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1. MICROBIOLOGY

1.1. Structure of the bacterial cell

Bacteria are single-celled prokaryotic microorganisms, and their DNA is not contained within a separate nucleus as in eukaryotic cells. They are approximately 0.1–10.0 mm in size (Fig. 1) and exist in various shapes, including spheres (cocci), curves, spirals and rods (bacilli) (Fig. 2). These characteristic shapes are used to classify and identify bacteria. The appearance of bacteria following a Gram stain is also used for identification. Bacteria which stain purple/blue are termed Gram-positive, whereas those that stain pink/red are termed Gram-negative. This difference in response to the Gram stain results from the composition of the cell envelope (wall) (Fig. 3).

Cytoplasmic membrane

A cytoplasmic membrane surrounds the cytoplasm of all bacterial cells and is composed of protein and phospholipid; it resembles the membrane surrounding mammalian (eukaryotic) cells, but it lack sterols. The phospholipids form a bilayer into which proteins are embedded, some spanning the membrane. The membrane carries out many functions, including the synthesis and export of cell-wall components, respiration, secretion of extracellular enzymes and toxins, and the uptake of nutrients by active transport mechanisms. Mesosomes are intracellular membrane structures, formed by folding of the cytoplasmic membrane. They occur more frequently in Gram-positive than in Gram-negative bacteria. Mesosomes present at the point of cell division of Gram-positive bacteria are involved in chromosomal separation; at other sites they may be associated with cellular respiration and metabolism.

Cell wall

Bacteria maintain their shape by a strong rigid outer cover, the cell wall (Fig. 3). Gram-positive bacteria have a relatively thick, uniform cell wall, largely composed of peptidoglycan, a complex molecule consisting of linear repeating sugar sub-units cross-linked by peptide side chains (Fig. 4a). Other cell-wall polymers, including teichoic acids, teichuronic acids and proteins, are also present.

Gram-negative bacteria have a thinner peptidoglycan layer and an additional outer membrane that differs in structure from the cytoplasmic membrane (Fig. 4b). The outer membrane contains lipopolysaccharides on its outer face, phospholipids on its inner face, and proteins and lipoproteins that anchor it to the peptidoglycan. Porins are a group of proteins that form channels through which small hydrophilic molecules, including nutrients, can cross the outer membrane. Lipopolysaccharides are a characteristic feature of Gram-negative bacteria and are also termed 'endotoxins' or 'pyrogen'. Endotoxins are released on cell lysis and have important biological activities involved in the pathogenesis of Gram-negative infections; they activate macrophages, clotting factors and complement, leading to disseminated intravascular coagulation and septic shock.

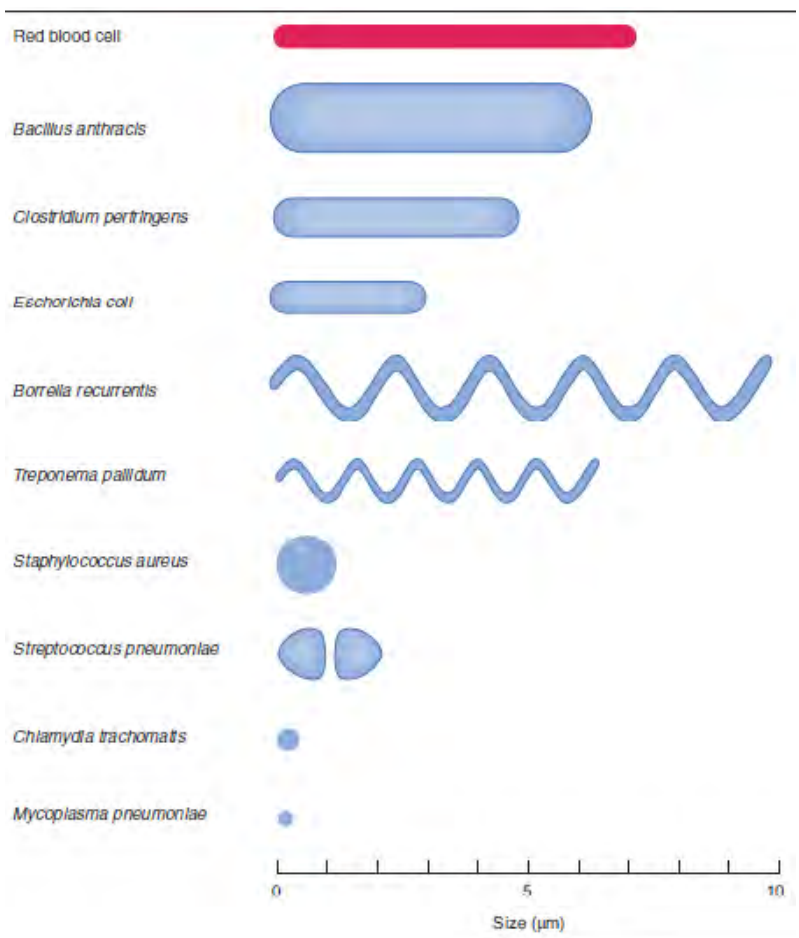


Figure 1. The shape and size of certain clinically important bacteria (*figure used with permission under Creative Commons license*)

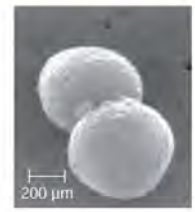
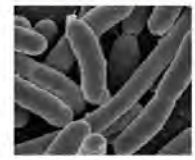
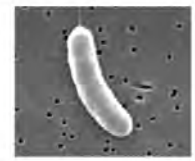
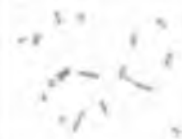
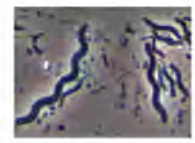
Common Prokaryotic Cell Shapes			
Name	**Description**	**Illustration**	**Image**
Coccus (pl. cocci)	Round		
Bacillus (pl. bacilli)	Rod		
Vibrio (pl. vibrios)	Curved rod		
Coccobacillus (pl. coccobacilli)	Short rod		
Spirillum (pl. spirilla)	Spiral		
Spirochete (pl. spirochetes)	Long, loose, helical spiral		

Figure 2. Various bacterial shapes (*figure used with permission under Creative Commons license*)

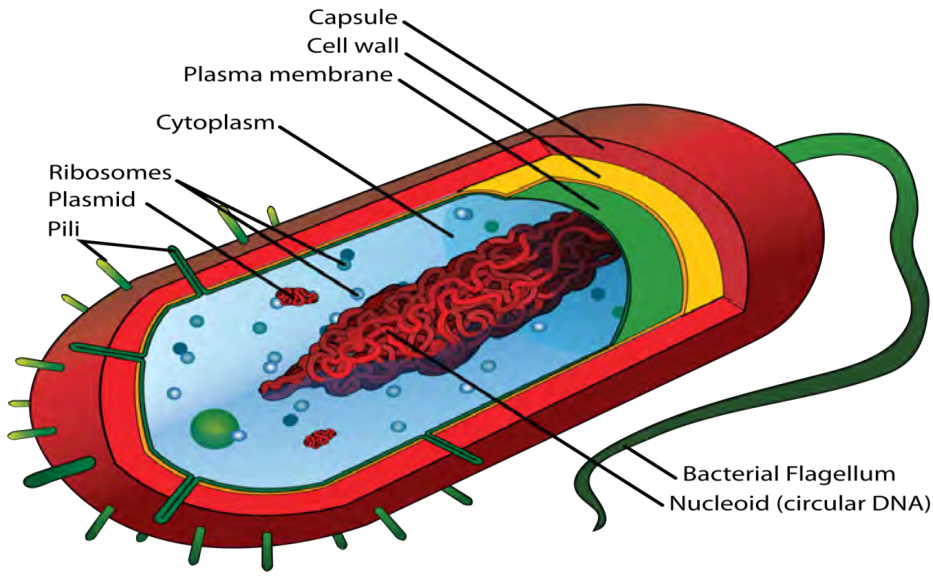


Figure 3. Section of a typical bacterial cell (*figure used with permission under Creative Commons license*)

Mycobacteria have a distinctive cell wall structure and composition that differs from that of Gram-positive and Gram-negative bacteria. It contains peptidoglycan but has large amounts of high molecular weight lipids in the form of long chain length fatty acids (mycolic acids) attached to polysaccharides and proteins. This high lipid content gives the mycobacteria their acid-fast properties (retaining a stain on heating in acid), which allows them to be distinguished from other bacteria (e.g. positive Ziehl-Neelsen stain). The cell wall is important in protecting bacteria against external osmotic pressure. Bacteria with damaged cell walls, such as after exposure to b-lactam antibiotics such as penicillin, often rupture. However, in an osmotically balanced medium, bacteria deficient in cell walls may survive in a spherical form called protoplasts.

Under certain conditions some protoplasts can multiply and are referred to as L-forms. Some bacteria, such as mycoplasmas, have no cell wall at any stage in their life cycle. The cell wall is involved in bacterial division. After the nuclear material has replicated and separated, a cell wall (septum) forms at the equator of the parent cell. The septum grows in, produces a cross-wall and eventually the daughter cells may separate. In many species the cells can remain attached, forming groups, such as staphylococci form clusters and streptococci form long chains (Fig. 5).

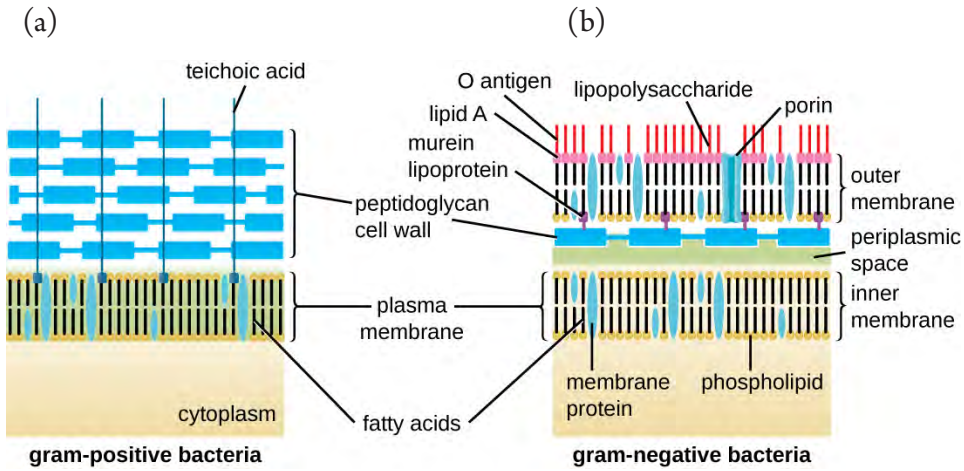


Figure 4. Cell wall and cytoplasmic membrane of (a) Gram-positive bacteria, (b) Gram-negative bacteria. The Gram-positive bacterial cell wall has a thick peptidoglycan layer with associated molecules (teichoic acids, teichuronic acids and proteins). The Gram-negative bacterial cell wall contains lipopolysaccharides, phospholipids and proteins in an outer membrane linked to a thin inner peptidoglycan layer. The mycobacterial cell wall contains long chain length fatty acids (mycolic acids) (*figure used with permission under Creative Commons license*)

Capsules

Some bacteria have capsules external to their cell walls. These structures are bound to the bacterial cell and have a clearly defined boundary. They are usually polysaccharides with characteristic compositions that can be used to distinguish between microorganisms of the same species (e.g. in serotyping). Capsular antigens can be used to differentiate between strains of the same bacterial species, such as in the typing of *Streptococcus pneumoniae* for epidemiological purposes. The capsules are important virulence determinants in both Gram-positive and Gram-negative bacteria, because they may protect the bacteria from host defences and, in some bacteria, aid attachment to host cells.

Bacterial slime and biofilm

Extracellular slime layers are produced by some bacteria. They are more loosely bound to the cell surface than capsules and do not form a clearly defined surface boundary. The slime layer is composed predominantly of complex polysaccharides (glycocalyx), which acts as a virulence factor through the formation of biofilm, for example, by facilitating the attachment of *Staphylococcus*

epidermidis onto artificial surfaces, such as intravascular cannulae, replacement joints and heart valves. Once formed, biofilms present a major problem for treatment and may require removal of the biomedical device.

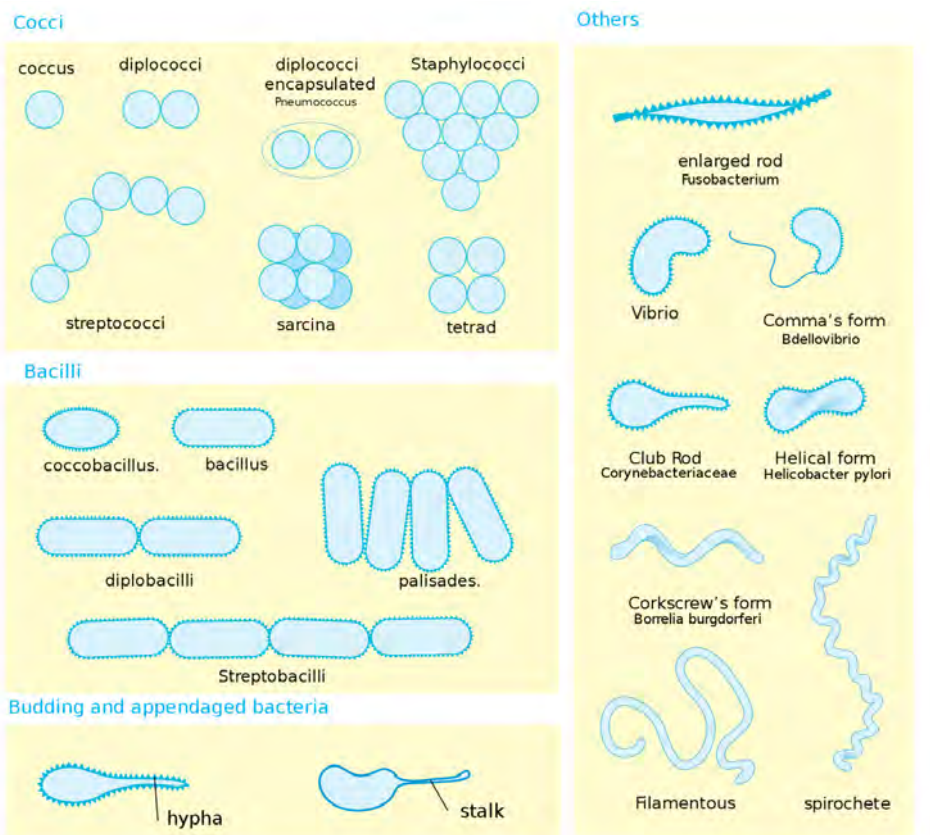


Figure 5. Various groups of bacteria (*figure used with permission under Creative Commons license*)

Flagella

Bacterial flagella are spiral-shaped surface filaments consisting mainly of the protein, flagellin. They are attached to the cell envelope as single (monotrichous) or multiple (peritrichous) forms (Fig. 6). Flagella facilitate movement (motility) in bacteria by rapid rotation. They can be observed under the light microscope with special stains. Flagella are usually detected for diagnostic purposes by observing motility in a bacterial suspension or by spreading growth on solid media. The antigenic nature of the flagella may be used to differentiate between and identify strains of *Salmonella sp*.

