TVET CERTIFICATE IV in Animal Health

MICRO ORGANISMS IDENTIFICATION

ANHMO401

Perform micro-organisms identification

Competence

Credits: 10

Learning hours: 100

Sector: Agriculture and Food processing

Sub-sector: Animal Health

Module Note Issue date: September, 2020

Purpose statement

This general module describes the performance outcomes, skills, knowledge and attitude required by the learner to identify micro-organisms. For the veterinary assistant involved in both veterinary and farming practices, this competence is very important to enable him to perform better his professional work. It is a prerequisite for all the specific modules of the qualification involving the manipulation and treatment of the different animal species. Upon completion of this module, the trainee will be able to:

- Classify different types of micro-organisms;
- Select material and equipment;
- Perform different methods of identification

Table of Contents

LU & PC is linked in LO inside the content

Total Number of Pages: 91

List of tables

List of figure

List of pictures

Learning Unit 1 – Classify different types of micro-organisms

LO 1.1 – Identify different types of micro-organisms

- Topic 1: Different types of microorganisms (structure)

A. Bacteria

A.1. General Structures

Cell wall: Thin, rigid cellulose covering that encloses the protoplasm of the cell and gives rigidity to the bacterial shape.

Cytoplasmic membrane: A semipermeable membrane which is located directly beneath the cell wall, and which governs osmotic activity.

Cytoplasm: The protoplasmic or vital colloidal material of a cell exclusive of the nucleus.

Nucleus: Diffused chromatin material responsible for replication of the cell. The bacterial cell does not have a nuclear membrane nor a well-defined nucleus.

General Structures **Special Structures**

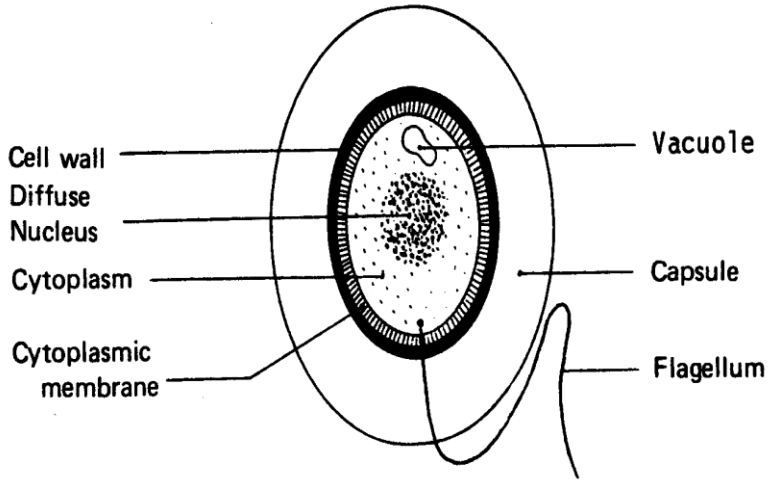


Figure 1: General and special structures of bacteria

A.2. Special Structures

Capsule: An accumulation of high-molecular-weight, excretory substances (slime layer) around a bacterium or bacteria. A capsule serves as a defense mechanism against phagocytosis by white blood cells and penetration by viruses.

Flagellum: A protoplasmic strand of elastic protein originating in the cytoplasm and extending from the body of the cell. A flagellum serves as an organ of locomotion. The arrangement of flagella (plural) is peculiar to the species.

Inclusion bodies (Vacuoles): Vacuoles of reserve or waste materials contained within the cytoplasm.

A.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 classification schemes most commonly used by clinicians and clinical microbiologists are discussed below.

Scientists interested in the evolution of microorganisms are more interested in taxonomic techniques that allow for the comparison of highly conserved genes among different species. As a result of these comparisons a phylogenetic tree can be developed that displays the degree of relatedness of different organisms. A relatively new application of this technology has been the recognition and characterization of no cultivatable pathogens and the diseases that they cause.

Criteria of classification of bacteria

Bacteria may be classified following different criteria such as:

- **Gram stain and bacterial morphology:** Of all the different classification systems, the Gram stain has withstood the test of time. Discovered by H.C. Gram in 1884 it remains an important and useful technique to this day. It allows a large proportion of clinically important bacteria to be classified as either **Gram positive or negative** based on their morphology and differential staining properties.

  Some bacteria such as mycobacteria (the cause of tuberculosis) are not reliably stained due to the large lipid content of the peptidoglycan. Alternative staining techniques are therefore used that take advantage of the resistance to distaining after lengthier initial staining.

- **Growth Requirements:** Microorganisms can be grouped on the basis of their need for oxygen to grow.
  - Facultatively 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 Clostridium 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 (e.g., the cause of gonorrhea) are examples of Microaerophilic bacteria.

- **Biochemical reactions:** Clinical microbiology laboratories typically will identify a pathogen in a clinical sample, purify the microorganism by plating a single colony of the microorganism on a separate plate, and then perform a series of biochemical studies that will identify the bacterial species.

- **Serologic systems:** Selected antisera can be used to classify different bacterial species. This may be based on either carbohydrate or protein antigens from the bacterial cell wall or the capsular polysaccharide. (Group A streptococcal M proteins or O and H polysaccharide antigens of salmonella).

- **Environmental Reservoirs:** When considering likely pathogens it is also important to know which of the different species are found in different locations. Environmental reservoirs are generally divided into those that are endogenous (*i.e.,* on or within the animal body) and exogenous (somewhere in the environment). When considering the likely cause of an infection the likely source of the infection is important in your differential diagnosis.
- **Form or shape:** Bacteria are classified into five groups according to their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes). They can exist as single cells, in pairs, chains or clusters

B. Virus

B.1. Structure of virus

Viruses are small obligate intracellular parasites, which by definition contain either a RNA or DNA genome surrounded by a protective, virus-coded protein coat. Viruses may be viewed as mobile genetic elements, most probably of cellular origin and characterized by a long co-evolution of virus and host. For propagation viruses depend on specialized host cells supplying the complex metabolic and biosynthetic machinery of eukaryotic or prokaryotic cells. A complete virus particle is called a virion.

The main function of the virion is to deliver its DNA or RNA genome into the host cell so that the genome can be expressed (transcribed and translated) by the host cell. The viral genome, often with associated basic proteins, is packaged inside a symmetric protein capsid. The nucleic acid-associated protein, called nucleoprotein, together with the genome, forms the nucleocapsid. In enveloped viruses, the nucleocapsid is surrounded by a lipid bilayer derived from the modified host cell membrane and studded with an outer layer of virus envelope glycoproteins.

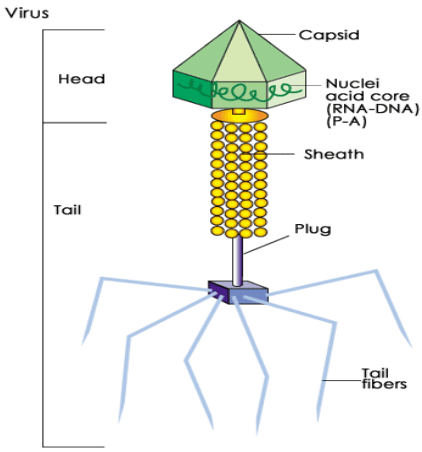


Figure 2 General structure of virus

B.2. Classification of Virus

Viruses are classified on the basis of morphology, chemical composition, and mode of replication.

Morphology

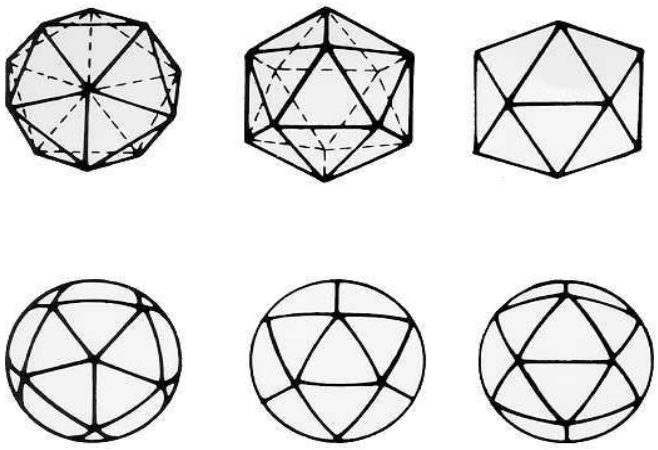
Helical Symmetry: In the replication of viruses with helical symmetry, identical protein subunits (protomers) self-assemble into a helical array surrounding the nucleic acid, which follows a similar spiral path. Such nucleocapsids form rigid, highly elongated rods or flexible filaments; in either case, details of the capsid structure are often discernible by electron microscopy.

In addition to classification as flexible or rigid and as naked or enveloped, helical nucleocapsids are characterized by length, width, pitch of the helix, and number of protomers per helical turn.

The most extensively studied helical virus is tobacco mosaic virus given above. Many important structural features of this plant virus have been detected by x-ray diffraction studies.

Icosahedral Symmetry: An icosahedron is a polyhedron having 20 equilateral triangular faces and 12 vertices. Lines through opposite vertices define axes of fivefold rotational symmetry: all structural features of the polyhedron repeat five times within each 360° of rotation about any of the fivefold axes. Lines through the centers of opposite triangular faces form axes of threefold rotational symmetry; twofold rotational symmetry axes are formed by lines through midpoints of opposite edges. An icosaheron (polyhedral or spherical) with fivefold, threefold, and twofold axes of rotational symmetry is defined as having 532 symmetry (read as 5, 3, 2).

Icosahedral models seen, left to right, on fivefold, threefold, and twofold axes of rotational symmetry



These axes are perpendicular to the plane of the page and pass through the centers of each figure. Both polyhedral (upper) and spherical (lower) forms are represented by different virus families.

Chemical Composition and Mode of Replication

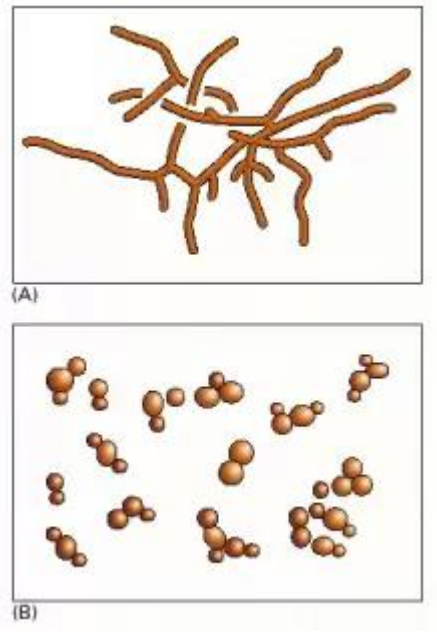
The genome of a virus may consist of DNA (Deoxyribo Nuclear Acid) or RNA (Ribo Nuclear Acid), which may be single stranded (ss) or double stranded (ds), linear or circular. The entire genome may occupy either one nucleic acid molecule (monopartite genome) or several nucleic acid segments (multipartite genome). The different types of genome necessitate different replication strategies.

C. Fungi

C.1. Structure of Fungi

The fungal branch of the eucaryotic kingdom includes both unicellular yeasts (such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and filamentous, multicellular molds (like those found on moldy fruit or bread). Most of the important pathogenic fungi exhibit dimorphism—the ability to grow in either yeast or mold form. The yeast-to-mold or mold-to-yeast transition is frequently associated with infection. *Histoplasma capsulatum*, for example, grows as a mold at low temperature in the soil, but it switches to a yeast form when inhaled into the lung, where it can cause the disease histoplasmosis

Dimorphism in the pathogenic fungus Histoplasma capsulatum



(A) At low temperature in the soil, Histoplasma grows as a filamentous fungus.

(B) After being inhaled into the lung of a mammal, Histoplasma undergoes a morphological switch triggered by the change in temperature. In this yeast-like form, it closely resembles Saccharomyces cerevisiae

C.2. Classification of Fungi

Fungi are usually classified according to biological taxonomy based upon the type of hypha, spore, and reproduction. There are four classes of fungi, whose characteristics are different:

Class Phycomycetes: The algal fungi: bread molds and leaf molds. The only known mycosis (fungal disease) caused by fungi of this class is mucormycosIs, a very rare fungal growth of the upper respiratory tract, bronchial mucosa, and lungs. It occurs largely as a complication of a chronic, debilitating disease, such as uncontrolled diabetes.

