ phage group	*Escherichia coli* bacteriophage λ	Bacteria
Podoviridae	T7 phage group	*Escherichia coli* T7 bacteriophage	Bacteria
Inoviridae	*Inovirus*	*Escherichia coli* bacteriophage fd	Bacteria
	Plectrovirus	*Acholeplasma* bacteriophage Ls1	Bacteria
Leviviridae	*Levivirus*	*Escherichia coli* bacteriophage MS2	Bacteria
	Oβ-SPvirus	*Escherichia coli* bacteriophage Oβ	Bacteria

Continued

Family	Group	Type species	Host
Myoviridae	T4 phage group	*Escherichia coli* T4 bacteriophage	Bacteria
Corticoviridae	*Corticovirus*	*Pseudomonas* bacteriophage PM2	Bacteria
Cystoviridae	*Cystovirus*	*Pseudomonas* bacteriophage Ø6	Bacteria
Lipothrixviridae	*Lipothrixvirus*	*Thermoproteus* TTV1	Bacteria
Microviridae	*Microvirus*	Ø174	Bacteria
Plasmaviridae	*Plasmavirus*	*Acholeplasma* bacteriophage L2	Mycoplasma
Fuselloviridae	*Fusellovirus*	*Fluidizationsplinter* SSV-1	Thermophilic bacteria

2 Characters

Bacteriophage consists of a nucleic acid genome enveloped by a protein coat named a capsid whose role is to shield the genetic material and to aid in the infection of a new host. Some phages may also carry other enzymes with nucleic acid inside the capsid. The coat consists of protein subunits arranged in highly ordered structures which give the phage a different shape. The number of different kind of protein that go to form the capsid may range from one in simple phage like MS2 to in a complicated phage like T4.

2.1 Three Morphological Shapes

There are three morphological traits related to bacteriophage structure. ①Icosahedron. This is an almost orbicular shape compose of 20 triangular faces. Icosahedrons are very general shapes in nature as they are very effective ways to form a sealed-in shell from subunits. ②Filamentous. Long protein tubes created by capsid proteins assembled into a helical structure. ③Complex. Some phage composed of icosahedral heads adhere to helical tails. In the case of a cluster of phages named the T-even phages, the head is actually an elongated icosahedron. The tails can be contractile or non-contractile, sheathed or non-sheathed and they may also have base plates and tail fibers associated with them. The role of tail is to aid the injection of the genetic material into the cell.

2.2 Size

Bacteriophage range in size from in the region of 25nm (icosahedron diameter) for MS2 to 110nm×85nm (head size) for T4 to which is linked to a 25nm×110nm tail. The filamentous phages such as M13 contain measurements about 6nm wide by 860nm long. A few bacteriophages, such as Ø6, contain membrance envelopes but they are not

as often as in animal viruses, generally.

The genetic material inside the phage capsid may be DNA or RNA, which can be double-stranded or single-stranded, circular or linear (Table 33-2).

Table 33-2 The nature of phage genomes (胡建和等, 2011)

Nucleic acid type		Structure	Example
DNA	Single-stranded	Circular	ØX174, M13
	Double-stranded	Linear Circular	T phage PM2
RNA	Single-stranded	Linear	MS2
	Double-stranded	Linear	Ø6

2.3 Single and/or Double-Stranded DNA

The nucleic acid molecule of bacteriophage is enclosed by a protein coat. Some phages also have lipid, but these are abnormal. Substantial variability is discovered in the nucleic acid of phage. Many phages have double-stranded DNA, others have single-stranded RNA, and some have single-stranded DNA. Uncommon bases such as hydroxymethylcytosine are occasional discovered in the phage nucleic acid. Many phages have special syringe-like structures that bind to cell surface receptors and inject the nucleic acid of phage into a host cell.