Fimbriae

Fimbriae (also termed pili) are thin, hair-like appendages on the surface of many Gram-negative, and some Gram-positive, bacteria. They are approximately half the width of flagella, and are composed of proteins called pilins. In some bacteria they are distributed over the entire cell surface. Fimbriae are virulence factors enabling bacteria to adhere to particular mammalian cell surfaces, an important initial step in colonisation of mucosal surfaces. For example, *Neisseria gonorrhoeae* produce fimbriae that bind to specific receptors of cervical epithelial cells, whereas *Streptococcus pyogenes* have fimbriae containing 'M' protein, which facilitates adhesion to human cells in the pharynx. Specialised fimbriae are involved in genetic material transfer between bacteria, a process called conjugation.

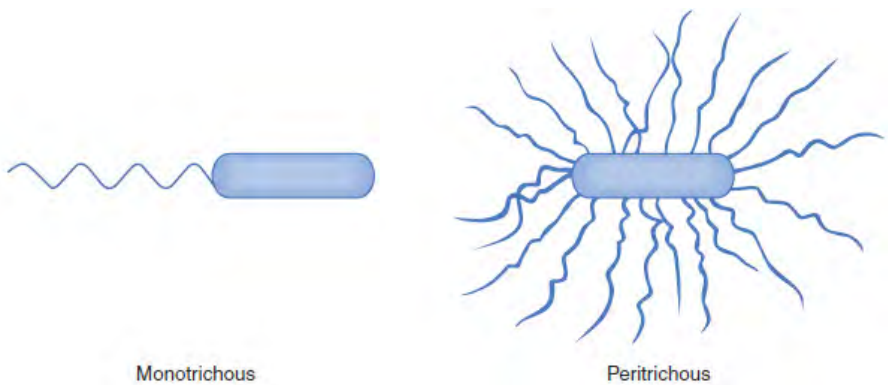


Figure 6. Arrangements of bacterial flagella (*figure used with permission under Creative Commons license*)

Figure 7. Size, shape and position of bacterial spores (from left to right): non-projecting, oval, central, e.g. *Bacillus anthracis*; projecting, spherical, terminal, e.g. *Clostridium tetani*; non-projecting, oval, sub-terminal, e.g. *C. perfringens* (*figure used with permission under Creative Commons license*)

Nuclear material

The bacterial chromosome consists of a single circular molecule of double-stranded DNA, which is maintained in a compact form within the cell by supercoiling. When released from the cell and uncoiled the DNA would be about

1 mm long (10 to 100-times the length of the cell). Additional, smaller extra-chromosomal DNA molecules, called plasmids, may also be present in bacteria. The chromosome usually codes for all the essential functions required by the cell; some plasmids control important phenotypic properties of pathogenic bacteria, including antibiotic resistance and toxin production. Extracellular nuclear material for encoding virulence and antibiotic resistance may also be transferred between bacteria and incorporated into the recipient's chromosome or plasmid. Transfer of genes encoding for virulence or antibiotic resistance may account for bacteria becoming resistant to antibiotics and for low-virulent bacteria becoming pathogenic.

Endospores

Endospores (spores) are small, metabolically dormant cells with a thick, multi-layered coat, formed intracellularly by members of the genera *Bacillus* and *Clostridium* (Fig. 8). They are highly resistant to adverse environmental conditions and may survive desiccation, disinfectants or boiling water for several hours. Spores are formed in response to limitations of nutrients by a complex process (sporulation) involving at least seven stages. When fully formed, they appear as oval or round cells within the vegetative cell. The location is variable, but is constant in any one bacterial species. Spores can remain dormant for long periods of time. However, they are able to revert to actively-growing cells (i.e. germinate) relatively rapidly in response to certain conditions, such as the presence of specific sugars, amino acids or bile salts. Spores also have an important role in the epidemiology of certain human diseases, such as anthrax, tetanus, gas gangrene and infection caused by *Clostridium difficile*. The eradication of spores is of particular importance in certain processes, for example the production of sterile products including pharmaceuticals and surgical instruments, in routine hospital ward and care centre cleaning, and in food preservation.

1.2. Bacterial growth

Most bacteria will grow on artificial culture media prepared from extracts from animal or plant tissues, which supply pre-formed nutrients and vitamins. However, some bacteria, e.g. *Mycobacterium leprae* (leprosy) and *Treponema pallidum* (syphilis), cannot yet be grown in vitro; other bacteria, e.g. *Chlamydia sp.* and *Rickettsia sp.*, only replicate intracellularly within host cells and are therefore grown in tissue culture.

Under suitable conditions (nutrients, temperature and atmosphere), a bacterial cell will increase in size and then divide by binary fission into two identical cells. These two cells are able to grow and divide at the same rate as

the parent cell, provided that conditions including nutrient supply remain stable. This results in an exponential or logarithmic growth rate. The time required for the number of bacteria in a culture to double is called the generation time, e.g. *Escherichia coli* has a generation time of about 20 minutes under optimal conditions. By contrast, *Mycobacterium tuberculosis* has a generation time of 24 hours.

Most bacteria of medical importance require carbon, nitrogen, water, inorganic salts and a source of energy for growth. They have various gaseous, temperature and pH requirements, and can utilize a range of carbon, nitrogen and energy sources. Some bacteria also require special growth factors, including amino acids and vitamins. Growth requirements are important in selecting the various culture media required in diagnostic microbiology and in understanding the tests for identifying bacteria.

Carbon, oxygen and nitrogen sources

Bacteria are classified into two main groups according to the type of compounds that they can utilise as a carbon source:

1. Autotrophs utilise inorganic carbon from carbon dioxide and nitrogen from ammonia, nitrites and nitrates; they are of minor medical importance.
2. Heterotrophs require organic compounds as their major source of carbon and energy; they include most bacteria of medical importance.

Bacteria require CO_2 for growth; adequate amounts are present in the air or are produced during metabolism by the microorganisms themselves. A few bacteria, however, require additional CO_2 for growth, such as *Neisseria meningitidis, Campylobacter jejuni.*

Bacteria can also be classified into four groups according to their O_2 requirements:

1. Obligate (strict) aerobes: grow only in the presence of oxygen, e.g. P*seudomonas aeruginosa*.
2. Microaerophilic bacteria: grow best in low oxygen concentrations, e.g. *Campylobacter jejuni*.
3. Obligate (strict) anaerobes: grow only in the absence of free oxygen, e.g. *Clostridium tetani*.
4. Facultative anaerobes: grow in the presence or absence of oxygen, e.g. *Escherichia coli*.

Temperature

Most pathogenic bacteria grow best at 37°C. However, the optimum temperature for growth is occasionally higher, e.g. for *C. jejuni*, it is 42°C. The ability of some bacteria to grow at low temperatures (0–4°C) is important in food

microbiology; *Listeria monocytogenes*, a cause of food poisoning, will grow slowly at 4°C and has resulted in outbreaks of food poisoning associated with cooked, chilled products. Bacteria can be classified into four major types on the basis of their temperature response:

1. Psychrophilic bacteria: grow just above the freezing temperature, and can cause contamination of food stored in refrigerators. Example – *Pseudomonas*.
2. Mesophilic bacteria: grow at normal temperature in water and food products, liberate gas and cause changes in texture. Example – *Lactobacillus*.
3. Thermophilic bacteria: can survive at higher temperatures and can withstand pasteurization. Example – *Clostridium*, *Bacillus*.
4. Thermophilic bacteria: can survive pasteurization but cannot grow at pasteurization temperature. Example – *Micrococcus*, *Streptococcus*.

pH

Most pathogenic bacteria grow best at a slightly alkaline pH (pH 7.2–7.6). There are a few exceptions: *Lactobacillus acidophilus*, present in the Gram-stain of *Clostridium sporogenes* (showing oval sub-terminal spores), and a *Clostridium tetani* with a terminal spore (arrowed). The vagina of post-pubescent females prefers an acid medium (pH 4.0). It produces lactic acid, which keeps the vaginal secretions acidic, thus preventing many pathogenic bacteria from establishing infections. *Vibrio cholerae*, the cause of cholera, prefers an alkaline environment (pH 8.5).

Growth in liquid media

When bacteria are added (inoculated) into a liquid growth medium, subsequent multiplication can be followed by determining the total number of live microorganisms (viable counts) at various time intervals. The growth curve produced normally has:

1. Lag phase (A): the interval between inoculation of a fresh growth medium with bacteria and the commencement of growth;
2. Log phase (B): the phase of exponential growth; the growth medium becomes visibly turbid at approximately 1,000,000 cells/ml;
3. Stationary phase (C): the growth rate slows as nutrients become exhausted, waste products accumulate, and the rate of cell division equals the rate of death; the total viable count remains relatively constant;
4. Decline phase (D): the rate of bacterial division is slower than the rate of death, resulting in a decline in the total viable count (Fig. 8).

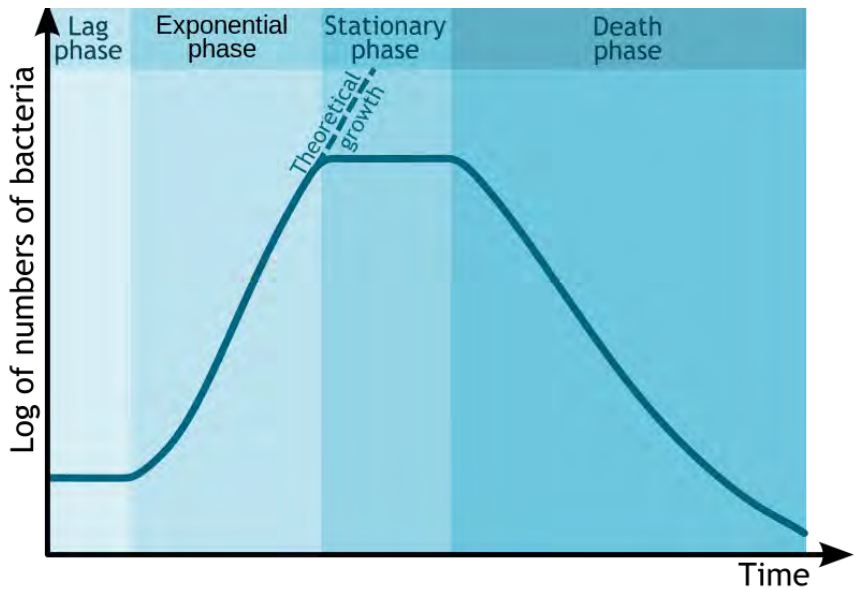


Figure 8. Bacterial growth curve showing the four phases: (A) lag; (B) log or exponential; (C) stationary, and (D) decline (death) – (*figure used with permission under Creative Commons license*)

Growth on solid media

Liquid growth media containing the nutrients needed for bacterial growth can be solidified with agar, a polysaccharide extracted from seaweed. Heating during sterilisation of the medium melts the agar, which then remains liquid until the temperature falls to approximately 40°C, when it produces a transparent solid gel. Solid media are normally set in Petri dishes ('agar plates'). When spread across the surface of an agar plate, most bacteria grow as visible colonies. Each colony comprises millions of bacterial cells that emanated from either a single cell or a cluster of cells. The appearance of the bacterial colony (colonial morphology) assists in identification.

Growth on laboratory media

To grow bacteria in vitro, the microbiologist has to take into account the physiological requirements. Various types of liquid and solid media have been developed for the diagnostic microbiology laboratory.

Simple media

Many bacteria will grow in or on simple media, for example nutrient broth/nutrient agar that contains 'peptone' (polypeptides and amino acids from the enzymatic digestion of meat), and 'meat extract' (water-soluble components of meat containing mineral salts and vitamins).

Enriched media

These contain additional nutrients for the isolation of more fastidious bacteria that require special conditions for growth, e.g. agar containing whole blood (blood agar) or agar containing lysed blood (chocolate agar).

Selective media

These are designed to facilitate growth of some bacteria, while suppressing the growth of others, and include: mannitol salt agar which contains increased NaCl (salt) concentration for the recovery of staphylococci; MacConkey agar, which contains bile salts and allows the growth of bile-tolerant bacteria only, and antibiotics, which are frequently added to media to allow only certain bacteria to grow while suppressing or killing others.

Indicator media

These are designed to aid the detection and recognition of particular pathogens. They are often based on sugar fermentation reactions that result in production of acid and the subsequent colour change of a pH indicator. For example, MacConkey agar contains lactose and a pH indicator (neutral red); lactose-fermenting bacteria (e.g. *Escherichia coli*) produce acid and form pink colonies, and non-lactose fermenting bacteria (e.g. *Salmonella spp.*) do not produce acid and form pale yellow colonies. This property facilitates the recognition of possible *Salmonella* colonies among normal bowel flora. Note that indicator media may also contain selective agents including antibiotics or substances such as bile salts and crystal violet to suppress growth of most Gram-positive microorganisms. MacConkey agar is therefore both a selective medium and an indicator medium.

1.3. Classification of bacteria

The classification of bacteria serves a variety of different functions. Because of this variety, bacteria may be grouped using many different typing schemes. The critical feature for all these classification systems is an organism identified

by one individual (scientist, clinician, epidemiologist), is recognized as the same organism by another individual. At present the typing schemes used by clinicians and clinical microbiologists rely on phenotypic typing schemes. These schemes utilize the bacterial morphology and staining properties of the organism, as well as O_2 growth requirements of the species combined with a variety of biochemical tests. For clinicians, the environmental reservoir of the organism, the vectors and means of transmission of the pathogen are also of great importance.

The Gram stain is a test used to identify bacteria by the composition of their cell walls. It is named for Hans Christian Gram, who developed the technique in 1884. Bacteria are first stained with a purple dye called crystal violet, which specifically binds to peptidoglycan, a complex structure of amino acids and sugars found in the cell wall. This is followed by a series of steps that ultimately remove any unbound or loosely bound crystal violet. Then the cells are stained with a second red-coloured dye called safranin. Gram-positive bacteria stain purple because their cell walls are rich in peptidoglycan. On the other hand, Gram-negative bacteria whose cells walls have two layers take on a red colouring. The outer layer of lipids does not bind strongly to crystal violet and the dye is easily washed away during the staining process. For example, *Streptococcus pneumoniae*, which causes pneumonia, is a Gram-positive bacterium, while *Escherichia coli* and *Vibrio cholerae*, which causes cholera, are Gram-negative bacteria.

Microorganisms can be grouped on the basis of their need for oxygen to grow. Facultative anaerobic bacteria can grow in high oxygen or low oxygen content and are among the more versatile bacteria. In contrast, strictly anaerobic bacteria grow only in conditions where there is minimal or no oxygen present in the environment. Bacteria such as bacteroides found in the large bowel are examples of anaerobes. Strict aerobes only grow in the presence of significant quantities of oxygen. *Pseudomonas aeruginosa*, an opportunistic pathogen, is an example of a strict aerobe. Microaerophilic bacteria grow under conditions of reduced oxygen and sometimes also require increased levels of carbon dioxide. *Neisseria* species (for example, those that cause gonorrhoea) are examples of microaerophilic bacteria.