Class Ascomycetes: The sac fungi: yeasts, mildews, and cheese molds. Fungi of this class are implicated in only three fungus diseases, all of which are rare.

Class Basidiomycetes: Mushrooms, toadstools, rusts, and smuts. The only pathogens in this class are the mushrooms of the genus Amanita, which cause severe systemic poisoning (sometimes death) when eaten.

Class Deuteromyceters. Fungi imperfecti: a heterogeneous collection of fungi without sexual reproduction. Most of the pathogens encountered in medical mycology belong to this class.

On the other hand, fungi may be classified as such:

Geographic grouping

Classification by geographic distribution.

Certain fungal diseases are considered endemic to particular areas Histoplasmosis - Central Mississippi Valley and Ohio Valley fever. Coccidioidomycosis - San Joaquin Valley fever

Epidemiologic grouping
Concerned with how fungal disease is transmitted.

Few are contagious - i.e. ringworm of the scalp. Some are inhaled; others must be directly introduced into deeper tissue such as by a puncture from a thorn.

Most mycoses are dependent on the susceptibility of the individual host.

Taxonomy grouping
Scientific grouping according to morphologic and cultural characteristics; varies somewhat, depending on author.

One example of a taxonomic grouping:

KINGDOM: Plantae

PHYLUM: Thallophyte - Entire plant is somatic; no roots, or leaves.

SUBPHYLUM: Schizomycotina

CLASS: Schizomycetes (bacteria)

ORDER: Actinomycetales

FAMILY: Actinomycetaceae

GENUS: Actinomyces

SPECIES: *A. isarelii*

A. bovis

A. naeslundii

GENUS: Nocardia

SPECIES*: N. asteroides*

N. brasiliensis

N. carviae

FAMILY: Streptomycetaceae

GENUS: Streptomyces

SPECIES*: S. madurae*

S. pelletierii

S. paraquavensis

S. somaliensis

SUBPHYLUM: Myxomycotina

CLASS: Myxomycetes (slime molds)

SUBPHYLUM: Eumycotina (true fungi)

CLASS: Phycomycetes (algae fungi)

GENUS*: Rhizopus sp.*

Mucor sp.

Others

CLASS: Ascomycetes

GENUS: *Penicillium sp.*

Aspergillus sp.

Many others

CLASS: Basidiomycetes

GENUS: *Amanita sp.*(many poisonous toadstools)

Agaricus sp. (Edible mushrooms)

CLASS: Deuteromycetes (fungi imperfecti)

Yeast that reproduce by budding.

GENUS & SPECIES:

Cryptococcus neoformans

Candida albicans

Diphasic yeast (yeast-like when grown at 37 degrees)

GENUS & SPECIES:

Blastomyces dermatitidis

Blastomyces brasiliensis

Histoplasma capsulatum

Coccidioides immitis

Sporotrichum schenkii

Filamentous fungi - no yeast stage

GENUS & SPECIES:

Geotrichum sp.

Microsporum sp.

Trichophyton sp.

Epidermophyton sp.

Topographic Grouping - Classification as to type of mycoses produced

1. **Superficial**

Confined to the outermost layers of the skin and hair.

No host cellular / inflammatory response due to organisms being remote from living tissue. Essentially no pathology; the disease is recognized purely on cosmetic basis.

2. **Cutaneous**

Have particular affinity for the keratin of the skin, nails, and hair.

Most cutaneous infections are caused by a closely related group of fungi, called the dermatophytes.

These keratinophilic prefer the non-living cornified layers. The disease caused by these organisms is called Adermatophytosis or "dermatomycosis". Host response is patchy scaling or eczema forming eruptions; inflammation may occur. They are classified according to the area of the body that is involved.

3. **Subcutaneous**

Involve the deeper layers of skin and often muscle tissue.

Man is an accidental host following inoculation of fungal spores via some form of trauma. This type of fungal infection is often tentatively identified by the presence of a characteristic tissue reaction or granule.

4. **Systemic**

Attack the deep tissues and organ systems; often create symptoms that resemble other diseases.

Two categories of systemic disease:

- Those caused by truly pathogenic fungi with the ability to cause disease in the normal human host when the inoculum is of sufficient size.

  Histoplasma capsulatum

  Blastomyces dermatitidis

  Coccidioides immitis

  Paracoccidioides brasiliensis

- Opportunistic fungi, low virulence organisms, require the patient's defences to be lowered before the infection is established.

Aspergillus sp.

Candida albicans

Cryptococcus neoformans

LO 1.2 – Characterize different types of bacteria of veterinary importance

- Topic 1: Characterization of Bacteria of Veterinary Importance

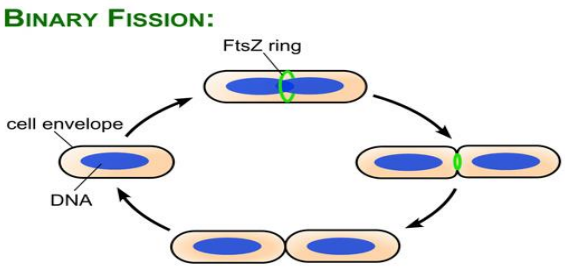
Like other bacteria, these bacteria have different characteristics from multiplication process to their resistance and death.

A. Multiplication of bacteria

Most bacteria rely on binary fission for propagation. Conceptually this is a simple process; a cell just needs to grow to twice its starting size and then split in two. But, to remain viable and competitive, a bacterium must divide at the right time, in the right place, and must provide each offspring with a complete copy of its essential genetic material. Bacterial cell division is studied in many research laboratories throughout the world.

These investigations are uncovering the genetic mechanisms that regulate and drive bacterial cell division. Understanding the mechanics of this process is of great interest because it may allow for the design of new chemicals or novel antibiotics that specifically target and interfere with cell division in bacteria.

Cell division in bacteria



Before binary fission occurs, the cell must copy its genetic material (DNA) and segregate these copies to opposite ends of the cell. Then the many types of proteins that comprise the cell division machinery assemble at the future division site. A key component of this machinery is the protein FtsZ (Filamenting temperature sensitive mutant Z).

Protein monomers of FtsZ assemble into a ring-like structure at the center of a cell. Other components of the division apparatus then assemble at the FtsZ ring. This machinery is positioned so that division splits the cytoplasm and does not damage DNA in the process. As division occurs, the cytoplasm is cleaved in two, and in many bacteria, new cell wall is synthesized. The order and timing of these processes (DNA replication, DNA segregation, division site selection and invagination of the cell envelope and synthesis of new cell wall) are tightly controlled.

B. Pathogenicity of Bacteria

Hemolytic or non hemolytic.

- Beta hemolytic-can complete hemolysis (dissolution) of red blood cells
- Alpha hemolytic-cause partial hemolysis of red blood cells.
- Gamma forms-do not cause hemolysis.

Production of toxins.

- Exotoxins are extremely potent poisons which are produced in bacterial cells, which diffuse freely into the cells of host tissues, causing severe systemic poisoning.
- Endotoxins are less potent than exotoxins, produced in bacterial cells, and diffuse into the host cells only after the bacterial cell disintegrates.

C. Resistance of bacteria

The speed and scale with which resistance has emerged in many bacterial pathogens has caught many veterinarians and physicians by surprise. Resistance has increased markedly in the last two decades.

In human medicine, the antibiotic resistance crisis has taken the approaches to treating some totally resistant bacterial infections back to the pre-antibiotic era, including surgery such as the amputation of infected limbs. The emergence and spread of highly resistant bacteria is a serious threat to modern medicine and surgery.

The potential for mutation and for genetic exchange between all types of bacteria, combined with the short bacterial generation time, is of major importance in limiting the use of antimicrobial drugs in controlling infection in animals and humans. The use of antimicrobial drugs does not induce resistance in bacteria but rather eliminates the susceptible bacteria and leaves the resistant bacteria already present in the population. The exposure of animals to antimicrobials is the basis of selection for the evolution and spread of resistance genes and resistant bacteria.

The genetic processes involved in the development of resistance in bacteria are precisely those involved in the evolution of bacteria generally; it is natural, Darwinian, selection for “fitness.”

Resistance to antimicrobial drugs can be classified as constitutive or acquired.

C.1. Constitutive Resistance

Microorganisms may be resistant to certain antibiotics because the cellular mechanisms required for antibiotic susceptibility are absent from the cell. Bacteria in the genus *Mycoplasma*, for example, are resistant to benzyl penicillin G because they lack a cell wall; similarly *E. coli* is resistant to penicillin G largely because the drug fails to penetrate into the cell.

C.2. Acquired Resistance

Acquired, genetically based, resistance can arise because of mutation or, more importantly, through horizontal gene transfer ("transferable drug resistance")—the acquisition of genetic material from other bacteria by different means (bacteriophages, plasmids, transformation, or transposons). Mutations, especially in the chromosome, tend to produce changes in bacterial cell structures, whereas horizontal gene transfer tends to encode synthesis of enzymes that modify antibiotics. Mutation-based resistance is often a gradual, stepwise process, whereas transferable resistance is often high-level, all-or-none, resistance.

D. Culture conditions

D.1. Temperature

The correct temperature is very important for the proper growth of bacteria since bacteria vary considerably with respect to temperature requirements.

- Some bacteria grow best in a temperature range of 10º to 20ºC; these bacteria are called **psychrophilic** (psychrotrophic) bacteria.
- Other bacteria have an optimum temperature in the 30º to 40ºC range; they are referred to as **mesophilic** bacteria.
- The **thermophilic** bacteria have an optimum temperature range from 50º to 60ºC.

The majority of human and animal pathogens are mesophilic, growing best at 37ºC and, for this reason, a constant temperature incubator adjusted to 37ºC (normal body temperature) and containing sufficient moisture satisfies the temperature requirement in a clinical bacteriology section. However, some authorities suggest that most incubators should be set at 35ºC, so that a temperature higher than 37ºC will be unlikely at any time.

D.2. Oxygen Requirements

It is essential that the proper gaseous environment be furnished when attempting to cultivate bacteria. The specific needs apply to oxygen and carbon dioxide. Often, the failure to isolate pathogenic microorganisms from clinical materials is due to inadequate provisions with respect to aeration. Bacteria are divisible into two broad groups on the basis of their oxygen requirements:

- Aerobic forms which require free oxygen for growth, and
- Anaerobic forms which require the absence of free oxygen for growth. Aerobic and anaerobic bacteria are further divided into several categories.

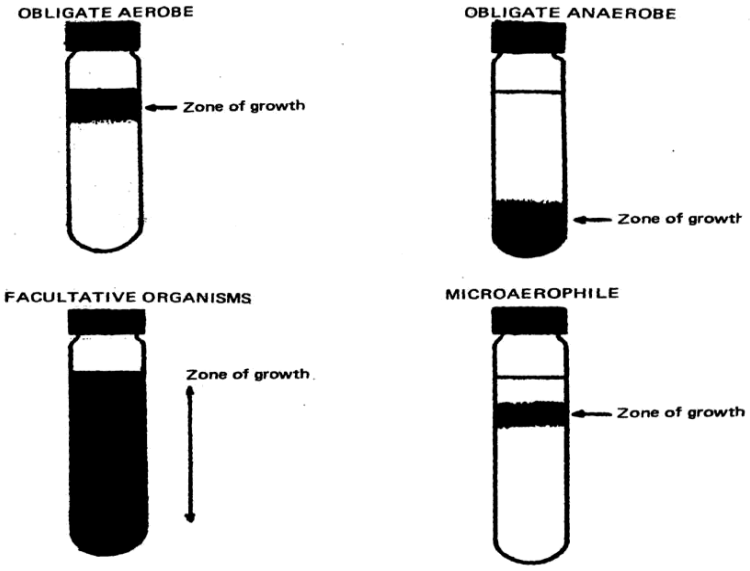


Figure 3: Aerobic and anaerobic bacteria growth zone

Aerobic and anaerobic bacteria divided into the following categories:

- **Obligate Aerobes.** An obligate aerobe must have free atmospheric oxygen in order to grow. These forms grow best on agar media in a normal atmosphere. They will also grow in the upper portion of a broth medium. Some members of the genus Bacillus are strict aerobes.
- **Microaerophiles.** For optimal growth these forms require a greatly reduced atmosphere of oxygen such as that supplied in the lower portion of a broth medium. These organisms will also grow well on an agar plate, when incubated in an atmosphere of increased carbon dioxide (candle jar). Haemophilus species and many streptococci are microaerophiles.
- **Facultative Organisms.** Those organisms capable of adapting to either presence or absence of atmospheric oxygen fall into this group.
- **Obligate Anaerobes.** These forms require the strict absence of atmospheric oxygen for growth. Free oxygen is toxic to obligate anaerobes because of resultant enzyme destruction or inactivation. Special measures must be taken to remove and exclude oxygen from these cultures during incubation. Some pathogenic bacteria are obligate anaerobes on primary isolation yet will adapt to aerobic conditions upon subculture. Examples of true obligate anaerobes are represented in the genus **Clostridium** and some **streptococci.**

D.3. Carbon Dioxide Requirements

Many microorganisms, aerobic and anaerobic, require a carbon dioxide concentration above normal atmospheric levels. Examples of aerobes requiring increased CO_2 are **Brucella** and **Mycobacterium**.