2.4 Different Mode of Propagation

Phages can be differentiated on account of their pattern of propagation. Lytic phages yield many copies themselves as they kill their host cell. The most thoroughly researchful lytic phages, the T-even (e.g., T2, T4) phages of *Escherichia coli*, have illustrated the need for accurately timed expression of viral genes in order to concert events associated with phage formation. Temperate phages are capable of enter a non-lytic prophage status in which duplication of their nucleic acid is attached to duplication of host cell DNA. Bacterium carrying prophage are termed lysogenic because a physiological signal can induce a lytic cycle lead to death of host cell and libration of many copies of the phage. The greatest characterized temperate phage is the *E. coli* phage λ. Genes that decide the lytic or lysogenic response to λ infection have been demonstrated and their intricate interactions studied in detail.

Filamentous phages, instanced by the well-characterized *E. coli* phage M13, are exceptive in several respects. Their filaments have single-stranded DNA complicated with protein and are extruded from their hosts, which are enervated but not killed by the phage infection. Engineering of DNA into phage M13 has offered single strands that are precious sources for DNA manipulation and analysis.

3 Replications

3.1 Adsorption (Attachment)

Phage infection of a host starts by bind to particular receptors on the surface of the cell. These receptors vary in nature, being polysaccharides or proteins, which may be exhibit on the surface the whole time or only yields under certain conditions. For example, phage T4 attaches to lipoproteins in the outer membrane of *E. coli* whereas phage λ attaches to a maltose-transport protein only exhibit in the outer membrane when bacterium is grown in maltose-containing medium.

3.2 Penetration

In general, only the genetic material of phage is injected into the host, leaving the empty protein coat on the surface of the cell. Lysozyme associated with the phage head is applied to dissolve the peptidoglycan. The mechanism of osmosis varies from phage to depending on its structure. In the case of T4, which contains a contractile tail, a highly sophisticated mechanism has evolved. After attachment by its long tail contact and fibers of the base plate with the cell wall, the tail sheath contracts and the DNA is injected into the cell. In many cases the incoming DNA may be degraded by endonucleases, which are part of the host's restriction-modification defense systems, but sometimes DNA molecules will survive to establish an infection. These defense endonucleases, termed restriction enzymes, bind to particular sequences in foreign DNA and cut the DNA at these sites. The bacteria's own DNA is modified, regularly by methylation, to prevent it being degraded by theses restriction enzymes. Some phages such as the T-even phages contain evolved mechanisms to change their own DNA by methylation or glycosylation to prevent them being degraded by restriction enzymes on injection.

3.3 Nucleic Acid Replication

Once the inside nucleic acid of the host is transcribed and translated (or just translated in the case of RNA viruses) to produce enzymes that guide the synthesis of new phage nucleic acids. These are occasionally called early proteins. Many phages disconnect the synthesis of host proteins and disintegrate the host genome, thus insuring that all the cellular biosynthetic machinery is directed to the yield of phage components. After a short time, there is often a switch to the yield of large number of phage scaffold proteins, structural proteins required for phage assembly and proteins required for lysis and phage release. These are referred to as late proteins, for example, the reproduction of single-stranded RNA occasionally.

3.4 Phage Assembly

Once enough phage nucleic acid and capsid components have been synthesized the new phage particles are assembled impulsively while, at the same time, packaging the nucleic acid into the capsid, for example bacteriophage T4 (Fig. 33-1).

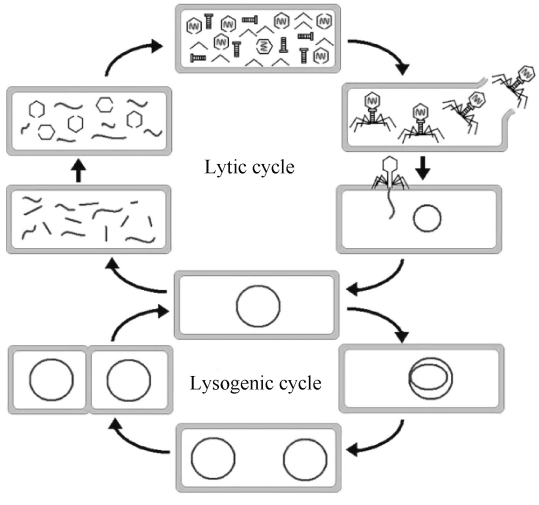


Fig. 33-1 The life cycle of bacteriophage (https://en.wikipedia.org)

3.5 Release

Many phages are released by splitting the bacterial cell wall which is why this life cycle is usually called the lytic cycle and the phage are known as virulent phage. Enzymes relent the cell wall and phages are released as bursts of 50-1,000 phages per cell. The time from infection to release is approximately 22min at 37℃ for T4. Other phage, such as the filamentous phage M13, release phage through the cell wall without destroying the cell so phage particles can be released over a very long period of time. The bacterium continues to grow but at a less rate.