There are three basic bacterial shapes, according to 'Mims Medical Microbiology'. Round bacteria are referred to as cocci (singular: coccus); cylindrical, capsule-shaped bacteria as bacilli (singular: bacillus); and spiral bacteria are aptly called spirilla (singular: spirillum). Cocci can also associate with one another in different configurations: combinations of two or diplococcus; a linear chain or streptococcus; and a cluster or staphylococcus. The shapes and configurations of bacteria are often reflected in their names.

In 1872, German scientist Ferdinand Cohn classified bacteria to 4 major types, depending on their shapes. Examples of differently-shaped bacteria include:

1. Coccus: These types of bacteria are unicellular, spherical or elliptical. They either remain as a single cell, or can aggregate together in various configurations.
2. Monococcus: Also called micrococcus and represented by a single, discrete round cell. Example: *Micrococcus flavus*.
3. Diplococcus: The cell of the Diplococcus divides ones in a particular plane and after division, the cells remain attached to each other. Example: *Diplococcus pneumonia*.
4. Streptococcus: Here the cells divide repeatedly in one plane to form chain of cells. Example: *Streptococcus pyogenes*.
5. Tetracoccus: Consists of four round cells, which defied in two planes at a right angles to one another. Example: *Gaffkya Tetragena*.
6. Staphylococcus: Here the cells divided into three planes forming a structured like bunches of grapes giving and irregular configuration. Example: *Staphylococcus aureus*.
7. Sarcina: These cells divide in three planes but they form a cube like configuration consisting of eight or sixteen cells but they have a regular shape. Example: *Sarcina Lutea*.
8. Bacilli: Rod-shaped or cylindrical bacteria which either remain singly or in pairs. Example: *Bacillus cereus*.
9. Vibrio: curved, comma-shaped bacteria represented by a single genus. Example: *Vibrio cholerae*.
10. Spirilla: spiral or spring-like, with multiple curvature and terminal flagella. Example: *Spirillum volutans*.

On the basis of nutrition, bacteria are classified as follows:

1. Autotropic bacteria: these bacteria are non-pathogenic, free living, self-sustaining in nature, which prepare their own food by utilisation of solar energy and inorganic components like carbon dioxide, nitrogen etc.
2. Photoautotrophs: these bacteria contain bacterio-chlorophyll and bacterioviridin and can prepare their own food by fixing carbon dioxide the nature by the utilisation of solar energy.
3. Chemoautotrophs: these bacteria prepare food by deriving the energy from oxidation of inorganic substances like nitrogen dioxide, carbon dioxide etc. They can also fix carbon dioxide and water for their nutrition.
4. Heterotrophic bacteria: this type of bacteria cannot fix inorganic Carbone but rather depend on external organic Carbone for their nourishment. They also can be classified on the basis of presence and absence of flight and on the basis of the media on which the bacteria are growing.

Classification of bacteria on the basis of number of flagella:

On the basis of their flagella, bacteria can be classified as:

1. Atrichos: These bacteria has no flagella. Example: *Corynebacterium diptheriae*.
2. Monotrichous: One flagellum is attached to one end of the bacteria cell. Example: *Vibrio cholera*.
3. Lophotrichous: Bunch of flagella is attached to one end of the bacteria cell. Example: *Pseudomonas*.
4. Amphitrichous: Bunch of flagella arising from both end of the bacteria cell. Example: *Rhodospirillum rubrum*.
5. Peritrichous: The flagella are evenly distributed surrounding the entire bacterial cell. Example: *Bacillus*.

The classification criteria mentioned thus far are based on physiological properties and morphology. However, classification of bacteria based on their evolutionary relationships to one another, that is to say, drawing a sort of family tree of all bacterial species, is a relatively new development. This type of phylogenetic classification became possible with the advent of nucleotide sequencing technology (the ability to read the order of nucleotides in DNA or RNA). Since ribosomes are present in all living organisms, one can look at similarities and differences in the RNA sequences that encode certain ribosomal proteins and determine the degree of relatedness of different organisms.

Classification of Bacteria Based on Metabolic Characteristics

Bacteria can be divided into 2 major groups, based on their metabolic properties. The two most important metabolic properties used to classify bacteria into groups include:

– How the organism deals with oxygen;
– What the organism used as a carbon and energy source.

Obligate aerobes have all the faculties to carry out oxidative phosphorylation to obtain energy with oxygen quite perfectly. They use glycolysis, the Krebs Cycle and the electron transport chain, just as we do, to obtain the energy they need for their metabolism. Noteworthy is the fact that they are unable to carry out anaerobic respiration, and thus they will definitely die in the absence of oxygen.

Bacteria that are obligate aerobes include:

1. *Nocardia*;
2. *Bacillus cereus*;
3. *Neisseria*;
4. *Pseudomonas*;
5. *Bordetella*;
6. *Legionella*;

7. *Brucella*;
8. *Mycobacterium*;
9. *Leptospira Interrogans*;
10. B*ranhame llacatarrhalis*;
11. *Burkholderiacepacia*;
12. *Francisella tularensis*;
13. *Spirillum minus*;
14. *Coxiellaburnetti*.

Facultative anaerobes are the closest analogy to humans. They are able to carry out aerobic respiration quite perfectly, possessing both superoxide disputes and catalase (not peroxidase). However, their most noteworthy feature is that they are also able to carry out anaerobic respiration. Thus, they are mainly aerobic, but they have the faculty to carry out anaerobic respiration. This is why they are called facultative anaerobes. When the need arises, they have the faculty to carry out fermentation to obtain energy in the absence of oxygen. This is very similar to the anaerobic respiration carried out by human muscle cells during strenuous activity, such as sprinting. Facultative Anaerobes include:

1. *Listeria*;
2. *Actinomyces*;
3. *Bacillus anthracis*;
4. *Coryne bacterium*;
5. *Staphylococcus*;
6. Most other gram negative rods.

Microaerophilic bacteria are aerobic bacteria that require only a very small amount of oxygen to survive, and are poisoned by excessively high oxygen tension. Microaerophilic bacteria include:

1. *Enterococcus*;
2. some *Streptococci* (although some species of *streptococci* are facultative anaerobes);
3. *Helicobacter pylori*;
4. *Spirochetes*;
5. *Treponema*;
6. *Borrelia*;
7. *Leptospira* (except *Leptospira interrogans*);
8. *Campylobacter*.

Obligate Anaerobes don't like oxygen. They have no electron transport chain, and have no enzymes to prevent against oxidative stress. Thus, if they are exposed to oxygen, they die. Obligate Anaerobes include:

1. *Clostridium*;
2. *Bacteroides*;
3. *Fusobacterium*;
4. *Streptobacillus moniliformis*;

5. *Porphyromonas*;
6. *Prevotella*;
7. *Veillonella*;
8. *Peptostreptococcus*.

There is also a division of obligate anaerobes, known as aerotolerant anaerobes. These bacteria require no oxygen as they respire anaerobically, but unlike obligate anaerobes, they can survive in the presence of oxygen.

1.4. Reproduction of bacteria

Most bacteria multiply by a process called binary fission. A single bacterial cell, the 'parent', makes a copy of its DNA and grows large in size by doubling its cellular content. The doubled contents are pushed out to either end of the cell. Then a small fissure emerges at the centre of the parent, eventually splitting it into two identical 'daughter' cells. Some bacterial species such as cyanobacteria and firmicutes reproduce *via* budding. During budding, the daughter cell grows as an offshoot of the parent. It starts off as a small nub, grows until it is the same size as its parent, and splits off.

The DNA found in parents and offspring after binary fission or budding is exactly the same. Therefore bacterial cells try to introduce some variation into their genetic material by integrating additional DNA into their genome. This is known as horizontal gene transfer, and the resulting genetic variation ensures that bacteria can adapt and survive as their environment changes. There are three ways by which this occurs: transformation, transduction and conjugation.

During transformation, bacterial cells integrate short fragments of DNA from their surrounding environment. These fragments may be released by nearby bacteria that have ruptured. On the other hand, transduction occurs when bacteria are infected by special viruses known as bacteriophages that can carry bacterial DNA.

Conjugation requires physical contact between two bacteria. Genetic material, usually a duplicated plasmid, will transfer from a donor to a recipient. This plasmid copy travels out through a physical extension called the pilus and enters the recipient bacterial cell. Donor bacteria contain a sequence of DNA called the F-factor that enables pilus formation. Conjugation can aid in the spread of antibiotic resistance genes.

1.5. Pathogenic bacteria

Pathogenic bacteria are bacteria that can cause infection. This article deals with human pathogenic bacteria. Although most bacteria are harmless or often beneficial, some are pathogenic, with the number of species estimated as fewer

than 100 that are seen to cause infectious diseases in humans. By contrast, several thousand species exist in the human digestive system. One of the bacterial diseases with the highest disease burden is tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, which kills about 2 million people a year, mostly in sub-Saharan Africa. Pathogenic bacteria contribute to other globally important diseases, such as pneumonia, which can be caused by bacteria such as *Streptococcus* and *Pseudomonas*, and foodborne illnesses, which can be caused by bacteria such as *Shigella*, *Campylobacter*, and *Salmonella*. Pathogenic bacteria also cause infections such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy. Pathogenic bacteria are also the cause of high infant mortality rates in developing countries.

Some pathogenic bacteria cause disease under certain conditions, such as entry through the skin *via* a cut, through sexual activity or through a compromised immune function. *Streptococcus* and *Staphylococcus* are part of the normal skin microbiota and typically reside on healthy skin or in the nasopharangeal region. Yet these species can potentially initiate skin infections. They are also able to cause sepsis, pneumonia or meningitis. These infections can become quite serious creating a systemic inflammatory response resulting in massive vasodilation, shock, and death. Other bacteria are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression or cystic fibrosis. Examples of these opportunistic pathogens include *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and *Mycobacterium avium*. Bacterial pathogens often cause infection in specific areas of the body. Others are generalists.

1.5.1. Bacterial toxins

There are two types of bacterial toxins:

– Lipopolysaccharides: associated with the cell walls of Gram- bacteria. The lipopolysaccharide (LPS) component of the Gram- bacteria outer membrane bears the name endotoxin because of its association with the cell wall of bacteria.

– Proteins: may be released into the extracellular environment of pathogenic bacteria. Most of the protein toxins are thought of as exotoxins, since they are 'released' from the bacteria and act on host cells at a distance.

The protein toxins are soluble proteins secreted by living bacteria during exponential growth. The production of protein toxins is specific to a particular bacterial species. For example, only *Clostridium tetani* produces tetanus toxin, and only *Corynebacterium diphtheriae* produces the diphtheria toxin. Usually, virulent strains of the bacterium produce the toxin (or range of toxins) while non-virulent strains do not. Toxin is the major determinant of virulence. Both Gram-positive and Gram-negative bacteria produce soluble protein toxins. Bacterial protein toxins are the most potent poisons known and may show activity at very high dilutions.

The protein toxins resemble enzymes. Like enzymes, bacterial exotoxins are: proteins denatured by heat, acid, proteolytic enzymes have a high biological activity (most act catalytically) exhibit specificity of action Bacterial protein toxins are highly specific in the substrate utilized and in their mode of action. Usually the site of damage caused by the toxin indicates the location of the substrate for that toxin. Terms such as 'enterotoxin', 'neurotoxin', 'leukocidin' or 'hemolysin' are used to indicate the target site of some well-defined protein toxins. Certain protein toxins have very specific cytotoxic activity (i.e. they attack specific cells, for example, tetanus or botulinum toxins). Some (as produced by *staphylococci*, *streptococci*, *clostridia*, etc.) have fairly broad cytotoxic activity and cause the nonspecific death of tissues (necrosis). Toxins that are phospholipases may be relatively nonspecific in their cytotoxicity. This is also true of pore-forming 'hemolysins' and 'leukocidins'. A few protein toxins cause death of the host and are known as 'lethal toxins' (e.g. anthrax toxin).

Protein toxins are strongly antigenic. *In vivo*, specific antibody (antitoxin) neutralizes the toxicity of these bacterial proteins. *In vitro*, specific antitoxin may not fully inhibit their enzymatic activity. Protein toxins are inherently unstable: in time they lose their toxic properties but retain their antigenic ones. Toxoids are detoxified toxins which retain their antigenicity and their immunizing capacity (first discovered by Ehrlich). The formation of toxoids can be accelerated by: treating toxins with a variety of reagents including formalin, iodine, pepsin, ascorbic acid, ketones, etc. The mixture is maintained at 37°C at pH range 6 to 9 for several weeks. Toxoids can be used for artificial immunization against diseases caused by pathogens where the primary determinant of bacterial virulence is toxin production. E.g. immunizing against diphtheria and tetanus that are part of the DPT vaccine.

Many protein toxins consist of two components: Sub-unit A, which is responsible for the enzymatic activity of the toxin, and Sub-unit B, which is concerned with binding to a specific receptor on the host cell membrane and transferring the enzyme across the membrane. The enzymatic component is not active until it is released from the native toxin. Isolated A sub-units are enzymatically Tertiary structure of the active and but lack binding and cell entry pertussis toxin produced by capability. Pertussis Isolated B sub-units that bind to target toxins are a member of the A–B bacterial toxin superfamily. These are cells that even block the binding of a hexameric protein comprising native A+B toxin. Endotoxins are part of the outer cell wall of bacteria. Invariably associated with Gram-bacteria as constituents of the outer membrane of the cell wall. Endotoxin: Occasionally used to refer to any 'cell-associated' bacterial toxin. But should be reserved for the lipopolysaccharide complex associated with the outer envelope of Gram- bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens. Lipopolysaccharide (LPS) participates: in a number of outer membrane functions essential for bacterial growth and survival, especially within the context of a host-parasite interaction.

The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). Toxicity is associated with the lipid component (Lipid A) and Immunogenicity (antigenicity) is associated with the polysaccharide components. The cell wall antigens (O antigens) of Gram- bacteria are components of LPS. LPS activates complement by the alternative (properdin) pathway and may be a part of the pathology of most Gram-negative bacterial infections. Most part of endotoxins remain associated with the cell wall until disintegration of the bacteria. In vivo, this results from autolysis, external lysis, and phagocytic digestion of bacterial cells. Small amounts of endotoxin may be released in a soluble form, especially by young cultures. Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as, superoxide, peroxide and hypochlorite degrade them. Endotoxins, although strongly antigenic, cannot be converted to toxoids.

Endotoxin lipopolysaccharides: are complex amphiphilic molecules with a mw of about 10 kDa, that vary widely in chemical composition both between and among bacterial species. In a basic ground plan common to all endotoxins, LPS consists of three components:

- Lipid A;
- Core polysaccharide;
- Polysaccharide.

Lipid A is the Lipid component of LPS. Contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram- bacteria. Among *Enterobacteriaceae* Lipid A is virtually constant.

Core (R) polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO – Hep – Hep – Glu – Gal – Glu – GluNAc. Two unusual sugars, present Heptose and 2-keto-3-deoxyoctonoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin). Core polysaccharide is common to all members of a bacterial genus with minor variations (for example, *Salmonella*). But it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores.