E. Types of bacteria

There many types of bacteria based on their different characteristics: based on form, based on elevation, based on margin and based on Gram staining.

Types of bacteria based on form

Punctiform 

Irregular 

Circular 

Rhizoid 

Filamentous 

Spindle 

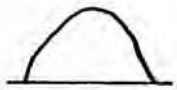
Bacillus 

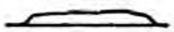
Coccus 

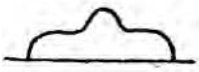
Vibrio 

Types of bacteria based on elevation

Flat

Pulvinate 

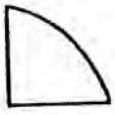
Raised 

Umbonate 

Convex 

Umbilicate 

Types of bacteria based on margin

Entire 

Erose 

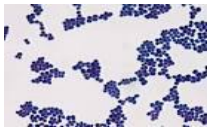
Undulate 

Filamentous 

Lobate 

Curled 

Types of bacteria based on Gram staining

Gram positive 

Gram negative 

F. Some bacteria with veterinary importance

Table 1: bacteria with veterinary importance

Group of Bacteria	Species	Host
Staphylococci	*Staphylococcus aureus*	All animal and Human
	Staphylococcus caprae	Goats
	S. lentus	
	S. delphini	Dolphins
	S. equorum	Horses
	S. felis	Cats
	S. gallinarum	Chicken
	S. hyicus	Pigs
	S. intermedius	Dogs
	S. pseudintermedius	
	S. simiae	Monkeys
Escherichia	*Escherichia coli*	Most of animals and Human
	E. albertii	Birds and human, Large animals are often less likely to be affected
	E. fergusonii	Farm animals, human
Salmonella	*Salmonella enterica and S. bongori*	Poultry, swine, cattle, horses, dogs, cats, wild mammals and birds, reptiles, amphibians, crustaceans
Bacillus	*Bacillus anthracis*	cattle, sheep, goats, horses, wild herbivorous animals; virtually all mammals and some birds

Brucella	*Brucella abortus*	Cattle, bison, water buffalo, African buffalo, elk, camels; other mammalian
	B. melitensis	Goats, sheep; other mammalian
	B. suis	Swine and wild pigs
	B. canis	Dogs; evidence of infection in wild canids including coyotes
Mycobacteria	*Mycobacterium avium Paratuberculosis*	Cattle, sheep, goats, camelids, deer, other ruminants; rabbits and other nonruminants
	Mycobacterium bovis	Cattle, bison, African buffalo, deer, opossums, badgers, kudu can be reservoirs; swine and many other mammals can be spillover hosts

LO 1.3 – Characterise viruses of veterinary importance

- Topic 1: Characterization of Viruses of Veterinary Importance

A. Multiplication of viruses

Viruses are a unique group of infectious agents whose distinctiveness resides in their simple, acellular organization and pattern of reproduction. A complete virus particle or **virion** consists of one or more molecules of DNA or RNA enclosed in a coat of protein, and sometimes also in other layers. These additional layers may be very complex and contain carbohydrates, lipids, and additional proteins. Viruses can exist in two phases: extracellular and intracellular.

- Virions, the **extracellular phase**, possess few if any enzymes and cannot reproduce independent of living cells.
- In the **intracellular phase**, viruses exist primarily as replicating nucleic acids that induce host metabolism to synthesize virion components; eventually complete virus particles or virions are released.

In summary, viruses differ from living cells in at least three ways:

- their simple, acellular organization;
- the presence of either DNA or RNA, but not both, in almost all virions (human cytomegalovirus has a DNA genome and four mRNAs); and
- their inability to reproduce independent of cells and carry out cell division as procaryotes and eucaryotes do. Although bacteria such as chlamydia and rickettsia are obligately intracellular parasites like viruses, they do not meet the first two criteria.

1. Multiplication of DNA Viruses

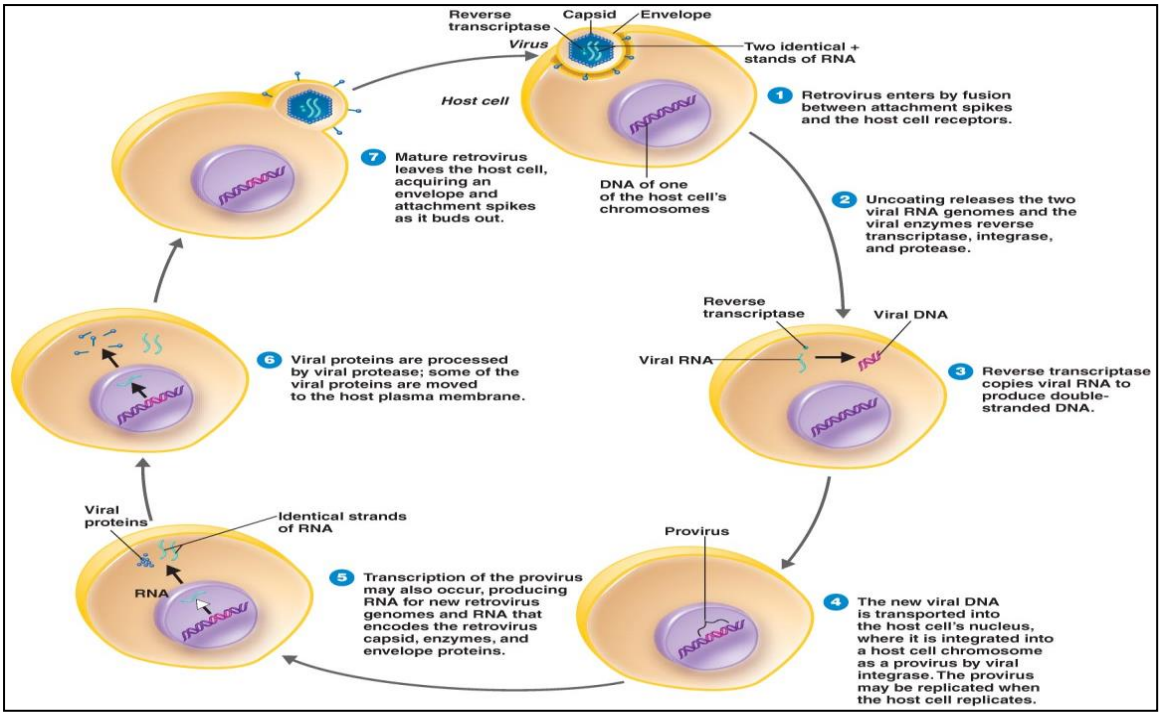


Figure 4: Multiplication of DNA Viruses

Multiplication of a Retrovirus (RNA virus)

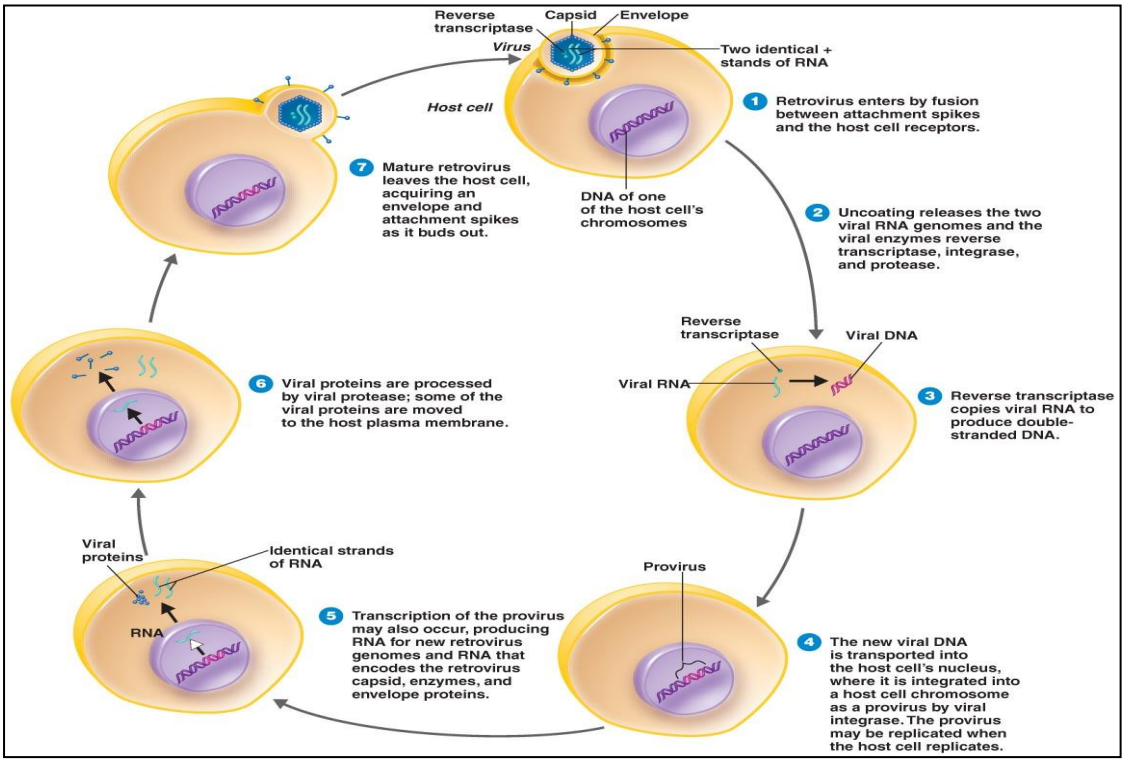


Figure 5: Multiplication of a Retrovirus (RNA virus)

B. <u>Pathogenicity of viruses</u>

Pathogenicity can be defined as the process (es) by which a virus produces disease in the host. The term "virulence" is used to describe the degree of pathogenicity of a virus—a virulent virus being the one that results in a significant disease.

The pathogenesis of viral infections is a multifactor process and involves both the status of the host and properties of the infecting virus.

The outcome of viral infection (i.e., the severity of disease or death) is, therefore, determined by the virulence of virus and susceptibility of the host.

Susceptibility of a host to a viral infection is determined by many factors including the genetic background of the host, species of the host animal affected, level of immunity, immune competency of the host, nutritional status, age, and presence of concurrent infection(s). Some of the viral virulence-related properties include production of cytotoxic effects by the virus (direct or indirect), replication strategies, tissue tropism, dose of the infecting virus, and route of exposure.

The host–virus interaction, as determined by these factors, can be examined at the cellular level and at the level of the host animal. The former relates to the ability of a virus to infect and replicate in a cell and effects of virus replication on that cell, while the latter is related to the cumulative effects of cellular infection on the animal host. Thus, what is seen at the host level is a reflection of the cellular effects of the virus.

C. Resistance of virus

Viral replication depends largely on the active participation of the metabolic pathways of the host cell, and the balance between preventing viral replication and wrecking cellular metabolism is dedicated. Only a relatively small number of useful drugs have been described, and their spectrum of antiviral activity is often narrow.

Antiviral drugs are generally only effective prophylactically or in the early stages of disease when viral replication is occurring. Rapid diagnosis is therefore required. Although no antiviral drugs have been approved for veterinary use. Among those drugs, some are immunomodulators (Acyclovir used for Herpes infections and Vidarabine used for Canine herpes are examples).

D. Culture conditions

Because they are unable to reproduce independent of living cells, viruses cannot be cultured in the same way as bacteria and eucaryotic microorganisms. For many years researchers have cultivated animal viruses by inoculating suitable host animals or embryonated eggs—fertilized chicken eggs incubated about 6 to 8 days after laying.

To prepare the egg for virus cultivation, the shell surface is first disinfected with iodine and penetrated with a small sterile drill. After inoculation, the drill hole is sealed with gelatin and the egg incubated.