4 Relationships of Bacteriophage and Host

There are alterations in how the entry of the phage genome into the host cell and how the mature virion leaving. Furthermore, there are various developmental pathways. The entry of some phages into the lytic pathway, resulting directly to the yield of more virus particles. Other phages can enter the lysogenic pathway, resulting in an extended quiescent state, named lysogeny. Phages that follow only the lytic pathway are known

as virulent; phages that can follow either pathway are termed temperate.

5 Applications

Bacteriophage is a significant research tool and best ideal material in study and research field of molecular organism, such as gene expression, virus replication, transmissibility, particle assembling and other activity. In recent years, it has become broadly recognized that bacteriophages have several potential roles in the food industry. They have been proposed as substitution to antibiotics in animal health, as biological preservatives in food and as tools for sensing pathogenic bacteria throughout the food chain. Bacteriophages are viruses that only infect and lyse bacterial cells. At present, they exhibit two exclusive features relevant in and appropriate for food safety. Scilicet, their safe use as they are less damage to mammalian cells and their better host specificity that allows proper starter performance in fermented products and keeps the natural microbiota peaceful.

5.1 Identifying and Typing Bacterium

Using phage to control *E. coli* O157: H7 has been a goal. Here we explored diverse phage treatments to decrease or remove *E. coli* O157: H7 *in vivo*. Experiments included testing the previously typical O157-particular lytic phage KH1 for its ability to restrict *E. coli* O157 carriage in sheep experimentally infected with an oral dose of *E. coli* O157: H7, the characterization and isolation of a novel lytic phage designated SH1, the use of a cocktail of the phages KH1 and SH1 to restrict *E. coli* O157: H7 carriage in a model of mouse, and the use of a new phage treatment that placed a mixture of KH1 and SH1 directly onto the bovine mucosa.

5.2 Precluding and Curing Transmissible Diseases

Using bacteriophage may exclude and cure *Poephogus grunniens* paratyphoid fever, swine paratyphoid fever, young stock *E. coli* bacteria and pullorum disease.

5.3 Determining Radiation Dose

Many reports that phage T7 and isolated T7 DNA thin films have been exposed to selected space cases: high vacuum (10^{-4}Pa) and intense UVC radiation (lambda=254nm) to detect the effects of DNA hydration, conformation and packing on UV radiation damage. Diagnostic variation in the absorption spectrum, in the electrophoretic pattern of DNA and the reduce of the amount of PCR products have been examined indicating the photo damage of isolated and intraphage DNA.

5.4 Checking Plant Pathogenic Bacteria

The use of phages for disease control is a rapid escalating area of plant protection with great potential to take the place of the chemical control measurements now prevalent. Phages can be applied validly as part of integrated disease management strategies. The relative ease of preparation of phage treatments and low cost of yield of these agents make them well candidates for extensive use in developing countries as well. However, the efficaciousness of phages, as is true of numerous biological control agents, depends mainly on prevailing environmental factors as well as on susceptivity of the target being. Great care is needful during development, yield and application of phage treatments. Additionally, continuous monitoring for the appearance of resistant bacterial strains is indispensable. Phage-based disease control measurement is a dynamic process with a need for constant adjustment of the phage preparation in order to effectively fight potentially adapting pathogenic bacteria.

Review Questions

1. Try to draw the morphological structure of the phage of *E. coli* T4 phage.
2. Try to describe the replication process of phage.
3. Illustrate the practical application of phage with examples.
4. Illustrate the pathogenicity of phage on bacteria with examples.
5. Try to describe measures to prevent the contamination of bacteriophage.

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