O polysaccharides (also referred to as the O antigen or O side chain) are attached to the core polysaccharide. Consists of repeating oligosaccharide sub-units made up of 3–5 sugars. The individual chains vary in length ranging up to 40 repeat units. O polysaccharide is much longer than the core polysaccharide

and it maintains the hydrophilic domain of the LPS molecule. A unique group of sugars, called dideoxyhexoses, occurs in the O polysaccharide. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide. Great variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria. LPS and virulence of Gram-negative bacteria Endotoxins are toxic to most mammals. They are strong antigens but they seldom elicit immune responses which give full protection to the animal against secondary challenge with the endotoxin. Endotoxins released from multiplying or disintegrating bacteria significantly contribute to the symptoms of Gram- bacteraemia and septicaemia important pathogenic factors in Gram-negative bacteria infections.

Regardless of the bacterial source all endotoxins produce the same range of biological effects in the animal host. Injection of living or killed Gram- cells, or purified LPS, into experimental animals wide spectrum of nonspecific pathophysiological reactions related to inflammation such as: fever changes in white blood cell counts disseminated intravascular coagulation tumour necrosis hypotension shock.

Physiological activities of endotoxins are mediated mainly by the Lipid A component of LPS. Its biological activity depends on a peculiar conformation determined by the glucosamine disaccharide, PO_4 groups, Acyl chains, and KDO-containing inner core. Lipid A is known to react at the surfaces of macrophages release cytokines that mediate the pathophysiological response to endotoxin.

1.5.2. Pathogenicity Islands

Pathogenicity Islands (PAI) are a distinct class of genomic islands which are acquired by horizontal gene transfer. Incorporated in the genome of pathogenic bacteria usually absent from non-pathogenic organisms of the same or closely related species. They occupy relatively large genomic regions ranging from 10–200 kb encode genes which contribute to virulence of the pathogen. Typical examples: adhesins, toxins, iron uptake systems, invasins, etc.

One species of bacteria may have more than one pathogenicity island. For example, in *Salmonella*, five pathogenicity islands have been identified. Found mainly in Gram negative bacteria, but have been shown in a few Gram-positives. Found in pathogens that undergo gene transfer by plasmid, phage, or a conjugative transposon and are typically transferred through mechanisms of horizontal gene transfer (HGT). May be located on the bacterial chromosome or may be a part of a plasmid. They are rich in Guanine + Cytosine content. They are flanked by direct repeats i.e. the sequence of bases at two ends are the same. Are associated with tRNA genes, which target sites for the integration of DNA. Have characteristics of transposons in that they carry functional genes e.g. integrase, transposase, or part of insertion sequences may move from one tRNA locus to another on the chromosome or plasmid.

Pathogenicity Islands play a vital role in the virulence of bacterial pathogens of humans, animals and plants. The availability of a large number of genome sequences of pathogenic bacteria and their non-pathogenic relatives identification of novel pathogen-specific genomic islands. PAI apparently -acquired during the speciation of pathogens from their non-pathogenic or environmental ancestors. The acquisition of PAI is an ancient evolutionary event that led to the appearance of bacterial pathogens on a timescale of millions of years May also represent a mechanism that contributes to the appearance of new pathogens. Pathogenicity Islands Knowledge about PAI, their structure, their mobility, and the pathogenicity factors they encode is helpful in gaining a better understanding of bacterial evolution and interactions of pathogens with eukaryotic host cells.

PAI can also have important practical implications such as providing delivery systems for vaccination, and tools for the development of new strategies for bacterial infection therapy.

Pathogenicity Islands represent distinct genetic elements encoding virulence factors of pathogenic bacteria. Belong to a more general class of genomic islands common genetic elements sharing a set of unifying features. Genomic islands have been acquired by horizontal gene transfer. In recent years many different genomic islands have been discovered in a variety of pathogenic as well as non-pathogenic bacteria. Because they promote genetic variability, genomic islands play an important role in microbial evolution.

1.5.3. Mechanism of pathogenesis

An infectious disease is a clinically evident deviation from health. It occurs when there is a parasitic relationship between a host and a microorganism. Several different factors influence a microorganism's relationship to its host and level of severity. These include:

– Pathogenicity: The ability to produce disease in a host organism.

– Virulence: The degree of pathogenicity of a microorganism. Determinants of virulence for a pathogen include a pathogen's genetic, biochemical, or structural features. For example, one strain of influenza may only cause a fever and sore throat, while another may cause pneumonia or other serious respiratory condition.

– Infectivity: The level at which a microorganism is able to infect or invade a host.

– Transmissibility: The measure of a microorganism's ability to spread from one host to the next. This can include both distance and number of affected individuals.

Over a century ago, Robert Koch established that infectious diseases were caused by microbes. He was looking for the causative agent for anthrax. Koch's

postulates are experimental criteria that are used to determine if a microbe caused a specific disease. The criteria include:

1. The microbe must be present in every case of the disease.
2. The organism must be grown in a pure culture from diseased hosts.
3. The same disease must be produced when a pure culture of the organism is introduced into a susceptible host.
4. The organism must be recovered from the experimentally infected hosts.

However, there are some exceptions to these criteria. These include:

1. Some organisms cannot be cultured in a lab and grown on artificial media.
2. Some pathogens can cause several disease conditions such as *M. tuberculosis*, which can cause lung disease and other diseases of the skin, bone, and internal organs.
3. There may be ethical reasons that do not allow testing, (i.e. human diseases with no animal model – smallpox, rubella).

Pathogenesis is the method by which a disease can develop. This can occur through foodborne intoxication where the causative agent produces toxins in the body (e.g. botulism). Another route is the colonization of an invading pathogen on the host surface, which allows the pathogen to increase in numbers and produce toxins that are damaging to the host's cells (e.g. *Vibrio* and *Corynebacterium*). Pathogenesis can also occur by pathogens invading and breaching the body's barrier in order to multiply. These organisms have mechanisms that will not allow macrophages (the body's defence against pathogens) to destroy them. They can also evade antibody detection (e.g. tuberculosis and plague). Finally, organisms can invade tissues within the body and produce toxins (e.g. *Shigella*). The relationship between a host and pathogen is dynamic. Production of disease occurs through a process of steps. The first five mechanisms make up a pathogen's invasiveness (i.e. ability to invade tissues).

Transmission

In order to begin infection and eventually cause disease, pathogens must find a transmission route. Transmission of an infectious agent can occur in many ways, but it is typically through exposed skin (e.g. a cut, abrasion, puncture, or wound) or mucous membranes (e.g. gastrointestinal tract, respiratory tract, or urogenital tract).

Adherence

Once the pathogen has gained access to the body, it must have some means of attaching itself to the host's tissues. This attachment is called adherence and is a necessary step in pathogenicity. Microbes contain ligands, which are projections that attach host receptors or surface proteins. Pathogens may have

specific adherence mechanisms to attach to cells or tissue surfaces. Examples of this include:

1. Tissue tropism (i.e. pathogens that prefer specific tissues over others),
2. Species specificity (i.e. pathogens that only infect certain species), and
3. Genetic specificity (i.e. surface mutations that occur so previous antibodies do not recognize the invading pathogen). If a microorganism cannot adhere to a host cell membrane, disease will not occur.

Invasion

At this point, microbes begin to invade the host and produce a bacteraemia (i.e. presence of bacteria in the bloodstream) or viremia (presence of a virus in the bloodstream). Microorganisms are exposed to many barriers after introduction into the host. Some bacteria are able to cause disease while remaining on the epithelial barriers, while many need to penetrate that barrier. Once this barrier has been penetrated, these pathogens can multiply without competition.

Colonization

Colonization is the multiplication of pathogenic organisms where toxins are produced and the normal flora are overcome. During this stage, pathogens compete with normal flora for space and nutrients. Pathogens usually colonize host tissues that are in contact with the external environment. During colonization, the host begins to show signs of septicaemia (i.e. blood infection where bacteria are reproducing). For infection to proceed an infectious dose should be determined. This is the minimal number of microbes necessary to establish infection. Certain pathogens are less contagious and therefore require larger numbers of pathogens to cause disease (i.e. 10–100 for *Shigella* and 1,000,000 for *Salmonella*).

Evasion of Host Defences

After colonization, pathogens circumvent the host's innate and adapted defences by phagocytosis. Multiple mechanisms are used by pathogens to evade a host's immune system. For the innate system this includes:

– Intracellular pathogens that live inside a host cell;
– Avoid phagocyte recognition by producing capsules prevents phagocytosis;
– Producing membrane damaging toxins which can kill phagocytes (e.g. leukocidins);
– Interfere with complement activation;
– Survive in the phagocyte.

Pathogens must also avoid adapted defences. Pathogens can produce proteases (i.e. allow each pathogen to avoid antibodies), or catalases (i.e. prevent the digestion of an engulfed pathogen). They can also utilize antigenic variation to alter the antigen structure. In addition, pathogens can mimic host molecules, which can cause disease-related damage.

Exiting the Host

A pathogen must exit the body. This occurs through various routes. Examples include sneezing, coughing, diarrhoea, coitus, pus, blood, or insect bites.

Survival Outside the Host

Finally, a pathogen must be able to survive in the environment long enough to be transmitted to another host. Some are hardy and can survive for several weeks before a new host is found. There are others that survive in animal reservoirs or require direct contact because they are fragile.

1.5.4. Antibiotic resistance

Antibiotics are typically used to treat bacterial infections. However in recent years, the improper or unnecessary use of antibiotics has promoted the spread of several strains of antibiotic-resistant bacteria.

Antibiotic resistance is a phenomenon where infectious bacteria are no longer susceptible to previously effective antibiotics. According to the CDC, each year in the United States, at least 2 million people are infected with antibiotic resistant bacteria, leading to the death of at least 23,000 each year.

One of the more notorious antibiotic resistant bacterial strains is methicillin-resistant *Staphylococcus aureus* (MRSA), which resists methicillin and other antibiotics used to treat *Staphylococcus* infections. It spreads primarily through skin contact. MRSA infections occur in health care settings such as hospitals and nursing homes, where it can lead to pneumonia or bloodstream infections. MRSA also spreads in the community, especially in situations where there is a lot of skin contact or the use of shared equipment; for example, among athletes, in tattoo parlours, or in day care facilities and schools. Community-acquired MRSA most often causes skin infections.

1.6. Viruses

The concept behind modern virology can be traced back to Adolf Mayer, Dimitri Ivanofsky and Martinus Beijerinck who, independently in the late 1880's, discovered what was later to be called tobacco mosaic virus (TMV). Their

discoveries led to the descriptions of filterable agents, too small to be seen with the light microscope, that could be grown in living cells and cause disease. The first filterable agent from animals, foot and mouth disease virus, was described by Loeffler and Frosch in 1898, and the first human filterable agent discovered yellow fever virus, discovered by Walter Reed in 1901. The term 'virus' derives from the Latin for slimy liquid or poison and was gradually introduced during this period to replace the term 'filterable agents'. The first virus to be visualized by x-ray crystallography and electron microscopy was TMV, reported in 1941 and 1939, respectively. These advances introduced the notion that viruses were structurally composed of repeating sub-units. Frederick Twort and Felix d'Herelle, working independently, are credited with the discovery of viruses which could infect and lyse bacteria in 1915. D'Herelle introduced the term 'bacteriophages' for these agents and also described the concepts of virus adsorption to its target, cell lysis and release of infectious particles. Over the next 35–40 years, work with phages led to numerous discoveries including how the introduction of DNA into a target cell could reproduce itself and the regulation of cellular macromolecular synthesis directed by viruses. In essence, the field of molecular biology was opened up during this period. Advances in animal virology were noted throughout the 20th century but the major breakthrough came through the development of tissue culture systems that led, for example, to the isolation of poliovirus by Enders et al. in 1949. This markedly facilitated detailed study of this agent and, most importantly, the development of poliovirus vaccines. The ensuing 60 years have seen diagnostic virology mature as a field with the discovery of new agents and diseases and the parallel determination of the importance of viruses in our understanding of molecular biology and cancer.

1.6.1. Virus structure, classification

A. Virus particle or virion. An infectious agent composed of nucleic acid (RNA or DNA), a protein shell (capsid) and, in some cases, a lipid envelope. Virions have full capacity for replication when a susceptible target cell is encountered.

– Capsid and capsomeres. The protein coat that surrounds the viral nucleic acid. This is composed of repeating protein sub-units called capsomeres. Generally, capsids have either helical or icosahedral symmetry.

– Nucleocapsid. The complete protein-nucleic acid complex.

B. Satellite or Defective Viruses. Viruses which require a second virus (helper virus) for replication. Hepatitis delta virus is the major human pathogen example. It requires the presence of hepatitis B virus to complete its replication cycle.

C. Viroids. Viroids are the smallest known autonomously replicating molecules. They consist of single-stranded, circular RNA, 240–375 residues in length and are plant pathogens.

D. Prions. Prions are not viruses but are often discussed within this microbiologic category. Prions are infectious protein molecules that contain

no definable nucleic acid and are responsible for the transmissible and familial spongiform encephalopathies: Creutzfeldt-Jakob disease, kuru, fatal familial insomnia, Gerstmann-Straussler-Sheinker syndrome, and bovine spongiform encephalopathy ('mad cow disease'). The pathogenic prion protein, PrPSc, is formed from a normal human protein, PrPC.

Viral classification has been confusing and oft-changing over the years. In the past, viruses were often classified by host, target organ or vector and these are still used vernacularly (e.g. the hepatitis viruses). Modern classification is based on the following three characteristics:

1. The type of viral nucleic acid (RNA or DNA, single-stranded or double-stranded).
2. Its replication strategy.
3. The capsid symmetry (icosahedral or helical).

DNA viruses

– Double-stranded DNA viruses include poxviruses, herpesviruses, adenoviruses, papova viruses and polyomaviruses.

– Single-stranded DNA viruses include parvoviruses. DNA viruses usually replicate in the nucleus of host cells by producing a polymerase that reproduces viral DNA. Viral DNA is not usually incorporated into host chromosomal DNA.

RNA viruses

RNA viruses possess a single strand of RNA and adopt different reproductive strategies:

– RNA sense (positive) may serve directly as mRNA and be translated into structural protein and an RNA-dependent RNA polymerase.

– RNA antisense (negative) contains an RNA-dependent RNA polymerase that transcribes the viral genome into mRNA. Alternatively, the transcribed RNA can act as a template for further viral (antisense) RNA.

– Retroviruses have single-stranded sense RNA that cannot act as mRNA. This is transcribed into DNA by reverse transcriptase and incorporated into host DNA. The subsequent transcription to make mRNA and viral genomic RNA is under the control of host transcriptase enzymes.

1.6.2. Pathogenesis of Viral Diseases

As with other infectious agents which cause human disease, the outcome of the interaction of a particular virus with the human host is dependent on both pathogen and host factors. Viral strains within a genus may have differential cell tropisms, replication capacities and cytopathogenic effects. As an example, strains of HIV may

preferentially target monocyte/macrophages or T-lymphocytes, may use different co-receptors (e.g. the chemokine receptors, CCR5 or CXCR4) on the cell surface, may replicate to different levels and may induce different degrees of cell killing. These traits have direct clinical correlates for HIV infected persons with respect to the rates of CD4 cell decline and progression to clinical AIDS. On the host side, the nature of the exposure and the host immune status are probably the two most important determinants of outcome. Thus, the key elements of the virus host interaction are:

1. Viral strain.
2. Inoculum size.
3. Route of exposure.
4. Susceptibility of the host (i.e. is there pre-existent immunity from past exposure or vaccination?).
5. Immune status and age of host.