Viruses may be able to reproduce only in certain parts of the embryo; consequently they must be injected into the proper region.

For example, the myxoma virus grows well on the chorioallantoic membrane, whereas the mumps virus prefers the allantoic cavity. The infection may produce a local tissue lesion known as a pock, whose appearance often is characteristic of the virus.

Bacterial viruses or **bacteriophages** (**phages** for short) are cultivated in either broth or agar cultures of young, actively growing bacterial cells. So many host cells are destroyed that turbid bacterial cultures may clear rapidly because of cell lysis. Agar cultures are prepared by mixing the bacteriophage sample with cool, liquid agar and a suitable bacterial culture. The mixture is quickly poured into a petri dish containing a bottom layer of sterile agar. After hardening, bacteria in the layer of top agar grow and reproduce, forming a continuous, opaque layer or "lawn." Wherever a virion comes to rest in the top agar, the virus infects an adjacent cell and reproduces. Eventually, bacterial lysis generates a plaque or clearing in the lawn.

LO 1.4 – Characterize Fungi of veterinary importance

- Topic 1:Characterization of Fungi of Veterinary Importance

Fungal diseases affect the health and consequently the survival of wild and domesticated animals. In this respect there is a close relationship between human and animal since they suffer from many of the same fungal pathogens

A. Multiplication of fungi

Since fungi can be found in virtually every conceivable habitat where there is adequate available organic material for survival, you might expect that a variety of reproductive mechanisms have evolved to perpetuate the species. Although many of the fungi possess the ability to reproduce both asexually and sexually, there is one large group (deuteromycetes) for which only asexual mechanisms are known.

In general, asexual reproduction is more effective, producing greater numbers of dispersal units than sexual reproduction. However, since it is an asexual process, the genetic variability among the progeny is low. The asexual (imperfect) stage of a fungus is termed the anamorph, while the sexual (perfect) stage is referred to as the teleomorph.

B. Pathogenicity of fungi

Pathogenesis is the ability of a microorganism to infect the host and produce disease resulting from interaction of pathogen with host via expression of certain factors on both sides.

Pathogenicity of a fungus depends on its ability to adapt to the tissue environment and to withstand the

lytic activity of the host's defences.

Several determinants including genes or gene products such as enzyme molecules known as virulence factors are involved in this relationship, producing superficial to invasive infections in animals.

For a fungus to produce disease in a patient, it must be actively invading tissues. Diseases caused by fungi without invasion of live tissues include mould allergies and cutaneous dermatophyte infections (ringworm), in which fungi invade and damage only the nonviable epidermis. Further, potentially lethal mycoses involving deep tissues result from fungal dissemination and invasion throughout the body.

Determinants of pathogenicity are called virulence factors. Pathogenic microbes often possess a number of virulence factors and mechanisms. These factors determine whether the organism (the host) lives or dies during host–microbe interactions. The factors can be inducible or constitutive, the direct product of genetic elements (proteins), or the products of complex biosynthetic pathways such as polysaccharides or lipid mediators.

Virulence factors must help the pathogen to grow at elevated temperatures, facilitate adherence, penetration and dissemination, or assist in resistance against innate immune defences, e.g., phagocytosis and complement, evasion from adaptive immune defences, or nutritional and metabolic factors, necrotic factors, or morphology variation.

The ability of a fungus to grow at 37^{o} C and physiological pH is a virulence factor for fungi that invade deep tissue, and the transition to parasitic form is essential for the pathogenicity of dimorphic fungi.

C. Resistance of fungi

Pathogenic fungi are the cause of life-threatening infections in an increasing number of immune compromised patients. The intrinsic resistance to antifungal therapy observed in some genera, along with the development of resistance during treatment in others, is becoming a major problem in the management of these diseases.

The mechanism of drug resistance in microorganisms traditionally takes the path of either identifying a cellular determinant that prevents entry of the drug or removes the drug from the cell or inactivates the drug or prevents the drug from inhibiting the target of various combinations of the above-mentioned pathways.

In fungi, mutation in gene encoding target proteins, up-regulations of expression of multidrug efflux pumps and drug target themselves, altering the stoichiometry of the inhibitor target ratio in favour of fungus are possible mechanisms. However, no fungus has yet been shown to have the ability to degrade an antifungal agent like beta lactamase in bacteria.

Multidrug resistance, called pleiotrophic drug resistance (POR) in *Saccharomyces cerevisiae* is possibly an ancient model for multidrug resistance that operates in pathogenic fungi through the efflux pump.

D. Culture conditions

Inoculation

- Plates - inoculated like a large "S", so that rapid growing fungi can remove.
- Slants - Inoculated with a straight line.

Incubation

- Aerobic (and anaerobic if Actinomycetes are suspected)
- Room temperature & also sometimes at 37□ if dimorphic fungus is expected.

General considerations

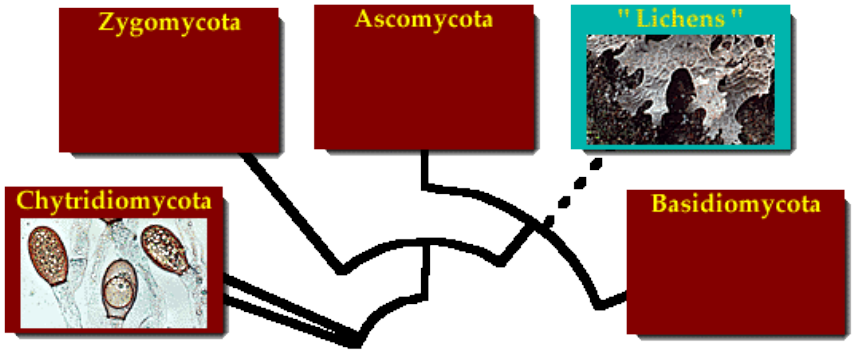
- Type of media used. Does it contain antibiotics?
- Growth rate & age of the culture.
- Routine cultures are kept for 4 weeks & should be examined every other day.
- Most systemic pathogens require 10 days to 2 weeks, while saprophytic fungi grow usually grow within 1 week.

Colony Morphology (macroscopic features)

- **Surface topography** - Some fungal colonies may be free growing, covering the entire surface of agar in a particular manner; others grow in a restricted manner.
- **Surface texture** -examples: cottony or wooly (floccose), granular, chalky, velvety, powdery, silky, glabrous (smooth, creamy), waxy, etc.
- **Pigmentation** - Fungi may be colorless or brightly colored. Color may be on fungus itself, on its sporulating apparatus, on the agar, or on the bottom of the colony (reverse pigmentation). Pigment color is due to the color of the sporulating apparatus. The pigment can be diffused into the agar. It is important to note the top pigment (obverse) and the discoloration of the agar medium (reverse).
- **Mycelium**

- Vegetative mycelium - provides nutrition
- Aerial mycelium – reproductive

E. Different types/classes of fungi



Fungi are usually classified in four divisions: the **Chytridiomycota** (chytrids), **Zygomycota** (bread molds), **Ascomycota** (yeasts and sac fungi), and the **Basidiomycota** (club fungi). Placement into a division is based on the way in which the fungus reproduces sexually. The shape and internal structure of the **sporangia**, which produce the spores, are the most useful character for identifying these various major groups.

There are also two conventional groups which are not recognized as formal taxonomic groups (i.e. they are polyphyletic); these are the Deuteromycota (fungi imperfecti), and the lichens. The **Deuteromycota** includes all fungi which have lost the ability to reproduce sexually. As a result, it is not known for certain into which group they should be placed, and thus the Deuteromycota becomes a convenient place to dump them until someone gets around to working out their biology**.**

F. Different forms of fungal colonies

Morphology of Fungi

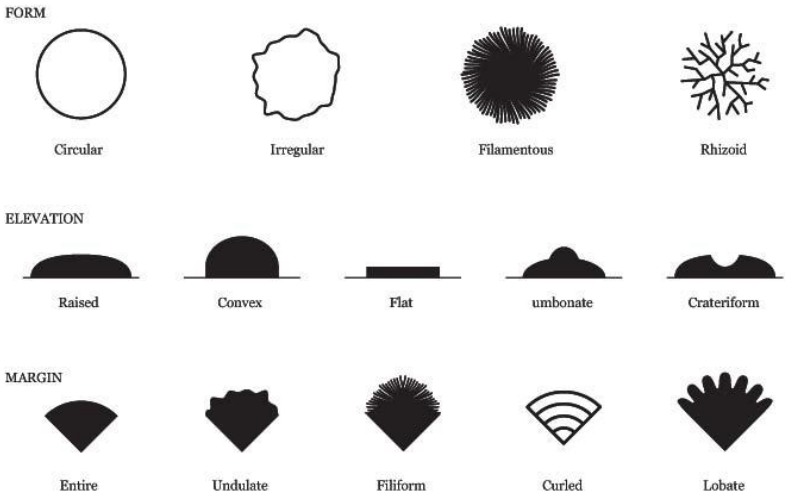


Figure 6: Morphology of Fungi

Learning Unit 2 – Select material, media and Equipment

L O 2.1 – Select material and equipment for sample collection

Materials and equipment are always selected depending on the purpose and the kind of analyses to be conducted.

- Topic 1: Material for sample collection

Care must be given to how the sample is collected; if not, interpretation of results may be difficult. Most infectious processes arise subsequent to the contamination of a compromised surface or site by microorganisms that are also a part of the flora occurring on a contiguous mucosal surface. In other words, microorganisms isolated from an affected site are often similar (if not identical) to those found as part of the normal flora of the patient.

Principles

Certain basic principles, therefore, should be followed at all times:

- First, instruments, containers, and other equipment in direct contact with specimens must be sterile (For example: test tubes, swabs, tissue forceps, disposable syringes and needles);
- Second, animal (specified areas) and veterinarian must be cleaned by using disinfectants to reduce contamination;
- Third, specimens should be properly labeled and dated (use permanent markers);
- Fourth, each collected sample must be kept in cool box to avoid transformation.

A. Equipment and material for collecting blood

Assemble equipment for collecting blood:

- Laboratory sample tubes for blood collection (sterile glass or plastic tubes with rubber caps, vacuum extraction blood tubes, or glass tubes with screw caps). EDTA tubes are preferred.
- Blood sampling systems (Needle and syringe system, vacuum extraction system with holder, winged butterfly system (vacuum extraction) or winged butterfly system (syringe))
- Tourniquet (single-use)
- Skin antiseptic solution: 70% isopropyl alcohol

Vacuum extraction blood tubes

Figure 7: Vacuum extraction blood tubes

B. Equipment and material for collecting urine

You should assemble the following material according to the types of urine collection:

- Urine Collection Containers (cups for collection and transport) Urine collection container cups come in a variety of shapes and sizes with lids that are either snap on or screw on
- Urine collection containers for 24-hour specimens come in a variety of shapes and colors, but most are of 3 liter (L) capacity1 and are amber colored (to protect light-sensitive analyses such as porphyrins and urobilinogen).
- Urinalysis Tubes Urine specimens are poured directly into urinalysis tubes with screw- or snap-on caps

Figure 8: Urine collection container cups **BD Vacutainer urine collection cup**

C. Equipment and material for collecting swabs

The use of swab stick is recommended: **Flocked Swabs** proven superior in sample uptake and release, compared to non-flocked swabs. Its quick sample collection and release ability is due to the unique spray type nylon pile implantation technology, allows the rapid diagnostic test.

Figure 9: Swab stick in a beaker

D. <u>Equipment and material for collecting faeces</u>

A freshly voided sample or one collected from the rectum is preferable to a rectal swab which often does not have enough faecal matter for agent detection. You will need to pick up one or more specimen containers, depending on which test(s):

- Plastic bags, lab gloves and plastic gloves can are used to collect stool in rectum of on the ground
- The sample is poured into small bottle containers with screw to transport in laboratory.

Figure 10: Bottle containers with screw

E. Equipment and material for collecting milk

Prepare in advance all the necessary material: teat dip, gloves and clean towels, alcohol swabs, sampling tubes, tube rack, marker, ice and cooler.

The tubes with cap should be used for Collection of milk sample. For a composite sample, draw the same amount of milk from each of the four quarters. Once the tube is filled (maximum of 3/4), put the cap back on.

Figure 11: Milk Collection tubes with cap

F. <u>Equipment and material for collecting mucus (sputum)</u>

Secretions can be collected directly into a vial or tube, or can be collected using swabs.