A generalized schema of viral infection leading to disease in the human host is as follows:

1. Depending upon the agent, the virus enters through the skin, mucous membranes, respiratory tract, gastrointestinal tract, *via* a transfusion or transplanted organ or *via* maternal-foetal transmission.
2. There is local replication at the site of the inoculation. Certain agents exhibit pathology at the skin or mucous membrane surface – e.g. herpes simplex virus, human papillomavirus.
3. For some neurotropic viruses there may be spread along peripheral nerve routes to ganglia (e.g. herpes simplex virus) or the central nervous system (e.g. rabies virus). For other neurotropic agents, the central nervous system is seeded following viremia.
4. For many agents, there is replication in regional lymph nodes with subsequent viremia and spread to target organs. Some viruses travel in the bloodstream free in plasma (e.g. picornaviruses); others are cell associated (e.g. cytomegalovirus (CMV).
5. Replication in target organs may lead to local damage and further rounds of viremia.
6. Non-specific and specific host immune responses come into play to try to control and downregulate the viral replicative process.

1.6.3. Immune Responses to Viral Infections

1. Innate (non-specific) immunity refers to those elements of the immune system that can clear virus or virus infected cells immediately upon or shortly after viral exposure and which are not dependent upon immunologic memory. Non-specific immunity may include:
   a) Phagocytic cells (neutrophils and monocyte/macrophages).
   b) Cytokines (e.g. interferons) and chemokines.

c) Natural killer cells.
d) Poorly defined antiviral factors that may exist in blood or body fluids.

2. Adaptive (specific) immunity refers to antigen specific B and T cell responses that lead to the development of antibodies, cytotoxic T cells and antibody dependent cellular cytotoxicity.

3. In some instances, an intense immunologic reaction to a viral agent can result in immunopathology and a serious clinical syndrome. A prime example is dengue haemorrhagic fever which is likely due to antibody dependent enhancement and T cell activation on re-exposure to dengue virus.

1.6.4. Mechanisms of Viral Persistence

Viruses may cause chronic, persistent infection with continuous viral replication in the face of an immune response. Examples include HIV, hepatitis B virus and hepatitis C virus. Some viruses may demonstrate persistent infection in immune compromised hosts. These include the herpesviruses, human papillomavirus and rubella virus, among others. Some viruses are able to cause latent infection. Latency is characterized by a quiescent or minimally transcriptionally active viral genome with periods of reactivation. Latent viruses include the herpesviruses (cytomegalovirus, Epstein-Barr virus, herpes simplex virus, varicella-zoster virus), human papillomavirus, human retroviruses. Recurrent herpes labialis (cold sores) or genital herpes due to HSV, or herpes zoster due to varicella zoster virus are classic examples of latency and reactivation. Viruses which exhibit latency may also exhibit chronic, persistent replication in the setting of immune compromise of the host.

Mechanisms of persistence of viruses which produce chronic infections include antigenic variation to escape antibody or cytotoxic T cell responses, downregulation of class I major histocompatibility antigens resulting in diminished recognition by cytotoxic T cells and modulation of apoptosis. Viruses which establish latent infection escape recognition by the immune system through decreased viral antigen expression and presentation. Sites of persistence include the nervous system (herpes simplex virus, varicella zoster virus, measles virus, poliovirus, JC virus), the liver (hepatitis B virus, hepatitis C virus), and leukocytes (HIV, cytomegalovirus, Epstein-Barr virus).

1.6.5. The Viral Life Cycle

All viruses depend on cells for reproduction and metabolic processes. By themselves, viruses do not encode for all of the enzymes necessary for viral replication. But within a host cell, a virus can commandeer cellular machinery to produce more viral particles. Bacteriophages replicate only in the cytoplasm, since prokaryotic cells do not have a nucleus or organelles. In eukaryotic

cells, most DNA viruses can replicate inside the nucleus, with an exception observed in the large DNA viruses, such as the poxviruses, that can replicate in the cytoplasm. RNA viruses that infect animal cells often replicate in the cytoplasm.

The Lytic Cycle

During the lytic cycle of virulent phage, the bacteriophage takes over the cell, reproduces new phages, and destroys the cell. T-even phage is a good example of a well-characterized class of virulent phages. There are five stages in the bacteriophage lytic cycle. Attachment is the first stage in the infection process in which the phage interacts with specific bacterial surface receptors (e.g. lipopolysaccharides and OmpC protein on host surfaces). Most phages have a narrow host range and may infect one species of bacteria or one strain within a species. This unique recognition can be exploited for targeted treatment of bacterial infection by phage therapy or for phage typing to identify unique bacterial subspecies or strains. The second stage of infection is entry or penetration. This occurs through contraction of the tail sheath, which acts like a hypodermic needle to inject the viral genome through the cell wall and membrane. The phage head and remaining components remain outside the bacteria.

The third stage of infection is biosynthesis of new viral components. After entering the host cell, the virus synthesizes virus-encoded endonucleases to degrade the bacterial chromosome. It then hijacks the host cell to replicate, transcribe, and translate the necessary viral components (capsomeres, sheath, base plates, tail fibres, and viral enzymes) for the assembly of new viruses. Polymerase genes are usually expressed early in the cycle, while capsid and tail proteins are expressed later. During the maturation phase, new virions are created. To liberate free phages, the bacterial cell wall is disrupted by phage proteins such as holin or lysozyme. The final stage is release. Mature viruses burst out of the host cell in a process called lysis and the progeny viruses are liberated into the environment to infect new cells.

The Lysogenic Cycle

In a lysogenic cycle, the phage genome also enters the cell through attachment and penetration. A prime example of a phage with this type of life cycle is the lambda phage. During the lysogenic cycle, instead of killing the host, the phage genome integrates into the bacterial chromosome and becomes part of the host. The integrated phage genome is called a prophage. A bacterial host with a prophage is called a lysogen. The process in which a bacterium is infected by a temperate phage is called lysogeny. It is typical of temperate phages to be latent or inactive

within the cell. As the bacterium replicates its chromosome, it also replicates the phage's DNA and passes it on to new daughter cells during reproduction. The presence of the phage may alter the phenotype of the bacterium, since it can bring in extra genes (e.g. toxin genes that can increase bacterial virulence). This change in the host phenotype is called lysogenic conversion or phage conversion. Some bacteria, such as *Vibrio cholerae* and *Clostridium botulinum*, are less virulent in the absence of the prophage. The phages infecting these bacteria carry the toxin genes in their genome and enhance the virulence of the host when the toxin genes are expressed. In the case of *V. cholera*, phage encoded toxin can cause severe diarrhoea; in *C. botulinum*, the toxin can cause paralysis. During lysogeny, the prophage will persist in the host chromosome until induction, which results in the excision of the viral genome from the host chromosome. After induction has occurred the temperate phage can proceed through a lytic cycle and then undergo lysogeny in a newly infected cell.

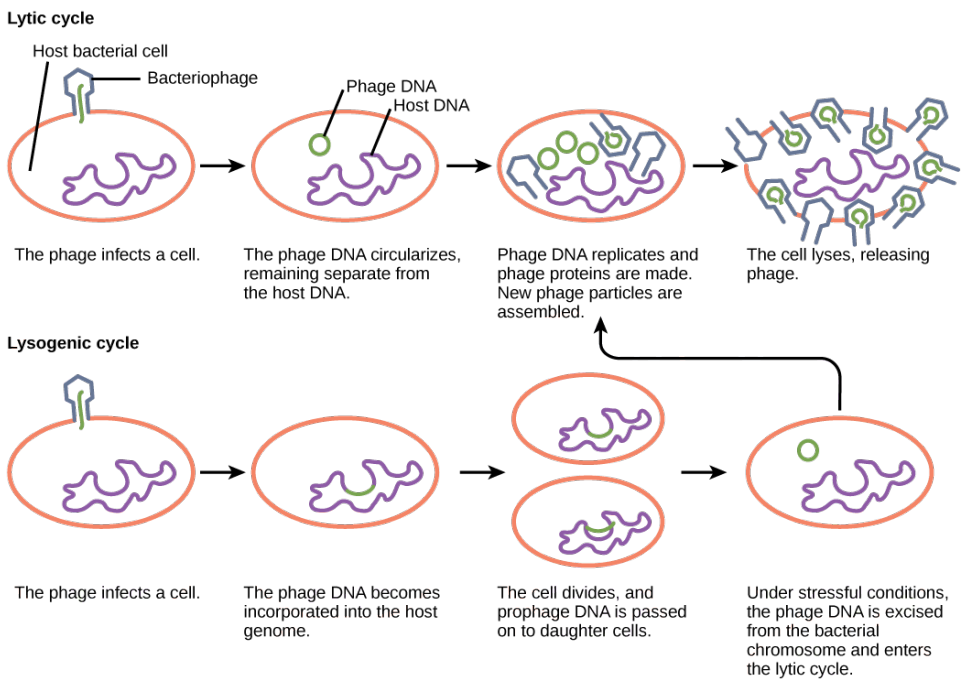


Figure 9. Scheme of both lytic and lysogenic cycles. In the lysogenic cycle, phage DNA is incorporated into the host genome, forming a prophage, which is passed on to subsequent generations of cells. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to be excised and enter the lytic cycle (*figure used with permission under Creative Commons license*)

Life Cycle of Viruses with Animal Hosts

Lytic animal viruses follow similar infection stages to bacteriophages: attachment, penetration, biosynthesis, maturation, and release. However, the mechanisms of penetration, nucleic-acid biosynthesis, and release differ between bacterial and animal viruses. After binding to host receptors, animal viruses enter through endocytosis (engulfment by the host cell) or through membrane fusion (viral envelope with the host cell membrane). Many viruses are host specific, meaning they only infect a certain type of host; and most viruses only infect certain types of cells within tissues. This specificity is called a tissue tropism. Examples of this are demonstrated by the poliovirus, which exhibits tropism for the tissues of the brain and spinal cord, or the influenza virus, which has a primary tropism for the respiratory tract.

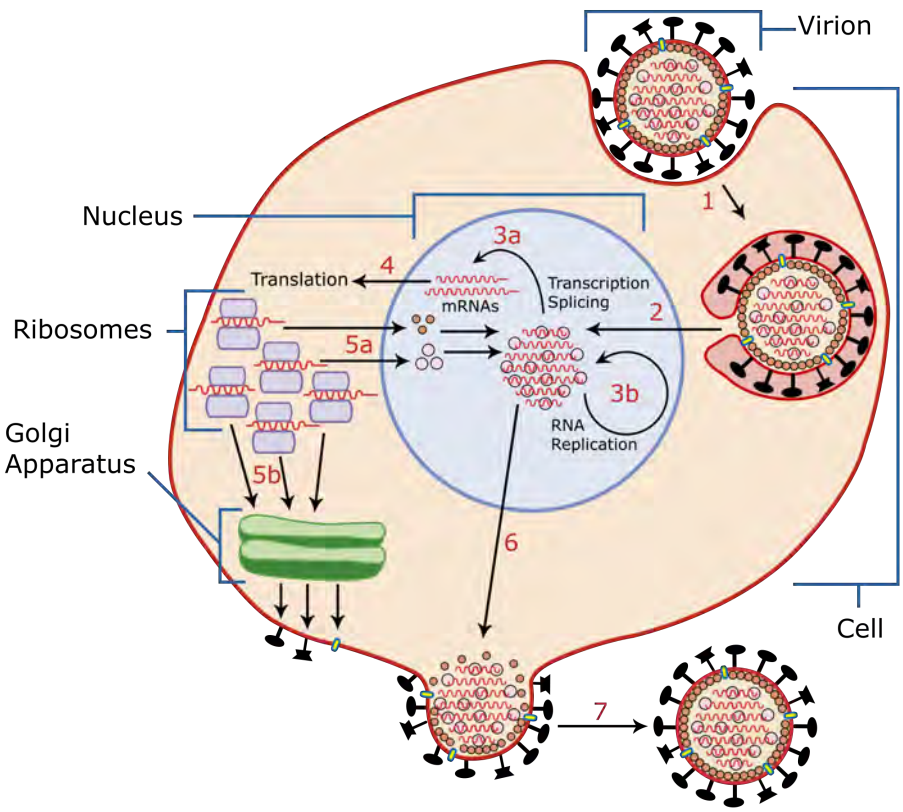


Figure 10. Virus cycle in animal cell. Viral glycoproteins attach the virus to a host epithelial cell. As a result, the virus is engulfed. Viral RNA and viral proteins are made and assembled into new virions that are released by budding (*figure used with permission under Creative Commons license*)

Animal viruses do not always express their genes using the normal flow of genetic information – from DNA to RNA to protein. Some viruses have a dsDNA genome like cellular organisms and can follow the normal flow. However, others may have ssDNA, dsRNA, or ssRNA genomes. The nature of the genome determines how the genome is replicated and expressed as viral proteins. If a genome is ssDNA, host enzymes will be used to synthesize a second strand that is complementary to the genome strand, thus producing dsDNA. The dsDNA can now be replicated, transcribed, and translated similar to host DNA.

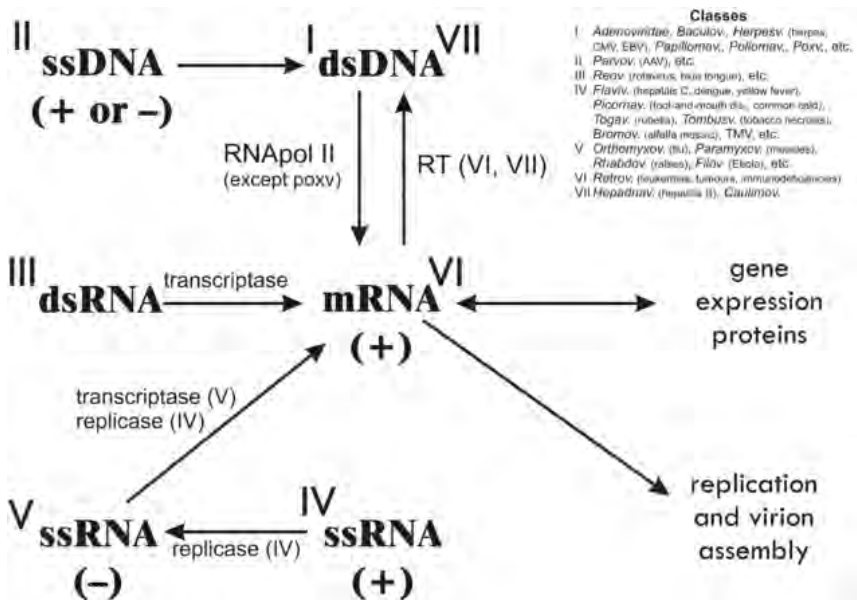


Figure 11. Animal virus classification (*figure used with permission under Creative Commons license*)

If the viral genome is RNA, a different mechanism must be used. There are three types of RNA genome: dsRNA, positive (+) single-strand (+ssRNA) or negative (–) single-strand RNA (–ssRNA). If a virus has a +ssRNA genome, it can be translated directly to make viral proteins. Viral genomic +ssRNA acts like cellular mRNA. However, if a virus contains a –ssRNA genome, the host ribosomes cannot translate it until the –ssRNA is replicated into +ssRNA by viral RNA-dependent RNA polymerase (RdRP). The RdRP is brought in by the virus and can be used to make +ssRNA from the original –ssRNA genome. The RdRP is also an important enzyme for the replication of dsRNA viruses, because it uses the negative strand of the double-stranded genome as a template to create +ssRNA. The newly synthesized +ssRNA copies can then be translated by cellular ribosomes.

An alternative mechanism for viral nucleic acid synthesis is observed in the retroviruses, which are +ssRNA viruses. Single-stranded RNA viruses such as HIV carry a special enzyme called reverse transcriptase within the capsid that synthesizes a complementary ssDNA (cDNA) copy using the +ssRNA genome as a template. The ssDNA is then made into dsDNA, which can integrate into the host chromosome and become a permanent part of the host. The integrated viral genome is called a provirus. The virus can now remain in the host for a long time to establish a chronic infection. The provirus stage is similar to the prophage stage in a bacterial infection during the lysogenic cycle. However, unlike prophage, the provirus does not undergo excision after splicing into the genome.

1.7. The action of physical and chemical agents on microorganisms

Sterilization is the killing or removal of all microorganisms, including bacterial spores which are highly resistant. Sterilization is an absolute term, i.e. the article must be sterile meaning the absence of all microorganisms.

Disinfection is the killing of many, but not all microorganisms. It is a process of reduction of number of contaminating organisms to a level that cannot cause infection, i.e. pathogens must be killed. Some organisms and bacterial spores may survive.

Disinfectants are chemicals that are used for disinfection. Disinfectants should be used only on inanimate objects.

Antiseptics are mild forms of disinfectants that are used externally on living tissues to kill microorganisms, e.g. on the surface of skin and mucous membranes.

Sterilization and disinfection are done by:

A. Physical Agents
    1. Heat
    2. Radiation
    3. Filtration
B. Chemical Agents

In practice, certain methods are placed under sterilization which in fact do not fulfil the definition of sterilization such as boiling for 1/2 h and pasteurization which will not kill spores.

1.7.1. Sterilization by heat

Heat is most effective and a rapid method of sterilization and disinfection. Excessive heat acts by coagulation of cell proteins. Less heat interferes metabolic reactions. Sterilization occurs by heating above 100°C which ensure lolling of bacterial spores. Sterilization by hot air in hot air oven and sterilization by autoclaving are the two most common method used in the laboratory.

Types of Heat treatment:
– Sterilization by moist heat;
– Sterilization by dry heat.

Sterilization by Moist Heat

Moist heat acts by denaturation and coagulation of protein, breakage of DNA strands, and loss of functional integrity of cell membrane. Boiling at 100°C for 30 minutes is done in a water bath. Syringes, rubber goods and surgical instruments may be sterilized by this method. All bacteria and certain spores are killed. It leads to disinfection.

I. Steaming

Steam (100°C) is more effective than dry heat at the same temperature as: (a) Bacteria are more susceptible to moist heat, (b) Steam has more penetrating power, and (c) Steam has more sterilizing power as more heat is given up during condensation. A steam Sterilizer works at 100°C under normal atmospheric pressure i.e. without extra pressure. It is ideally suitable for sterilizing media which may be damaged at a temperature higher than 100°C. It is a metallic vessel having 2 perforated diaphragms (Shelves), one above boiling water, and the other about 4 feet above the floor. Water is boiled by electricity, gas or stove. Steam passes up. There is a small opening on the roof of the instrument for the escape of steam. Sterilization is done by two methods:

Single Exposure for 11/2 hours. It leads to disinfection.

Tyndallization (Fractional Sterilization). Heat labile media like those containing sugar, milk, gelatine can be sterilized by this method. Steaming at 100°C is done in steam sterilizer for 20 minutes followed by incubation at 37°C overnight. This procedure is repeated for another 2 successive days. That is 'steaming' is done for 3 successive days. Spores, if any, germinate to vegetative bacteria during incubation and are destroyed during steaming on second and third day. It leads to sterilization.

II. Sterilization above 100°C

Autoclaving is one of the most common methods of sterilization. Principle: In this method sterilization is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. The temperature of boiling depends on the surrounding atmospheric pressure. A higher temperature of steaming is obtained by employing a higher pressure. When the autoclave is closed and made air-tight, and water starts boiling, the inside pressures increases and now the water boils above 100°C. At 15 ib per sq. inch pressure, 121°C temperatures is obtained. This is kept for 15 minutes for sterilization to kill spores. It works like a pressure cooker.

III. Sterilization below 100°C

1. Pasteurization.

Pasteurization is heating of milk to such temperature and for such a period of time so as to kill pathogenic bacteria that may be present in milk without changing colour, flavour and nutritive value of the milk. *Mycobacterium bovis*, *Salmonella species*, *Escherichia coli* and *Brucella* species may be present in milk. It does not sterilize the milk as many living organisms including spores are not destroyed.

Methods of Pasteurization:

- Flash Method. It is 'high temperature – short time method'. Heating is done at 72°C for 15 seconds.
- Holding Method. Heating is done between 63°C and 66°C for 30 minutes.

2. Inspissation. Inspissation is done between 75°C to 80°C. Inspissation means stiffening of protein without coagulation as the temperature is below coagulation temperature. Media containing serum or egg is sterilized by heating for 3 successive days. It is done in a Serum Inspissator machine.

Sterilization by Dry Heat

Dry heat at 160°C (holding temperature for one hour is required to kill the most resistant spores). The articles remain dry. It is unsuitable for clothing which may be spoiled.

1. Red Heat. Wire loops used in microbiology laboratory are sterilized by heating to 'red' in a Bunsen burner or spirit lamp flame. Temperature is above 100°C. It leads to sterilization.

2. Flaming. The article is passed through flame without allowing it to become red hot, e.g. scalpel. Temperature is not high to cause sterilization.

3. Sterilization by Hot Air. It is one of the most common method used for sterilization. Glass wares, swab sticks, all-glass syringes, powder and oily substances are sterilized in hot air oven. For sterilization, a temperature of 160°C is maintained (holding) for one hour. Spores are killed at this temperature. It leads to sterilization. Hot Air Oven is an apparatus with double metallic walls and a door. There is an air space between these walls. The apparatus is heated by electricity or gas at the bottom. On heating, the air at the bottom becomes hot and passes between the two walls from below upwards, and then passes in the inner chamber through the holes on Me top of the apparatus. A thermostat is fitted to maintain a constant temperature of 160°C.

1.7.2. Filtration

Many of the biological fluids (liquids) or gases that need to be sterilized cannot be done so by the application of heat. Their sterilization is achieved by filtration.

Filtration of Biological Fluids (Biological Filters)

When ingredients of a culture medium are thermolabile, i.e. easily destroyed by heat, the use of heat sterilization is not practicable. For instance, biological fluids such as solutions of antibiotics, vitamins, tissue extracts, animal serum, etc. come under this category. In such cases, however, the process of filtration is used. The filters suitable for the purpose are Seize filter (Asbestos filter), Chamberland-Pasteur filter (Porcelain filter), Berkefeld filter (Diatomaceous earth filter) and Membrane or Molecular filter. The first three filters are bacteriological filters, i.e. they allow liquid to pass but retain bacteria. Contrary to this, the membrane filters retain all forms of organisms whatever small they may be (even viruses). The mean pore diameter in these filters ranges from one to several micrometres. These filters do not merely serve the mechanical prevention but other factors such as electric charges of the filter, electric charge of the microorganisms, and the nature of the fluid being filtered.

Important biological filters

1. Seitz Filter (Asbestos filter)

This filter consists of 2–6 mm compressed asbestos fibre filter sheet. A variety of filter sheets containing different pore sizes are available in discs or squares ready for use and work satisfactorily only for a few hours. The medium to be filtered (sterilized) is poured into the funnel-like structure and drawn through filter sheet by vacuum. When the filtration is complete the filter sheet is discarded and the filtrate is obtained. A modified Seitz filter in which vacuum-drawn filtrate technique has been replaced by centrifugal technique is also used now-a-days where the filter is mounted on a centrifuge which forces the filtrate into the tube.

2. Chamberland-Pasteur Filter. (Porcelain Filter)

These filters consists of hollow unglazed cylinders having a short open end. The cylinders are composed of oxides of silicon, aluminium, potassium and sodium with traces of oxides of iron, calcium and magnesium (the mixture commonly called 'porcelain'). The cylinders are baked at a temperature as high as possible without sintering the porcelain. These filters are prepared to various degrees of porosity from 0.65 to 15 um and are used to remove bacteria and other coarse materials.

Membrane or Molecular Filter

A new type of filter called 'membrane' or 'molecular' filter has been developed in recent years. Unlike bacteriological filters which retain only bacteria, membrane filter retains all forms of microorganisms whatever their size be. These filters, are made up of biologically inert cellulose esters, and are prepared as

circular membranes of about 150 μm diameter consisting of millions of pores of an uniform and specifically predetermined size. Membrane filters were originally manufactured by the Millipore Filter Corporation (USA) and therefore they are also known as 'Millipore' or 'Ultra filters'. At the time of filtration, membrane filters of various porosity are used on the principle of 'graded filtration' and finally fluid free of all organisms larger than 10 μm is obtained.

1.7.3. Radiation

Radiation refers to the transmission of energy in a variety of forms through space or through a medium. The most effective type of radiation to sterilize or reduce the microbial burden in almost any substance is through the use of electromagnetic radiation. Various types of electromagnetic radiations are separated within the electromagnetic on the basis of their wavelengths. The radiations of shorter wavelengths are more damaging to microorganisms. Thus, two types of radiations of primary interest in sterilization are—electromagnetic waves and streams of minute particles. The electromagnetic waves in decreasing orders of wavelengths are infrared, ultra-violet light, X-rays and gamma rays, whereas the streams of minute particles of matter are alpha and beta radiation. However, the sterilization by electromagnetic radiation is commonly called 'cold sterilization' and is ideal for disposable materials made up of plastics, wool, cotton, etc., which can be sterilized using a high dose of irradiation without altering the material. For others, complete sterilization is difficult without causing changes in colour and flavour of the materials which occur at higher doses of radiations. Microbial cells possess various vital molecules, which are made up of atoms. An atom consists of a small nucleus surrounded by planetary electrons. When the electromagnetic rays and radiation particles pass through the matter, they give energy called radiant energy to the electrons of constituent atoms.

1.8. Bacteria as a potential tool in bioterrorism

Bacterial pathogens have been identified as agents that have been, or could be, used as weapons of biological warfare and/or biological terrorism. These agents are relatively easily obtained, prepared and dispersed, either as weapons of mass destruction or for more limited terrorist attacks.

According to the U.S. Centers for Disease Control and Prevention (CDC), bioterrorism is the deliberate release of viruses, bacteria, toxins or other harmful agents to cause illness or death in people, animals, or plants. These agents are typically found in nature, but could be mutated or altered to increase their ability to cause disease, make them resistant to current medicines, or to increase their ability to be spread into the environment. Biological agents can be spread

through the air, water, or in food. Terrorists tend to use biological agents because they are extremely difficult to detect and do not cause illness for several hours to several days. Some bioterrorism agents, like the smallpox virus, can be spread from person to person and some, like anthrax, cannot.

Bioterrorism is an attractive weapon because biological agents are relatively easy and inexpensive to obtain, can be easily disseminated, and can cause widespread fear and panic beyond the actual physical damage. Military leaders, however, have learned that as a military asset, bioterrorism has some important limitations; it is difficult to deploy a bioweapon in a way that only affects the enemy and not friendly forces. A biological weapon is useful to terrorists mainly as a method of creating mass panic and disruption to a state or country. However, technologists such as the American Bill Joy, co-founder of Sun Microsystems, have warned of the potential power that genetic engineering might place in the hands of future bio-terrorists. Setting up a laboratory in which bio-agents can be prepared does not require great financial or practical effort. The equipment and reagents for producing bio-agents are fully available on the civilian market (even from Internet shops), and are not always sold under the supervision of relevant security services. A properly prepared homemade laboratory can have the dimensions of a cargo container, or caravan. Additionally, the production of bio-agents can also be incorporated into the daily activity of analytical medical or scientific laboratories, as well as within the infrastructure of the cosmetic and pharmaceutical industries. Production costs for bio-agents are exceptionally low, and for this reason they are known as the 'Arms of the poor'. Calculations performed by The United Nations show that the financial cost of causing 1 km^2 of human casualties using conventional arms is about USD 2,000. For nuclear weapons it's USD 800; chemical weapons USD 600, and for biological weapons just USD 2. Bioweapons are easy to hide, and in comparison to chemical weapons, are colourless and odourless. In addition, actual attacks can initially appear to be untypical of mass terror actions, with the first symptoms of an attack even occurring sometime after their deployment. For these reasons it can be difficult to find the source of the attack. Finally, biological agents remain relatively stable after dissemination, at least for periods long enough to infect humans.

The use of agents that do not cause harm to humans but disrupt the economy has been discussed. A highly relevant pathogen in this context is foot-and-mouth disease (FMD), which affects cloven-hoofed animals is capable of causing widespread economic damage and public concern (as witnessed in the 2001 and 2007 FMD outbreaks in the UK), whilst having almost no capacity to infect humans.

The CDC defines bioterrorism as "deliberate release of viruses, bacteria or other germs (agents) used to cause illness or death in people, animals, or plants." The Biological Weapons Convention (BWC), opened for signature in 1972, was the first disarmament treaty, which banned development, production, and storage of this entire category of weapons of mass destruction.

1.8.1. The CDC categories

The CDC maintains a list of potential critical biological agents, including those usable in bioweapons. These are classified into three categories – A, B and C – based on their ease of transmission, morbidity and mortality rates, and likelihood of actually being used.

Category A

Anthrax

Anthrax is a non-contagious zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*. *Bacillus anthracis* is a gram-positive, rod-shaped bacterium, and one of the most likely agents to be used in a biological attack. Bacillus anthracis, the only obligate pathogen within the genus bacillus, has a large size at 1.0–1.5 μm width and 3–10 μm length. *B. anthracis* is an aerobic bacterium, meaning that in anaerobic conditions it is able to survive only a few days. However, its spores are highly resistant to adverse environmental conditions including heat, both ultraviolet and ionizing radiation, high pressure and chemical agents. It has been established that spores are able to survive in soil for up to 40 years.