Vesicular fluids provide a highly concentrated source of pathogen for diagnostic testing, and can be collected from unruptured vesicles using a sterile needle and syringe, and immediately transferred to a securely sealed vial or tube.

Specifically developed sampling tools, such as probang cups, can be used for collecting cellular material and mucus from the pharynx of livestock. Cotton ropes that animals are allowed to mouth and chew have been validated for use in collecting saliva specimens from domestic swine.

Figure 12: Mucus collection cup with screw lid

G. Equipment and material for collecting scabs

The specimens should be collected aseptically and preserved as specified for the intended test(s). Deep skin-scrapings obtained using the edge of a scalpel blade are useful. Epithelial tissues, particularly those associated with vesicular lesions and collected into viral transport media, can be critical in the laboratory diagnosis of specific viral infections such as foot and mouth disease.

Materials needed:

- 26 Gauge needle
- Sterile screw-capped plastic vials with O-ring (1.5 to 2 mL)
- Multiple—Alcohol wipes

H. Equipment and material for collecting lymph

Reagents

The following reagents should be used:

- Dulbecco PBS,
- D-MEM/F12 supplemented with antibiotic mixture (Invitrogen) to achieve a final concentration of 100 U penicillin/100 mg streptomycin/ml ketamine/xylazine,
- 1XProtease inhibitor mixture Fentanyl/,
- droperidol, diazepam,
- OVA-conjugated
- Alexa Fluor 647,
- artificial CSF (Cerebro-Spinal Fluid),
- Superfrost Plus Slides,
- Fisherbrand Cover Glasses 50 3 22 no. 1,
- Alexa Fluor 488–conjugated rat anti-mouse Lyve1,

- Armenian hamster anti-mouse CD31,
- Alexa Fluor 594–conjugated goat anti–Armenian hamster ,
- DAPI and ProLong Gold Antifade Mountant (Invitrogen).

Equipment

The following equipment should be used:

- stereomicroscope (SMZ1270; Nikon),
- glass pipette (part number GCP-40-60; Living Systems Instrumentation),
- 1-ml syringes (catalog no. 305620; Becton Dickinson),
- nylon thread (part number THR-G; Living Systems Instrumentation),
- scissors (ROBOZRS5882), forceps (Dumont no. 5 tip size, 0.25 3 0.01 mm tip size),
- forceps (Dumont no. 5 forceps, 0.05 3 0.01 mm tip size),
- Vannas Scissors (SCS-VAN-ST-2.5MM; Living Systems Instrumentation),
- petri dishes (catalog no. 350044; Becton Dickinson), standard 0.65-ml plastic tubes (catalog no. 87003-290; VWR International),
- Hamilton syringe (5 ml, 33G; Hamilton), and
- Leica CM3050 S Cryostat Olympus FV1200 Confocal Laser Scanning Microscope custom-made cannulation chambers

I. <u>Equipment and material for collecting organs and tissues</u>

Samples stored using LN_2 must have vials that are rated for use with cryogenic temperatures, preferably made of polypropylene with screw-top caps. Glass vials are problematic when frozen because of their fragility when handling and vulnerability to cracking when stressed, and their use with LN_2 is unacceptable because leakage into the vials can lead to the vessels exploding.

Pop-off lids are not recommended because this vial type can easily open on its own.

Vials can have threads located internally or externally on the vial opening; both vial types exhibit advantages and disadvantages.

- Externally-threaded closures promote more sterile conditions because internal threading can allow contaminants to enter if the cap is placed on an unclean surface when removed, but these vials can be susceptible to cracks and loss of air-tightness, which can lead to sample desiccation or oxidation.
- Internally-threaded vials might allow increased storage capacity, depending on the vial selected, but users also suggest that material can be trapped within the threads of this vial type.

Some manufacturers suggest that vial caps that incorporate a silicone gasket or O-ring (internally or externally threaded) are ideal for vapor-phase LN_2 freezing because the seal is enhanced; however, there are some concerns:

- Care must be taken if the vial cap is over-tightened because the gasket can become distended.
- The presence of gaskets or O-ring might improve the initial performance of seals but can be problematic when used with some alcohols (e.g., ethanol) because some gasket material, such as silicone, is vapor permeable.

When working with liquid-phase nitrogen cold storage, extra caution must be taken because the accidental entrapment of liquefied nitrogen inside the vial leads to pressure build up and, upon removal, rapid vaporization of the liquid can result in leakage or even explosion.

Note: As a precaution, vial manufacturers recommend that samples not be immersed in LN_2 unless they have secondary containment. Heat-sealing vials into flexible polyethylene tubing is recommended for safe storage in the liquid-phase environment.

J. Assemble equipment for preventing infections

- Long-sleeved, cuffed gowns (if in hospital) or disposable coverall suit (if in rural area) Note: Tasks where contact with blood or body fluid could happen, Impermeable gown or a plastic apron over the non-impermeable gown are recommended.
- Face protection: Face shield or "goggles and mask"
- For Hand Hygiene: use Alcohol-based handrubOR Clean, running water Soap Disposable (paper) towel.
- Personal Protective Equipment (PPE): Several pairs of disposable gloves (non-sterile, ambidextrous, single layer);One pair of gloves for blood collection; One additional pair as a replacement if they become damaged or contaminate
- Footwear: wear shoes with puncture-resistant soles or rubber boots; If in rural setting or patient home wear rubber boots or shoes with puncture-resistant soles with disposable overshoes secured around the shoes to prevent direct contact with ground and infected bodily fluid spills.

K. For waste management materials

- Leak-proof and puncture resistant sharps container
- Two leak-proof infectious waste bags: one for disposable material (destruction) and one for reusable materials (disinfection)

- Topic 2: Material for sample transportation

The sooner the specimen is processed in the microbiology laboratory, the better results after analysis. Realistically, the time between sample collection and processing may range from minutes to hours to days.

Major factors that compromise specimens and lead to inaccurate diagnosis:

- Drying (all microorganisms) and
- Exposure to a noxious atmosphere (oxygen for obligate anaerobes).

For this reason, it is important that the specimen be kept moist and, if conditions warrant, air excluded.

Swabs should always be placed in transport medium, regardless of the time elapsed between processing and collection.

- Fluids that may contain anaerobic bacteria (e.g., exudate from draining tracts, peritoneal and pleural effusions, and abscess material) should be inoculated onto appropriate media immediately.
- If this material is contained in a syringe, then the air should be expelled and a sterile stopper placed over the needle. If a swab is used to collect the sample, it should be placed in an anaerobic transport medium.
- If a syringe full of sample cannot be processed immediately, the syringe should be emptied into an anaerobic transport medium and held at room temperature.
- Swabs for bacterial culture should be submitted in bacterial transport medium

It is best not to refrigerate samples suspected of containing anaerobes because some species do not tolerate reduced temperatures.

You must make sure that the samples are packaged safely and responsibly so that they not only do they arrive safely in our lab but that they also comply with the relevant legislation.

- Topic 3: Material for sample conservation

Materials for sample conservation are depending on the nature of sample, temperature required and the time in which the sample will be kept before examination.

A. Blood

The sample vial must be stored in an ice-box till it is transferred to a refrigerator.

For transferring to another laboratory for analysis, it should be properly packed in a compartment that will prevent any mechanical damage during transport, and that will ensure a low temperature

B. Faeces or dung

The zip lock can be placed in an ice box for transport or kept at 4°C for short-term storage in the laboratory.

C. Horn /Ivory/Bone

The sample can next be stored at 4°C in a refrigerator for laboratory analysis

D. Milk

The sample can be placed in an ice box for transport or kept at 4°C for short-term storage in a refrigerator in the laboratory for analysis. Refrigeration: T° < 4°C – maximum of 24 hours and Freezing: T° < -20°C – maximum of 1 month.

Note: Testing for mycoplasms requires unfrozen milk.

E. Organ and tissues

Tissues should be preserved in:

- Sterile media to preserve tissue: Phosphate buffered saline /Dulbecco's Modified Eagle's medium/Normal Saline.
- Sterile test tubes/vials/containers with sterile media to preserve tissues.

F. Swabs

Swab each collection site with a separate sterile swab and place each swab into separate tubes. Swab samples can be stored in the refrigerator (4°C) for up to one week. Swab samples can be stored frozen (≤ 60.0 ° C) indefinitely. Swabs must be sent in a sterile viral with appropriate transport medium such as phosphate-buffered saline or physiologic saline in chilled.

G. Urine

If urine sample cannot be analyzed in within 1 hour, you should put the container in a sealed plastic bag then store it in the fridge at around 4C. Do not keep it for longer than 24 hours.

The bacteria in the urine sample can multiply if it is not kept in a fridge. If this happens, it could affect the test results.

H. Lymph

The duration of each period of lymph collection was varied according to the experimental situation. It was found that lymph could be collected for periods of up to 24 hr. without gross degenerative changes occurring in the appearance of the cells in stained films. However if the lymph cells were required for in vitro experiments or injection into other animals the collection period was ended as soon as sufficient cells had been collected and where possible was not allowed to exceed 8 hr.

Adequate specimens for phase or electron microscopy were collected over periods of a few minutes.

I. Mucus (sputum)

The conservation is done as follow:

- Sputum Culture, Stain: For best results, return the sample to the clinic within two hours. If this is not possible, keep the sample at room temperature and return it within 24 hours
- AFB Culture, Stain: Keep the samples in the refrigerator. Return all of the samples to the clinic at one time
- Fungus Culture: Keep the samples in the refrigerator. Return each sample to the clinic within two days of collection.
- Fungus/Pneumocystis, Stain: Keep the sample in the refrigerator. Return the sample to the clinic within 24 hours.
- Sputum for Cytology: Do not keep the samples in the refrigerator. Return all of the samples to the clinic at one time on the third day of collecting the samples.

L O 2.2 – Select material and media for culture

Materials and culture media are always selected depending on the purpose and the kind of microorganisms to be isolated.

- Topic 1: Material for sample preparation

During sample preparation, materials depend on sample nature, type of microorganism needed to be isolated, availability of resources and protocol. Some material can be available:

- Preparation tables
- Aseptic preparation area
- Incubation equipment
- Alcohol
- PPE
- Beaker and Erlenmeyer
- Measuring cylinder
- Test tubes
- Rack and stands
- Oats and cotton
- Aluminum papers
- Petri dishes
- Inoculation loops
- Waste disposal bins

- Water
- Analytical Balance
- Autoclave
- Bunsen burner
- Centrifuge
- Colony Counter
- Deep Freezer
- Homogenizer
- Hot plate
- Hot air oven
- Incubator
- Laminar Air Flow/ Laminar Hood
- Magnetic Stirrer
- Microscope
- pH Meter
- Spectrophotometer
- Vortex Mixture/ Vortexer
- Water Bath
- Water Distiller

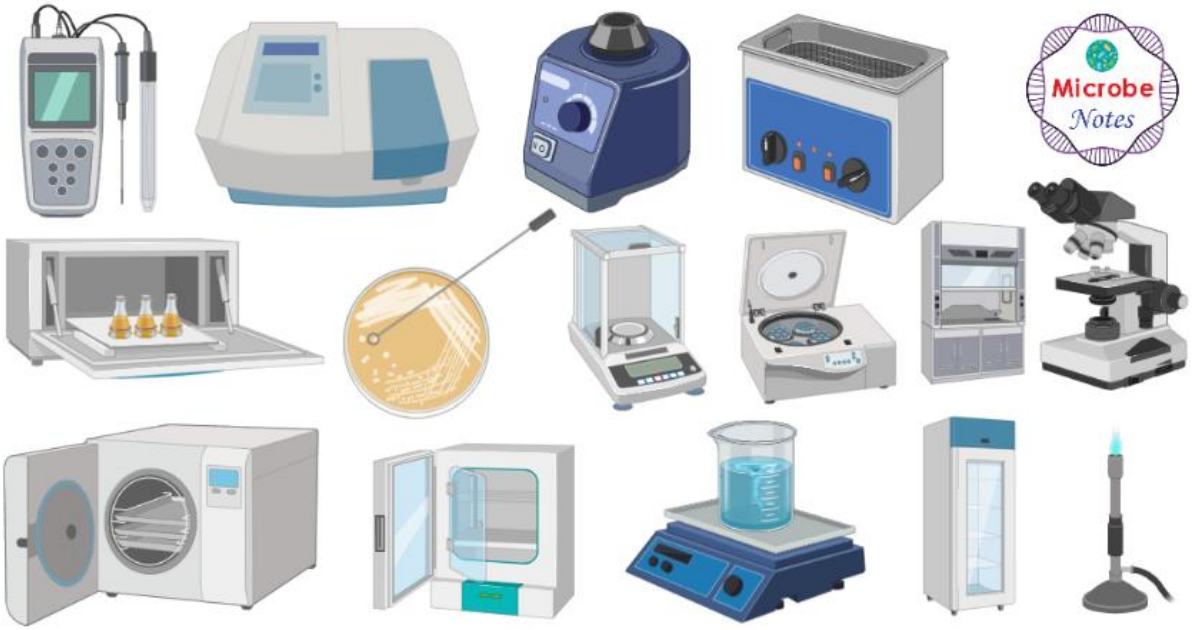


Figure 13: A set of some material for sample preparation

Analytical Balance

As they are highly precise and based on advanced technology, analytical balances are explicitly used in laboratories for the effective completion of tasks like weighing test materials and sampling amounts, formulation, density determination, purity analysis, quality control testing, and material and conformance testing.