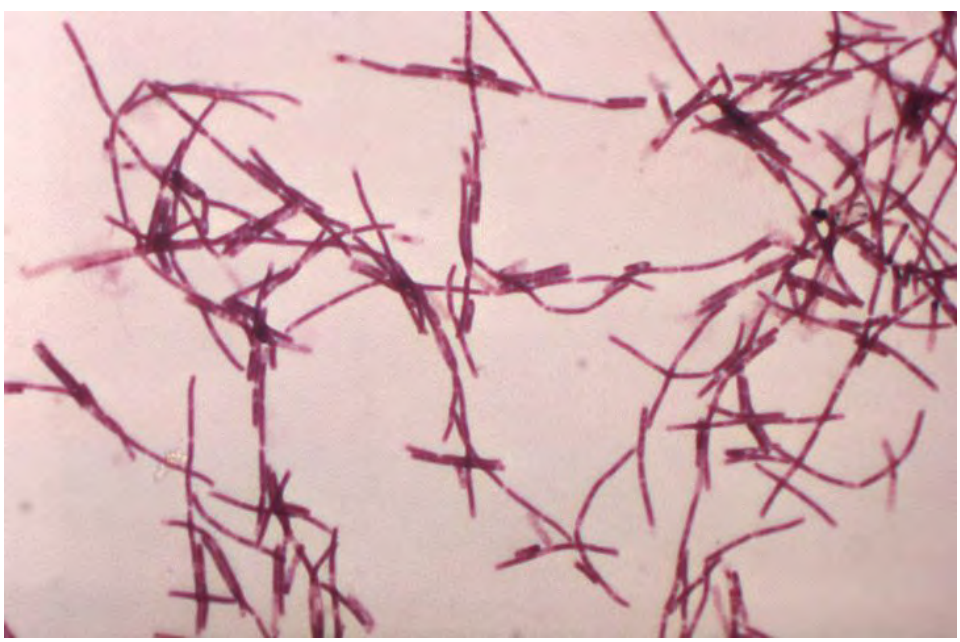


Figure 12. *Bacillus anthracis* (*figure used with permission under Creative Commons license*)

Anthrax takes three different forms: cutaneous anthrax, which is acquired through cuts and lesions in the skin; gastrointestinal anthrax, and pulmonary (inhalation) anthrax. The most dangerous and potentially useful bioterror form is pulmonary anthrax. It is caused by alveolar deposits of Anthrax spores, which are less than 5 mm in size. This mainly affects the lungs but can also be accompanied by meningitis. Inhaled spores are phagocytosed by macrophages and carried to local mediastinal lymph nodes, wherein it is sufficient to absorb 8,000 to 50,000 spores of *B. anthracis* to induce the disease. The spores then germinate into vegetative forms, replicate, and produce haemorrhagic mediastinitis. General symptoms include fever, coughing and chest pains about 12–24 hours after infection. There then follows a sudden increase in shortness of breath, swelling of the neck area and the appearance of the first signs of sepsis. Next comes necrosis of the lymph nodes, pulmonary oedema and a characteristic mediastinal widening, with fluid in the pleural cavity. In the absence of treatment, mortality is 97% and death usually occurs 3 days after the onset of symptoms. The only way to cure anthrax is to administer the antibiotics (such as ciprofloxacin) within 12 hours of infection. However, in this short period the symptoms of the disease may not yet even present. The pulmonary form of anthrax, even if treated with antibiotic therapy, has a 75% mortality rate. An anthrax vaccine does exist but requires many injections for stable use. In the US there is an anthrax vaccine, Anthrax Vaccine Adsorbed (AVA), which requires five injections for a stable result.

The first modern incidence of anthrax use in biological warfare occurred in 1916 when Scandinavian 'freedom fighters', supplied by the German General Staff, used anthrax (with unknown results), against the Imperial Russian Army in Finland. In 1993, the Japanese Aum Shinrikyo cult deployed anthrax in an unsuccessful attack in Tokyo, which had zero fatalities. Anthrax was again used in a series of attacks on the offices of several United States Senators in late 2001. This anthrax was sent in a powder form and delivered by the US mail, infecting 22 people, including 12 postmen, and killing five. Additionally, there was one documented accidental use in Sverdlovsk, Russia, in 1979, when approximately 1 or 2 grams of spores in aerosol form leaked into the atmosphere. The wind then carried them into the (fortunately) sparsely-populated suburbs of the town, killing 68 of 79 infected people.

There is no doubt that *Bacillus anthracis* is one of the most dangerous pathogens with potential use in bioterrorism. Its greatest advantages are that it is easy to acquire natural strains, as well as its ease of handling and low associated costs. Production of 1kg of spores can cost just USD 50. Based on scientific calculations by the World Health Organization, release of 50 kg of dried anthrax powder by aerosolization for two hours in a city of 500,000 inhabitants would cause 95,000 deaths and incapacitation of 125,000 individuals. The strain on medical resources would be tremendous, leading to a need for bed space

for 12,500 individuals (10% of those incapacitated), 60-day antibiotic courses for 125,000, and the disposal of 95,000 corpses. This would almost certainly lead to a rapid total breakdown in medical resources and civilian infrastructure.

Tularemia or 'rabbit fever'

Tularemia has a very low fatality rate if treated, but it can severely incapacitate those infected. The disease is caused by the *Francisella tularensis* bacterium, contracted through contact with fur, inhalation, ingestion of contaminated water or insect bites. This pathogen is a gram-negative, rod-shaped coccobacillus. *Francisella tularensis* is highly infectious and requires only a small number of organisms (about 10–50) to cause disease. However, in comparison to other potential bio-agents it is very sensitive to chemicals and high temperatures. Even the sun's rays can kill the bacteria within 30 minutes. But at low temperatures it exhibits considerable resistance, and can last up to 3 years.

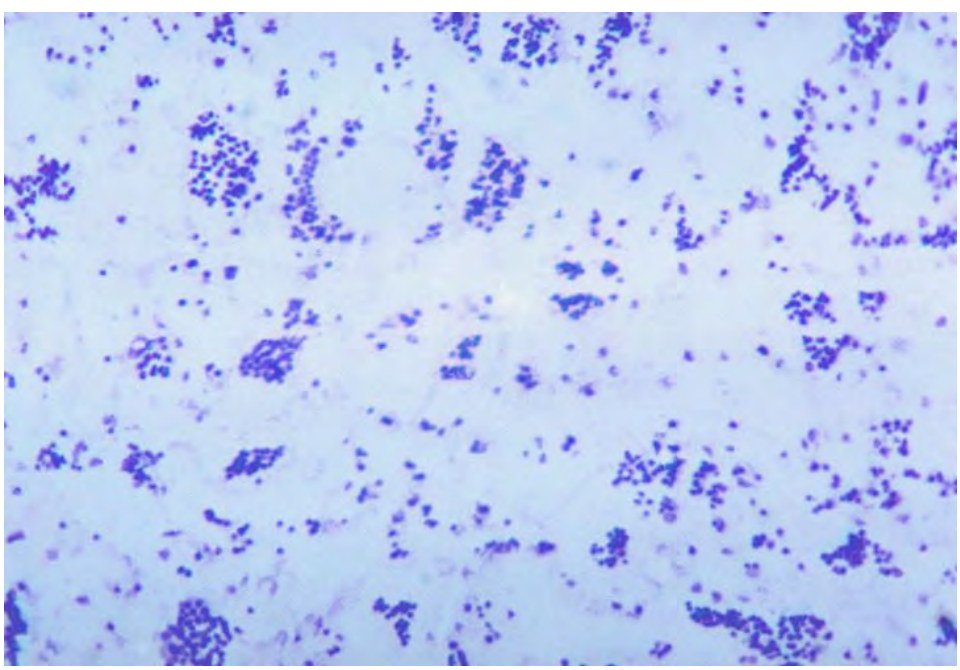


Figure 13. *Francisella tularensis* (*figure used with permission under Creative Commons license*)

F. tularensis has been classified as a Tier 1 Select Agent by the U.S. government, along with other potential agents of bioterrorism such as *Yersinia pestis*, *Bacillus anthracis* and the Ebola virus. The bacteria that cause tularemia occur widely in nature and can be isolated and grown in quantity in a laboratory,

although manufacturing an effective aerosol weapon to deploy them would require considerable sophistication.

There are several types of tularemia. The most common is transmitted *via* skin contact or bites by infected ticks, and this is ulceroglandular tularemia. However, if *F. tularensis* is used as a weapon, the bacteria would likely be made airborne for exposure by inhalation. People who inhale it as an infectious aerosol would generally experience severe respiratory illness, including life-threatening pneumonia and systemic infection. The pneumonic form is the most potentially lethal form of tularemia, and is caused by inhalation of the bacteria. Besides the general symptoms, patients with the pneumonic form also experience chest pain, bloody sputum and can have trouble breathing – and even sometimes stop breathing. The mortality rate of this form is about 30%.

WHO simulations have shown that bioterrorism using *F. tularensis* would be very effective. Dispersion of 50 kg of bacteria in a city of 5,000,000 inhabitants would cause the deaths of 19,000 people and infection of 250,000 people. In simulations run by Kaufmann et al., the financial costs of infecting 100,000 people ran to USD 5.4 billion.

Smallpox

Smallpox was a severe human disease caused by the variola virus (VARV), which was both highly lethal and highly contagious. VARV is a member of the genus Orthopoxvirus. A characteristic feature of this virus is its strict specificity to humans. It is transmitted easily through the atmosphere and has a high mortality rate (20–40%). It was once one of the most devastating diseases known to mankind. The first symptoms of smallpox usually appear 10 to 14 days after infection. During the incubation period of 7 to 17 days, the infected looks and feels healthy and is themselves non-infectious. Smallpox generally begins with fever, headaches, body aches, and weakness on day 1. Then in days 2–3 small, round pox (blisters) appear and spread on the face, arms, legs, and inside the mouth. By day 7 the pox turns into bigger blisters that fill with pus. On day 12 the blisters crust over; stomach pain and confusion can also occur. By week 3–4 the blisters have turned into scabs and fall off, leaving pitted scars on the skin. There is no proven treatment for smallpox once the rash appears, but research is underway to find an effective anti-viral medication. Those who are ill with smallpox can benefit from supportive care in a hospital setting. People who recover from smallpox usually have severe scars, especially on the face, arms and legs. In some cases, smallpox can also cause blindness.

The virus can be transmitted in several ways, including directly, from person to person. Direct transmission of the virus requires fairly prolonged face-to-face contact. The virus can also be transmitted through the air by droplets that escape when an infected person coughs, sneezes or speaks. Indirectly from an infected

person. In rare instances, the airborne virus can spread farther, possibly through the ventilation system in a building, infecting people in other rooms or on other floors. Smallpox can also infect through contaminated items, including contaminated clothing and bedding, although the risk of infection from these sources is less common. Historically, people have received smallpox by touching or inhaling the smallpox virus. Smallpox is not spread by insects or animals, and there is no naturally-occurring smallpox as it was eradicated in the world in the 1970s, thanks to a global vaccination program. However, some virus samples are still available in Russian and American laboratories. Some believe that after the collapse of the Soviet Union, cultures of smallpox have become available in other countries. Although people born pre-1970 will have been vaccinated for smallpox under the WHO program, the effectiveness of vaccination is limited as the vaccine provides a high level of immunity for only 3 to 5 years. Revaccination protection lasts longer. So as a biological weapon, smallpox is dangerous because of the highly contagious nature of both the infected and their pox. Also, the infrequency with which vaccines are administered among the general population since the eradication of the disease generally would leave most people unprotected in the event of an outbreak.

In the case of terrorist use in a public setting, the virus would be spread by:

– Breathing in the virus from a cough, sneeze, or saliva (spit) of a smallpox carrier;

– Touching skin that has smallpox blisters;

– Touching contaminated body fluids or objects, such as bedding or clothing;

– Breathing in the virus in a room, bus, or train shared with someone who has smallpox;

– Sharing a razor, tableware or toothbrush with someone who has smallpox.

Even if all the stocks of naturally-occurring, lab-stored smallpox virus are destroyed, it is now possible to genetically engineer a similar viral agent in a laboratory setting. This capability requires that the medical and public health communities maintain smallpox preparedness into the foreseeable future.

Plague

Plague is a disease caused by the *Yersinia pestis* bacterium. This bacteria is a Gram-negative, rod-shaped coccobacillus, a facultative anaerobic organism which does not produce spore forms, but has very high vitality. It can survive up to 6 months within the bodies of dead animals, in water for less than a month, and for a few days in dry conditions. *Y. pestis* is maintained in nature as a zoonotic infection in rodent hosts and their fleas in large areas of Asia, Africa and the Americas. Rodents are the normal host of plague, and the disease is transmitted to humans by flea bites and occasionally by aerosol in the form of pneumonic plague. This was the disease that caused the 'Black Death' in Medieval Europe.

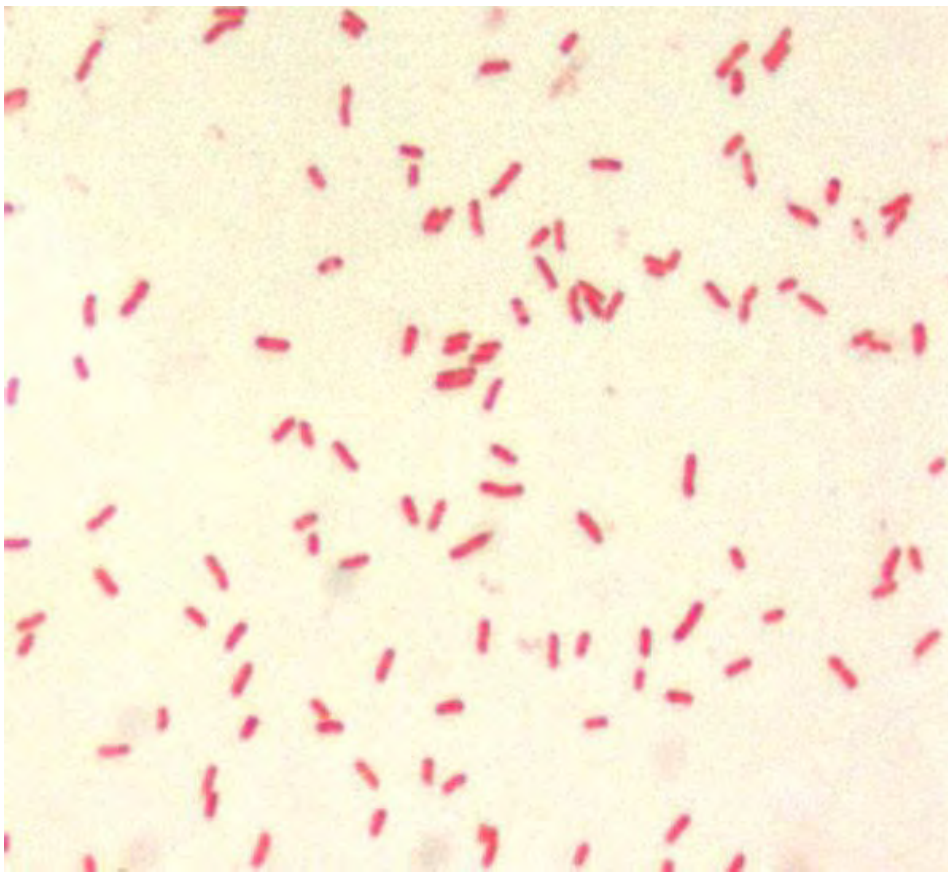


Figure 14. *Yersinia pestis* (*figure used with permission under Creative Commons license*)

There are three clinical forms of this disease: classical bubonic plague; primary septicaemic plague, and pneumonic plague, determined largely by the way the pathogen enters the body. The weaponized threat comes mainly in the form of pneumonic plague (infection by inhalation). The pneumonic plague occurs when *Y. pestis* infects the lungs. Pneumonic plague can spread from person to person through the air. Transmission occurs when someone breathes in aerosolized bacteria, which could happen during a bioterrorism incident. The most obvious symptom of pneumonic plague is coughing, often accompanied by haemoptysis (coughing up blood). With pneumonic plague, the first signs of illness are fever, headache, weakness and rapidly-developing pneumonia, characterised by shortness of breath, chest pains, coughing and sometimes bloody or watery sputum. The pneumonia plague progresses for two to four days and can cause respiratory failure and shock. This is a very aggressive infection

requiring early antibiotic treatment (within 24 hours of presentation of the first symptoms, in order to reduce the risk of death). Without therapy, the mortality rate approaches 100%.

The disease has a history of use in biological warfare dating back many centuries. This pathogen has great potential as a very useful bio-agent. It is naturally occurring and can be isolated and grown in bulk in a laboratory. Because of the delay between being exposed to the bacteria and becoming sick, carriers can travel over large areas before becoming contagious and possibly infecting others. World Health Organisation simulations has shown that bioterrorism using *Y. pestis* would be very effective. Dispersion of 50 kg of bacteria in a city of 5,000,000 inhabitants would causes 36,000 deaths, and plague infection of 150,000. Y. pestis can also be used to contaminate soil, food and water. Antibiotic-resistant strains of the bacteria, isolated in the 2014 outbreak in Madagascar, are very dangerous.

Viral haemorrhagic fevers

This includes haemorrhagic fevers caused by members of the Filoviridae family (Marburg virus and Ebola virus), and by the Arenaviridae family (for example, Lassa virus and Machupo virus). There are all families of negative-stranded and lipid-enveloped ribonucleic acid (RNA) viruses. The Ebola virus in particular has caused high fatality rates, ranging from 25–90%, with a 50% average. Ebola is one of the classic zoonotic diseases, with index transmission occurring from animals to human hosts. The Ebola virus is also transmitted through contact with the full range of bodily fluids of infected individuals (blood, urine, saliva, sweat, faeces, vomit, breast milk and semen). Ebola viruses enter the human body *via* mucosal surfaces, abrasions and lesions in the skin. The incubation period for Ebola ranges from 2 to 21 days, normally occurring after 4–10 days. The first signs are flu-like symptoms (fever, myalgia, chills), vomiting and diarrhoea. The disease can rapidly evolve into a severe state with a rapid clinical decline. Lethal Ebola cases generally succumb between days 6 and 16 from the onset of symptoms. Death from Ebola is commonly due to multiple organ failure and hypovolemic shock. No cure currently exists, although vaccines are being developed. The Ebola virus is useful as a bio-agents thanks to its high virulence. Studies performed on rhesus monkeys have shown that a low dose of virus introduced into the body in aerosol form rapidly leads to an almost 100% death rate. Additionally, the first symptoms are not typical of serious diseases. This significantly delays the detection of infection, and consequently initiation of treatment and the possibility of the outbreak spreading further. The Soviet Union investigated the use of filoviruses in biological warfare, and the Japanese Aum Shinrikyo cult unsuccessfully attempted to obtain cultures of Ebola virus.

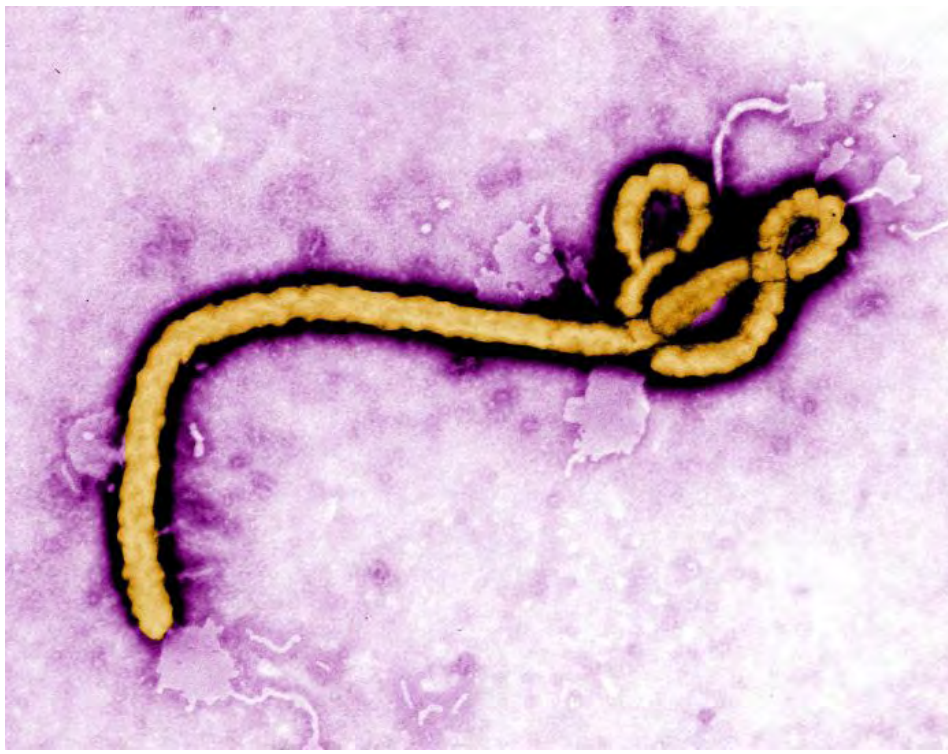


Figure 15. Ebola virus (*figure used with permission under Creative Commons license*)

Marburg virus (MV) was first discovered in Marburg, Germany. The source of infection was traced back to African green monkeys (*Chlorocebus aethiops*) that had been imported from Uganda. During the initial outbreak, 31 people became infected, seven of whom died. To date there have been a total of 452 cases and 368 documented deaths due to Marburg virus. Initial contraction of the virus comes *via* exposure to infected animals: either a reservoir host (several bat species) or a spill-over host such as NHPs as described in the first MV outbreak. MV has an incubation period ranging from 3 to 21 days (typically 5 to 10 days), which is likely modulated by factors such as infectious dose and possibly by route of infection. The onset of illness begins with generic flu-like symptoms; a characteristic high fever, severe headache, chills, myalgia, prostration and malaise. For many patients (50–75%) this is followed by rapid debilitation characterized by gastrointestinal symptoms including anorexia, abdominal pain, severe nausea, vomiting, and watery diarrhoea. Many of the initial symptoms may persist in the early organ phase, and patients may sustain a high fever. They may additionally display neurological symptoms including encephalitis, confusion, delirium, irritability, and aggression. Patients can also develop dyspnea and abnormal vascular permeability, particularly

conjunctival injection and oedema. During the latter part of this phase, more than 75% of patients present with some form of clear haemorrhagic manifestation such as petechiae, mucosal bleeding, melena, bloody diarrhoea, hematemesis, and ecchymoses. Fatalities typically occur 8-16 days following the onset of symptoms, with death usually resulting from shock and multi-organ failure. No treatments currently exist, aside from supportive care.

The arenaviruses have a somewhat reduced case-fatality rate compared to the filoviruses, but are more widely distributed, chiefly in central Africa and South America. Lassa, Machupo, and Lujo viruses are all associated with secondary person-to-person and nosocomial (healthcare setting) transmission. This occurs when a person infected by exposure to the virus from the rodent host spreads the virus to other humans. This can occur in a variety of ways. Person-to-person transmission is associated with direct contact with the blood or other bodily fluids containing the virus particles of infected individuals. Airborne transmission has also been reported in connection with certain viruses, as has contact with objects contaminated by these materials, such as medical equipment.

Category B

Category B agents are moderately easy to disseminate and have low mortality rates.

– Brucellosis (*Brucellaspecies*);
– Food safety threats (for example, *Salmonella* species, *E. coli* O157:H7, *Shigella*, *Staphylococcus aureus*);
– Glanders (*Burkholderia mallei*);
– Melioidosis (*Burkholderia pseudomallei*);
– Psittacosis (*Chlamydia psittaci*);
– Q fever (*Coxiella burnetii*);
– Typhus (*Rickettsia prowazekii*);
– Viral encephalitis (alphaviruses, for example, Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis);
– Water supply threats (for example, *Vibrio cholerae*, *Cryptosporidium parvum*).

Category C

Category C of the highest-priority bio-agents includes emerging pathogens that could be engineered for mass dissemination. These agents are readily available, easy to produce and disseminate, and have potentially high morbidity and mortality rates, as well as a major impact on public health. The main bio-agents in this category include:

– Nipahvirus;

- Hantavirus;
- SARS;
- H1N1 (a strain of influenza);
- HIV/AIDS;
- Multi-drug-resistant tuberculosis;
- Tick-borne haemorrhagic fever viruses;
- Tick-borne encephalitis virus;
- Yellow fever virus.

Hantavirus

This bunyavirus infection is transmitted to humans from rodents and causes either a haemorrhagic fever with renal failure or hantavirus pulmonary syndrome. The disease occurs widely throughout the world. Person-to-person spread does not appear to take place. The incubation period is 2–3 weeks, followed by fever, headache, backache and injected conjunctiva and palate. Hypotension, shock and oliguric renal failure follow. The mortality rate is about 5%.

Nipah and Hendra virus

Nipah virus, a paramyxovirus, causes severe disease in humans and animals. It is found in South Asia and causes febrile encephalitis with a high mortality rate. The reservoir is probably fruit bats, with human infection from contact with bats or an intermediate animal host such as pigs. Person-to-person spread occurs. The related, rarer Hendra virus is also acquired from bats and causes an influenza-like syndrome or encephalitis.

Influenza viruses

Influenza virus is an enveloped orthomyxovirus (100 nm) that contains a negative single-stranded RNA genome divided into eight segments. This structure facilitates genetic re-assortment, which allows the virus to change its surface antigens and the influenza virus will take up genetic material from avian and pig influenza strains. The virus expresses seven proteins, three of which are responsible for RNA transcription. The nucleoprotein has three antigenic types that designate the three main virus groups, influenza A, B and C. Of the three types, influenza A and, more rarely, influenza B undergo genetic shift. The matrix protein forms a shell under the lipid envelope with haemagglutinin and neuraminidase proteins expressed as 10-nm spikes on the envelope, which interact with host cells. Virus immunity is directed against the haemagglutinin (H) and neuraminidase (N) antigens.

Yellow fever

Yellow fever virus is a flavivirus, an enveloped positive-sense RNA virus, transmitted by Aedes aegypti. Yellow fever is a zoonosis in which humans are an accidental host (sylvatic disease), but an urban cycle results in periodic human epidemics.

Human Immunodeficiency Virus

HIV is a spherical, enveloped RNA virus. It is a retrovirus, using reverse transcriptase to produce a DNA copy from viral RNA that is incorporated into the host nucleus to become the template for further viral RNA. Three genes are required for viral replication: gag, pol and env. HIV is classified as a lentivirus. There are two types that are pathogenic for humans: HIV-1, which is most common; and HIV-2, which is found mainly in West Africa and appears to be less virulent.

Infection with HIV has spread worldwide, transmitted by the parenteral and sexual routes. Infection is most common in individuals at high risk of sexually transmitted diseases, especially those where genital ulceration is common. In developed countries, the main risk groups are intravenous drug users and men who have sex with men; heterosexual transmission is less common but does occur. In developing countries, HIV spreads mainly by heterosexual transmission and through unscreened transfusions or use of contaminated medical equipment. Infection can be transmitted from mother to foetus.

The virus principally infects cells with a CD4 receptor (e.g. T cells and macrophages). Viral replication results in progressive T-cell depletion and diminished cell-mediated immunity. Different virus strains display varying affinities for cells that express particular chemokine receptors. Lacking T-cell help, B-cell function is also reduced. HIV causes damage to neural cells and stimulates cytokine release that may also cause neurological damage. Many of the clinical signs of AIDS are caused by secondary infections, which occur when the CD4 count falls.

Multi-drug resistance (MDR) pathogens

One of the biggest modern public health issues that could be exploited by bioterrorists is the resistance of pathogens to antibiotic therapy. The resistance of various pathogens to different antimicrobial drugs has emerged as a threat to public health all over the world at a terrifying rate. Almost all infectious agents (bacteria, fungi, viruses and parasites etc.), have developed high levels of multi-drug resistance (MDR) with enhanced morbidity and mortality. These are what are known as 'super bugs'. Antibiotic-resistant pathogens possess great potential

for use in bioterrorism attacks and have a high mortality rate. This is the single most common source of hospital infections, and in 2007 it was estimated that 94,360 people in the U.S. were affected, 18,650 of whom died.

1.8.2. Genetic modificated pathogens

The new era of bio-terrorism is also part of the development of new, simpler methods of genetic engineering. Some pathogens can also be subjected to special proliferation procedures, followed by genetic manipulation, thereby exacerbating their virulence. Existing bioengineering techniques can be used to improve existing non-pathogenic biological agents, making them excellent biological weapons. These technologies allow genetic material to be added, removed, or altered at particular locations in the genome. One example of this type of technique is CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats). CRISPR-Cas9 is a unique technology that enables geneticists and medical researchers to edit parts of the genome by removing, adding or altering sections of the DNA sequence.

CRISPR-Cas9 is a unique technology that enables to edit parts of the genome by removing, adding or altering sections of the DNA sequence. It is currently the simplest, most versatile and precise method of genetic manipulation and is therefore causing a buzz in the science world. The CRISPR-Cas9 system consists of two key molecules that introduce a change into the DNA. The first is enzyme Cas9, which acts as a pair of 'molecular scissors' that can cut the two strands of DNA at a specific location in the genome, so that bits of DNA can then be added or removed. The next part of this genetic engineering system is a small molecule of RNA (about 20 bases long), located within a longer RNA scaffold. The scaffold part binds to DNA and the pre-designed sequence 'guides' the Cas9 to the right part of the genome. This makes sure that the Cas9 enzyme cuts at the right point in the genome.

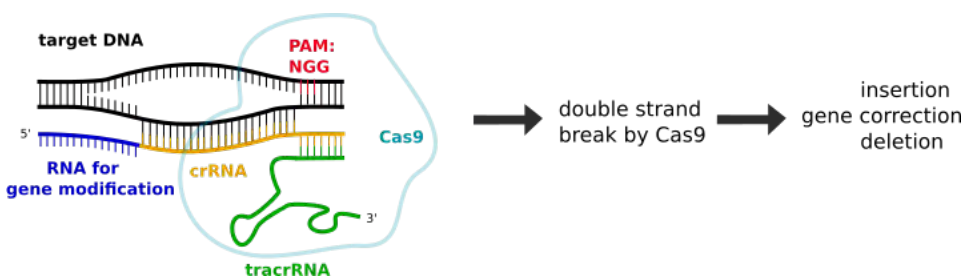


Figure 16. CRISPR-Cas9 mechanism (*figure used with permission under Creative Commons license*)

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