Autoclave

Autoclaves are mostly used for the sterilization of medical or laboratory equipment with the capacity of sterilizing a large number of materials at once.

They are commonly used for the preparation of culture media during laboratory applications.

Bunsen burner

It is commonly used for processes like sterilization, combustion, and heating. In medical or microbiology laboratories, it is commonly used for micro-loop sterilization.

Centrifuge

The primary application of a centrifuge is the separation of particles suspended in a suspension. It can be used for the separation of cell organelles, nucleic acid, blood components, and separation of isotopes.

Colony Counter

A colony counter is primarily used for counting the number of colonies present on a culture plate to estimate the concentration of microorganisms in liquid culture.

Deep Freezer

A deep freeze can be used for the preservation of different things used in the laboratories for a very long period of time. Deep freezers are used in laboratories to store and preserve medical equipment, food items, blood samples, medicines, and injections, etc. for a more extended period of time.

Homogenizer

It is used in the preparation step before the extraction and purification of different macromolecules like proteins, nucleic acids, and lipids.

Hot plate

In a laboratory, hot plates are used to heat glassware and their components. They are used over water baths as in water baths might be hazardous in case of any spills or overheat.

Hot air oven

Hot air oven can be used to sterilize materials like glassware, metal equipment, powders, etc. It allows for the destruction of microorganisms as well as bacterial spores.

Incubator

Incubators have a wide range of applications including cell culture, pharmaceutical studies, hematological studies, and biochemical studies. Incubators can also be used in the steam cell research area.

Laminar Air Flow/ Laminar Hood

Laminar Hood is commonly used to conduct processes that are sensitive to contamination. It is used for experiments related to plant tissue culture and for the experiments of genetic transformation.

Magnetic Stirrer

It is usually used for mixing various liquid components in a mixture in a chemical or microbiology laboratory.

This device is used in place of other stirrers as it is noise-free and because the size of the stir bar is so tiny, there is less chance of contamination.

Microscope

Based on the type of microscopes, different microscopes are used for different purposes. They are primarily used for the observation of minute particles which cannot be observed with naked eyes.

pH Meter

A pH meter is primarily used to measure the acidity of pharmaceutical chemicals, cultures, soil, and water treatment plant. It can be used to measure the acidity level in wine and cheese during their production.

Spectrophotometer

In a microbiology laboratory, a spectrophotometer is applied for the measurement of substance concentration of protein, nucleic acids, bacterial growth, and enzymatic reactions.

Vortex Mixture/ Vortexer

Vortex mixer is mostly used for the mixing of various sample fluids in the sample tubes and also allows for the homogenization of cells and cell organelles.

Water Bath

Water baths are primarily used for heating samples under a controlled temperature. These are suitable for heating chemicals that might be flammable under direct ignition.

Water Distiller

It is used to obtain distilled water required for many lab tests as well as for the preparation of culture media.

- Topic 2: Material for sample culturing

Culture medium or growth medium is a liquid or gel designed to support the growth of microorganisms. There are different types of media suitable for growing different types of cells. Here, we will discuss microbiological cultures used for growing microbes, such as bacteria or yeast.

A. Types of culture media

Culture media are solutions containing all of the nutrients and necessary physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium and, in fact, many can't grow in any known culture medium. Organisms that cannot grow in artificial culture medium are known as obligate parasites. Mycobacterium leprae, rickettsias, Chlamydias, and Treponema pallidum are obligate parasites.

Bacterial culture media can be distinguished on the basis of composition, consistency and purpose.

A.1. Classification based on consistency

Media are classified into solid, semi-solid and broth (liquid) media.

Solid medium

Solid medium is media containing agar (at a concentration of 1.5-2.0%) or some other, mostly inert solidifying agent. Solid medium has physical structure and this allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks).

Solid medium is useful for isolating bacteria or for determining the characteristics of colonies. e.g. Plate Count Agar, Potato Dextrose Agar, Baird Parker Agar, etc.

Semi solid media

They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of microaerophilic bacteria or for determination of bacterial motility. e.g. Rappaport Vassiliadis Agar.

Liquid (Broth) medium

These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. eg. Sugar fermentation tests, MR-VR broth.

A.2. Classification based on the basis of composition

Synthetic or chemically defined medium

A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known. For microorganisms, it provides trace elements and vitamins required by the microbe and especially a defined carbon and nitrogen source. Glucose or glycerol are often used as carbon sources, and ammonium salts or nitrates as inorganic nitrogen sources

Non synthetic or chemically undefined medium

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Synthetic medium may be simple or complex depending up on the supplement incorporated in it. A simple non-synthetic medium is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors whereas complex non-synthetic medium support the growth of more fastidious microorganisms.

It has some complex ingredients, such as yeast extract, which consists of a mixture of many, many chemical species in unknown proportions. Undefined media are sometimes chosen based on price and sometimes by necessity – some microorganisms have never been cultured on defined media.

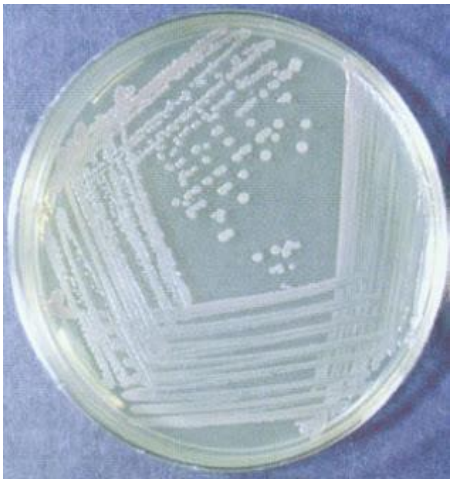
A.3. Classification based on the basis of purpose/ functional use/ application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

They are classified as follows:

General purpose media/ Basic media

Basal media are basically simple media that supports most non-fastidious bacteria. **Peptone water, nutrient broth** and **nutrient agar** considered **basal medium**. These media are generally used for the primary isolation of microorganisms.



Picture 1: Nutrient agar plate

Addition of extra nutrients in the form of blood, serum, egg yolk to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope etc are few of the enriched media.

Blood agar is preparing by adding 5-10% (by volume) to a basal medium such as nutrient agar or other blood agar bases. Chocolate agar is also known as heated blood agar or lysed blood agar.

Selective and enrichment media

Selective and enrichment media are designed **to inhibit unwanted commensal or contaminating bacteria** and **help to recover pathogen from a mixture of bacteria**. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose.

Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.

Selective medium

Principle: Differential growth suppression

Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., they select for certain microbes). Solid medium is employed with selective medium so that individual colonies may be isolated.

Examples of selective media include:

- Thayer Martin Agar used to recover N.gonorrhoeae contains Vancomycin, Colistin and Nystatin.
- Mannitol Salt Agar and Salt Milk Agar used to recover S.aureus contain 10% NaCl. Potassium tellurite medium used to recover C.diphtheriae contains 0.04% Potassium tellurite.

- McConkey's Agar used for Enterobacteriaceae members contains Bile salt that inhibits most gram positive bacteria

Picture 2: McConkey's Agar with culture

- Pseudosel Agar (Cetrimide Agar) used to recover P.aeruginosa contains cetrimide (antiseptic agent).
- Crystal Violet Blood Agar used to recover S.pyogenes contains 0.0002% crystal violet.
- Lowenstein Jensen Medium used to recover M.tuberculosis is made selective by incorporating Malachite green.
- Wilson and Blair's Agar for recovering S.typhi is rendered selective by the addition of dye Brilliant green.
- Selective media such as TCBS Agar used for isolating V. cholerae from fecal specimens have elevated pH (8.5-5.6), which inhibits most other bacteria.

Enrichment culture/ Medium

Enrichment medium is used **to increase the relative concentration of certain microorganisms** in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as broth medium.

Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. Selenite F broth, tetrathionate broth and alkaline peptone water are used to recover pathogens from fecal specimens.

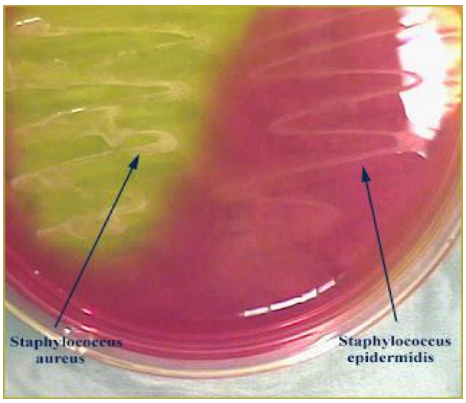
Differential/ indicator medium: Differential appearance

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies.

Such media are called differential media or indicator media Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Examples of differential media include:

- Mannitol salts agar (mannitol fermentation = yellow)



Picture 3: Mannitol salts agar (mannitol fermentation = yellow)

- Blood agar (various kinds of hemolysis i.e. α, β and γ hemolysis)
- Mac Conkey agar (lactose fermenters, Pink colonies whereas non- lactose fermenter produces pale or colorless colonies.
- TCBS (Vibrio cholera produces yellow colonies due to fermentation of sucrose)

Transport media

Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria.

Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors:

- Cary Blair medium and Venkatraman Ramakrishnan (VR) medium are used to transport feces from suspected cholera patients.
- Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.
- Pike's medium is used to transport streptococci from throat specimens.

Anaerobic media

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients.

Picture 4: Anaerobic medium

Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.

Assay media

These media are used for the assay of vitamins, amino acids and antibiotics. E.g. antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique.

Other types of medium includes Media for Enumeration of Bacteria, Media for characterization of Bacteria and Maintenance media.

- Topic 3: Selection of solid culture media for enumeration
  - Blood Agar. Most commonly used medium. 510% defibrinated sheep or horse blood is added to melted agar at 45-50°C. Blood acts as an enrichment material and also as an indicator.
  - Nutrient Agar. It is solid at 37°C. 2.5% agar is added in nutrient broth. It is heated at 100°C to melt the agar and then cooled
  - Chocolate Agar or Heated Blood agar: Prepared by heating blood agar. It is used for culture of pneumococcus, gonococcus, meningococcus and Haemophilus. Heating the blood inactivates inhibitor of growths.
  - MacConkey Agar. Most commonly used for enterobac-teriaceae. It contains agar, peptone, sodium chloride, bile salt, lactose and neutral red. It is a selective and indicator medium
  - Mueller Hinton Agar. Disc diffusion sensitivity tests for antimicrobial drugs should be carried out on this media as per WHO recommendation to promote reproducibility and comparability of results.

- Topic 4: Selection of culture media for isolation

These are solid media that contain substances that inhibit the growth of all but a few bacteria but at the same time facilitate isolation of certain bacteria.

A. Solid culture media

- Tellurite Blood Agar. It is used as a selective medium for isolation of Cotynebacterium diphtheriae
- Sorbitol MacConkey Agar: Isolation of verocytotoxin-producing (enterohemorrhagic) E. coli of 0157 type (as it fails to ferment D-sorbitol)
- MacConkey Bile Salt Lactose Agar, Brilliant MacConkey Agar, Leifson's Deoxycholate-Citrate Agar (DCA), Wilson and Blair's Brilliant Green Bismuth- Sulphite Agar (BBSA), Taylor's Xylose Lysine Deoxycholate Agar (XLD), Hektoen Enteric Agar, Salmonella–Shigella Agar: Differential and media for isolation of Salmonella and Shigella from stool specimens
- Lowenstein-Jensen Medium and Middlebrook Media: Selective media for isolation of Mycobacterium tuberculosis from sputum and other samples

B. Semi-solid media

Semisolid Agar (0.05% to 0.1% Agar): Prevents convection current and allows the growth of anaerobic and micro-aerophilic organisms

C. Liquid culture media

- Tetrathionate Broth. This medium is used for isolating Salmonella from stool. It acts as a selective medium.
- Selenite F Broth. Uses and functions are same as that of tetrathionate broth.
- Gram-Negative Broth and Selenite-F Broth: Enrichment media for isolation of Shigella and Salmonella from stool samples
- Peptone Water · Basal media for preparation for carbohydrate fermentation media, To ascertain whether a bacteria is motile or non-motile, Basis for Indole test
- Modified Korthoff's Medium: Liquid media for isolation of Leptospira

- Topic 5: Selection of culture media for differentiation identification

Differential media or indicator media distinguish one microorganism type from another growing on the same media. This type of media uses the biochemical characteristics of a microorganism growing in the presence of specific nutrients or indicators (such as neutral red, phenol red, eosin y, or methylene blue) added to the medium to visibly indicate the defining characteristics of a microorganism.

A. Solid culture media

- Blood agar (used in strep tests), which contains bovine heart blood that becomes transparent in the presence of hemolytic.

- MacConkey Agar Differential media for Enterobacteriacaeae (i.e., lactose fermenting and non-lactose fermenting)
- EMB (Eosin-methylene blue) Agar. A selective and differential medium for enteric Gramnegative rods.
- Triple Sugar Iron Agar (TSI) Medium: Differentiation of various members of Enterobacteriaceae
- KIA (Kligler Iron Agar). This is a differential slope medium used in the identification of enteric bacteria

B. Semi-solid media

- Mannitol salt agar (MSA), differential for mannitol fermentation; Selective and indicator media for S. aureus
- Motility Indole Urea (MIU) Medium. This is used to differentiate enterobacteria species by their motility, urease, and indole reactions.

C. Liquid culture media

- Peptone Water Sugar Media. These indicator media are used to study 'Sugar fermentation'. 1 % solution of a sugar (lactose, glucose, mannitol etc) is added to peptone water containing Andrade's indicator in a test tube.
- Hiss's Serum Water Medium. This medium is used to study the fermentation reactions of bacteria which cannot grow in peptone water sugar media, e.g. pneumococcus, Neisseria, Corynebacterium.
- Proteose Peptone-Yeast Extraction Broth: Media for carrying out biochemical tests for anaerobes

Thioglycollate Broth and Trypticase Soy Broth: All purposes enrichment broth for anaerobes, aerobes, micro-aerophilic, and fastidious organisms

- Topic 6: Selection of material for inoculation

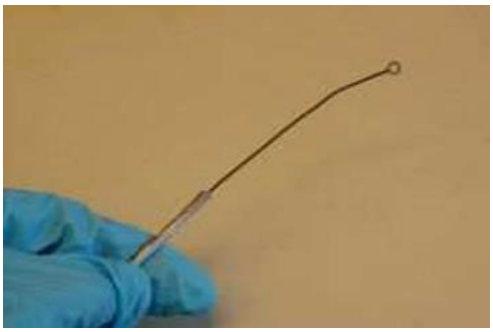
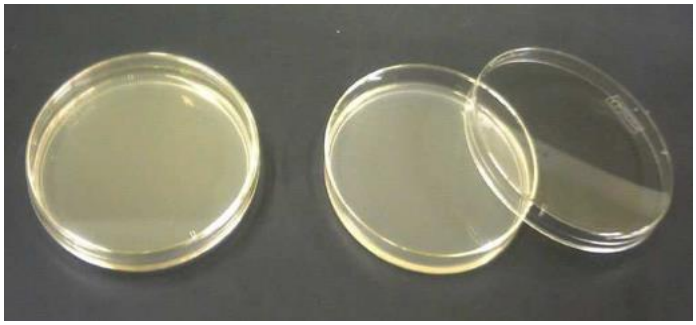
Inoculum: Suspension of microorganisms

Inoculation: Introduction of microbes into culture medium. Suspension of M.O is put in the plate Then agar is poured or M.O is mixed with agar at suitable temperature.

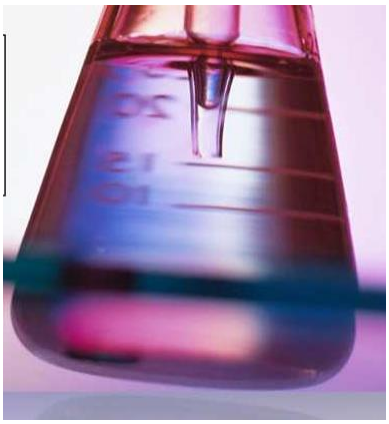
Different material should be selected such as:

- Inoculation loop
- Petri dishes
- Test tubes with or without lid
- Incubators
- Tube racks
- Prepared culture media
- Suspensions (inoculum)

- Bunsen burner (sterilization of inoculation loop)

Picture 5: Inoculation loop and Petri dishes




Picture 6: Prepared culture media and Tubes with lids

L O 2.3 – Select material and equipment for sterilization

Sterilization is necessary for the complete destruction or removal of all microorganisms (including spore-forming and non-spore forming bacteria, viruses, fungi, and protozoa) that could contaminate pharmaceuticals or other materials and thereby constitute a health hazard.

Sterilization methods

There are many methods used in sterilization of material, media and equipment for microorganism isolation and identification purposes.

Heat

In any discussion of the effect of heat upon bacteria, it must be realized that a time-temperature relationship exists in all cases. In this regard, if vegetative bacterial cells are exposed to a temperature of 55º to 58ºC for 30 minutes, all psychrophiles and mesophiles will be destroyed, while some thermophiles will survive. The process of pasteurization is based upon this time-temperature relationship. Heat may be applied in a dry or wet form.

Dry Heat: The use of dry heat to control bacteria is frequently employed in the bacteriology laboratory. The flame of the Bunsen burner is used to sterilize bacteriological loops and needles.

Contaminated materials which will burn are destroyed by burning. The hot air oven is used to dry glassware and to sterilize glassware and metalware. Sterilization is brought about by employing a temperature of 170º to 180ºC for 1 to 2 hours.

Heat labile substances such as culture media, paper, rubber, plastic items, and non-heat-resistant glassware cannot be sterilized by using the hot air oven.

Moist Heat: Control systems based upon the use of moist heat are used extensively. Moist heat rather than dry heat is used in the sterilization of culture media.

- Boiling water. The most basic system using moist heat to control bacteria is seen in the practice of placing surgical instruments in boiling water. This procedure does not always provide sterile conditions. Ideally, boiling water provides a temperature of 100ºC, and although vegetative bacterial cells are destroyed by such treatment, spores of bacteria are resistant to boiling water. Accordingly, boiling is not a recommended procedure, and it is not used in the bacteriology laboratory to establish sterile conditions.
- Free-flowing steam. Free-flowing steam finds limited use in the bacteriology laboratory as a means of sterilizing media. It must be remembered that in terms of temperature, steam is formed at 100ºC. Any sterilization procedure based upon the utilization of free-flowing steam will be limited to the destruction of vegetative cells. For this reason, media to be sterilized by tyndallization, the name given to the procedure of sterilization by the use of free-flowing steam, must be treated on three successive days. Most spores present in the media would germinate, and the resultant vegetative cells would be susceptible to destructive action of free-flowing steam. The Arnold sterilizer is based upon the free-flowing steam principle.

- Steam under pressure. At this point it should be apparent that if spores are to be destroyed along with vegetative cells, a temperature higher than 100ºC is required. Temperatures higher than 100ºC are made possible by placing steam under pressure; the autoclave is the name of the instrument whose operation is based upon steam under pressure. It is the increased temperature which destroys microorganisms; the pressure acts to increase the temperature of the steam. Usually the items to be sterilized are exposed to a temperature of 120ºC for 15 minutes at a setting of 15 pounds pressure. (Exposure time starts after the desired temperature level and pressure are reached). It must be remembered, however, that the increased temperature of the

autoclave may result in the breakdown of thermolabile substances such as urea and carbohydrates. When media containing these substances are to be sterilized, adjustments in the sterilization procedure are required. Autoclaving is the most extensively used method for sterilizing culture media.

Filtration

Sterilization by filtration represents a mechanical means of removing bacteria from liquids. When the relative size of bacteria and spores is recalled, it should be apparent that the porosity of the filter that is used must be extremely small. The Seitz filter and the membrane filter are examples of very fine filters that are used in the bacteriology laboratory. The use of these filters is recommended for the sterilization of liquids containing thermolabile substances such as carbohydrates, urea, and sera.

Radiation

Bacteria, like all living systems, are susceptible to the lethal effects of radiation. In the laboratory, ultraviolet (UV) light sources are sometimes built into isolation hoods. Special UV lighting devices are sometimes installed in rooms where highly infectious specimens, such as those from tuberculosis patients, are handled. Exposure of specimens or equipment must be direct and sufficiently prolonged. (Severe damage to the eyes can result from even a short exposure to ultraviolet rays.

Highly penetrating rays such as x-rays and gamma rays are not routinely used in the medical bacteriology laboratory).

- Topic 1: Material for culture media sterilization

Sterilization is defined as the complete destruction or elimination of all viable organisms (in or on an object being sterilized). There are no degrees of sterilization: an object is either sterile or not.

A. Heat

Heat is the most important and widely used method. For sterilization, the type of heat, time of application and temperature required to ensure destruction of all microorganisms must always be considered. Endospores of bacteria are the most thermo-resistant of all cells so their destruction usually guarantees sterility.

B. Incineration

In this process, organisms are burned and physically destroyed. It is widely used for needles, inoculating wires, glassware, tubes etc. and objects that cannot be destroyed in the incineration process.

C. Boiling

Boiling is done at >100˚C for 20-30 min. It kills everything except for some endospores. To kill endospores and therefore perfectly sterilize the solution, very long or intermittent boiling is required.

D. Autoclaving

Autoclaving is the process of using steam under pressure in an autoclave or pressure cooker. It involves heating at 121˚C for 15-20 min under 15 psi pressure and can be used to sterilize almost anything. However heat labile substances will be denatured or destroyed. Sterilization of nutrient media is usually done using this process.

E. Dry Heat (Hot Air Oven)

The process involves heating at 160˚C for 2 hours or at 170˚C for 1 hour. It is used for glassware, metal and objects that will not melt.

- Topic 2: Material for glassware sterilization

A. Hot Air Oven for Sterilization

It is used for sterilization of glassware's, such as test tubes, pipettes and petri dishes. Such dry sterilization is done only for glassware's. Liquid substances, such as prepared media and saline solutions cannot be sterilized in oven, as they lose water due to evaporation.

The glassware's are sterilized at 180°C for 3 hours. In a modern oven, there is a digital temperature display and automatic temperature controller to set the desired temperature easily. Time is set by a digital timer. After loading the glassware's, the door is closed and oven switched on.

B. Drying Oven

For preparation of certain reagents, the glassware's, after proper cleaning and rinsing with distilled water, are required to be dried. They are dried inside the drying oven at 100°C till the glassware's dry up completely.

C. Autoclaving

It is used not only to sterilize liquid substances such as prepared media and saline (diluents) solutions, but also to sterilize glassware's, when required.

- Topic 3: Equipment for sterilization

The following are sterilization equipment:

- **Bunsen burner:** used to work aseptic on the bench
- **Autoclave:** used for sterilization of glass ware and media

- **Hot Air Oven:** It is used for sterilization of glassware's, such as test tubes, pipettes and petri dishes.
- **Drying Oven:** For preparation of certain reagents, the glassware's, after proper cleaning and rinsing with distilled water, are required to be dried.

Learning Unit 3 – Apply different methods of identification

L O 3.1 – Collect sample

All samples must be collected under aseptic conditions from animals following appropriate sample collection procedure and transported to the laboratory.

- Topic 1: Blood sample collection

The collection of blood must be performed in such a manner that the animal's health is not compromised. Collection of excessive volumes of blood can lead to severe decreases in blood pressure, shock, and death. Hemostasis must be ensured.

Procedure:

- Localization of the site;
- Disinfection of the site;
- Samples must be collected aseptically by using Venoject needle and Venoject test tubes with anticoagulant (heparin);
- Shake up and down the test tubes in quick to mix blood with anticoagulant;
- Transportation of samples to the laboratory for testing;
- Proper labeling should be applied to the collection tubes.

- Topic 2: Urine sample collection

As with any type of laboratory specimen, there are certain criteria that need to be met for proper collection and transportation of urine specimens. This will ensure proper stability of the specimen and more accurate test results.

- All urine collection and/or transport containers should be clean and free of particles or interfering substances.
- The collection and/or transport container should have a secure lid and be leak-resistant. Leak-resistant containers reduce specimen loss and healthcare worker exposure to the specimen while also protecting the specimen from contaminants.

- It is good practice to use containers that are made of break-resistant plastic, which is safer than glass.
- The container material should not leach interfering substances into the specimen.
- Samples should be transported within an ice box (cool box)
- Specimen containers should not be reused.
- Proper labeling should be applied to the collection container or tubes.

- Topic 3: Collection of swabs

A sterile swab is used to collect the sample from the ear, genital tract, nasal, etc areas.

Procedure:

- Using a sterile swab collect sample aseptically;
- Put the swab in a diluent solution (if needed) or in a preservation appropriate for the selected test;
- Transportation of samples to the laboratory for testing;
- Proper labeling should be applied to the collection tubes.

- Topic 4: Collection of faeces (stool) sample

To minimize sample contamination, the sample would be taken as follows:

- With a sterile grove collect the sample from rectum
- Put the sample in sterile and appropriate container
- Keep the sample in low temperature (cool box) to reduce transformation and multiplication of microorganisms

- Topic 5: Milk sample collection

During milk sample collection, this procedure must be used:

- Wash teats only if necessary;
- Disinfect teat with 70 % ethyl alcohol or teat dip solutions;
- Discard first few squirts of milk and milk into sterilized test tubes;
- Label all samples with a permanent marker
- Samples are kept in a cool box for transportation;
- Store samples in a refrigerator at $4^{o}C$ if the culturing is not done on the same day.

- Topic 6: Collection of mucus sample (sputum)

For the sample to be accurate it must come from deep within the lungs. This is usually thick and sticky, unlike saliva from your mouth which is watery and thin.

Procedure:

- Write the identification of an animal on the sample container.
- Wash your hands with soap and water.
- Rinse the mouth with water.
- When an animal coughed up some mucus, open the sample container. Carefully collect the mucus by swab into the container.
- Screw the lid on.
- Repeat Steps 3 to 5 every 10 to 15 minutes over the next hour.
- Make sure the lid is screwed on tightly.
- Write time you started collecting samples on the sample container.
- Transport the sample to the laboratory as soon as possible.

- Topic 6: Collection of lymph sample

The use of small plastic cannulae enabled to collect lymph from the major lymph ducts of unanaesthetised rats. Surgical techniques for the construction of chronic lymphatic fistulae in sheep so that, mammary, intestinal, hepatic and thoracic duct lymph could be collected for long periods of time, under physiological conditions.

Procedure:

- After shaving the fur from the designated anatomical area, an incision is made through the skin, exposing the underlying fascia and muscle layers.
- Collecting lymphatic vessels are identified as the pale vessels running in parallel to small arterial and vein vessels under the dissecting microscope.
- Fat tissue, particularly abundant around the mesenteric vessels, but less so in other locations, is carefully removed, and ~1-cm length of the lymphatic vessel is securely tied/ligated at both the proximal and distal ends using nylon thread
- Once the lymphatic is securely ligated, the vessel is cut at the proximal and distal ends above the ligatures and then transferred to the custom-made vessel chamber which holds a glass pipette (tip diameter of 40–60 mm) connected to the polyethylene tubing attached to the male Luer stop.
- The chamber as well as the glass pipette are prefilled with warm 13 PBS supplemented with 13 protease inhibitor mixture solution for proteomic, lipidomic, and metabolomic studies or 13 MEM with 10% FBS for FACS analysis of immune cells.

Two strategies were used to collect lymphatic fluid:

- In the first strategy, the knot/suture in the proximal end of the vessel is removed, and the glass pipette inserted into the vessel to flush out the lymph after removing the distal knot.
- Alternatively, the lymphatic vessel is transferred into a petri dish and, upon removal of the knots, at the proximal and distal ends, the vessel is held toward the plate, and the lymphatic fluid pressed out.

Using the cannulation method, we were able to successfully collect lymphatic fluid

- Topic 7: Collection of organs and tissues sample

Samples can be send with bacterial or virus transport medium depending on the diagnostic technique, the nature of the sample and the suspected disease. It is important to contact the laboratory or to refer to the Manual of Standards for diagnostic tests and Vaccines from the OIE to know what medium to use.

Commonly used transport medium include:

- 0.04M phosphate buffer (pH 7.6),
- Glycerol-phosphate buffer.

Samples should be refrigerated at 4°C and sent on ice. Freezing samples is not recommended unless immunohistochemistry is used.

Tissue samples not more than 1 cm x 2 cm and 5 mm thick should be cut and placed into at least 10 times the volume of neutral buffered formalin. Immunohistochemistry can be done on frozen fixed tissues but samples for histopathology should not be frozen.

Procedure:

- Ensure that gloves are worn
- Make sure that all instruments and surfaces are sterilized (or use disposable products) between sub-samples
- Collect sample as soon after euthanasia as possible
- Fill two sets of tissue vials in the field: one for current project and one for institutional collection (rather than subsampling one vial later).
- Only place one tissue type in each vial

- Epigenetic studies require same tissue type for all samples in study (i.e., all muscle)
- Cut/slice up tissue sample, small size allows permeation by preservative (Note: read specific recommendations for preservative provided by manufacturer.)
- Fresh preservative may be needed if in field for extended period of time
- Don't over-fill vial with tissue, or tissue and preservative (expansion, can interfere with vial threads, etc.)

- Topic 8: Scabs sample collection

Collection - usually by physician or nursing staff:

- Skin - cleaned with 70% alcohol to remove dirt, oil and surface saprophytes
- Nails - cleaned same as for skin. Usually clipped; need to be finely minced before inoculating to media
- Hair - obtained from edge of infected area of scalp. Use a Wood's lamp (fluorescence) to help locate infected hair. Hair can be obtained by plucking, brushing, or with a sticky tape.
- Body fluids - normal sterile collection procedures

L O 3.2 – Isolate bacteria

- Topic 1: Preparation of material for culture

During isolation of bacteria, all materials and supplements for culture media must be sterilized (free from any microorganism). We already presented different methods used in sterilization of materials in glass, metal, culture media, etc.

- Topic 2: Preparation of culture media

Culture media are dehydrated (powder form) chemicals used in pre-enrichment, enrichment, isolation and enumeration of microorganism. The composition of the dehydrated media may vary a little among the different manufacturers.

Also, the media should be prepared according to the manufacturer's description. Some media are poured into petri plates (petri dishes) after sterilization (autoclaving) and others there is no further sterilization is required.

Procedure:

- Agar medium should be prepared from a commercially available dehydrated base according to the manufacturer's instructions.
- Immediately after autoclaving, allow it to cool in a 45 to 50°C water bath.

- Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 2 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.
- The agar medium should be allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C).
- Plates should be used within seven to days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar.
- A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer.

- Topic 3: Dilution series

Dilution series or decimal dilutions is the technique that is used in microorganism isolation and enumeration. The role of dilution is to help us in counting by diluting the number of microorganisms contained in the samples. The number of dilutions varies according to the type of the sample to analyze and how much this sample is contaminated.

Procedure:

- You take 1ml or 1gm of the original sample and pour it in 9ml of sterilized buffered peptone water in the first tube and this constitutes 10^{-1} dilution.
- Thereafter using separate sterile pipet, we took 1ml of 10^{-1} dilution and pour it in 9ml of buffered peptone water (0.1%) and this made dilution 10^{-2}.
- The process may continue till a dilution of 10^{-7} depending on the concentration of microorganisms in your sample. Each dilution with its own pipet.

- Topic 4: Inoculation of sample on culture media

Disease-producing bacteria usually occur in specimens in association with other bacteria, rather than in the pure state. In laboratory identification of microorganisms it is necessary that pure cultures (cultures containing only a single species) be studied.

To secure a pure culture of a given organism from a specimen or sample containing mixed flora, it is necessary to isolate a single cell from all other cells present. The cell is cultivated in such a manner that its collective progeny remain isolated.

Two common methods used to inoculate specimens or broth cultures to agar media, are the **streak plate method** and the **pour plate method**.

A. Streak Plate

The plate is kept in an inverted position during processing and incubation. Only the part of the Petri dish containing the medium is picked up when working with the dish in order to prevent contamination of the surface of the plating medium and therefore contamination of the specimen. This is done whether the streaking is for isolation or for sensitivity studies.

The Petri dish should be labeled with the specimen number or patient's name, and the date and time of inoculating.

Procedure:

- A loopful of inoculum is collected on a flame-sterilized wire loop and streaked over approximately one-quarter of the agar surface.
- After flaming the loop again and without collecting more inoculum, the plate is rotated slightly and another quadrant of the agar surface is streaked, overlapping the original quadrant as shown in figure below:

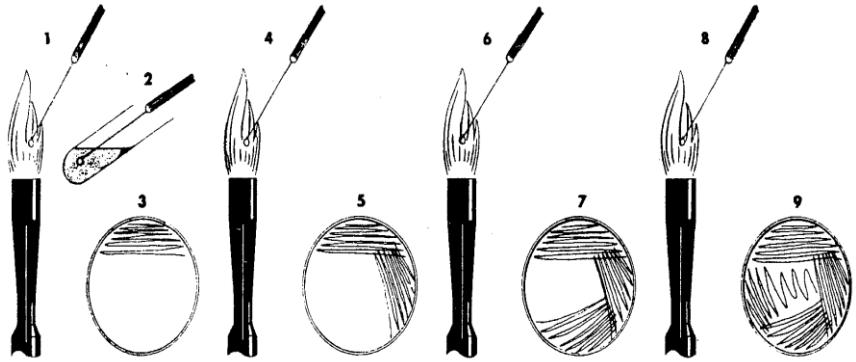


Figure 14: Streak Plate

- This process of diluting and spreading the inoculum over the medium is continued until the entire agar surface is covered. As the streaking continues, fewer and fewer cells remain on the loop, and finally single cells are deposited on the agar. Each isolated cell will give rise to a visible colony under suitable environmental conditions.
- If clinical materials on cotton swabs are to be cultured, the swab is rolled over a small area of the agar surface at the edge of the plate. With a wire loop the inoculum is spread over the four quadrants of the agar surface in the previously described manner (steps 1-9).

Interpretation of results

After inoculation, the plates are ordinarily incubated at 37ºC for 18-24 hours in the inverted position. Growth of isolated colonies is examined grossly and microscopically for characteristics of various genera and species.

Using a wire needle, pure cultures are obtained by picking growth from the center of the colony and subculturing to suitable broth or agar media.

A. Pour Plate Method

The medium is inoculated by using spread plate method; 1 ml of each dilution series is poured on the surface of the medium and spread by using a sterile spreader.

Procedure:

- Test tubes containing dilutions are placed near the flame (Bunsen burner)
- With a sterile pipet, pour 1ml from each test tube into agar plate
- By using a sterilized glass rod (spreader) spread the sample to the entire surface of the plate
- Each dilution is corresponding with its plate or in duplicate to increase the chance to have accurate results
- Incubation of plates at the favorable temperature of microorganisms to be isolated

Interpretation of results

Colonies grown on the plates after incubation may be examined morphologically, biochemically and microscopically for characteristics of various genera and species.

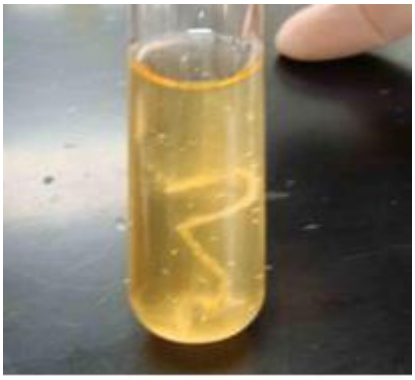
B. Test Tube Cultures

After isolated colonies are obtained by the streak plate or pour plate technique, it is often necessary to subculture growth to tubed media to permit further study.

- Tubed media are prepared by dispensing broth or agar media into appropriate test tubes.
- The tubes are plugged with small sections of rolled cotton which are bent in the middle and inserted in the tube 2.5 cm and should project at least 2 cm outside the lip of the tube. Cotton plugs should fit snugly, but not so tight that difficulty will be experienced in removing and replacing the plug during bacteriological manipulations.

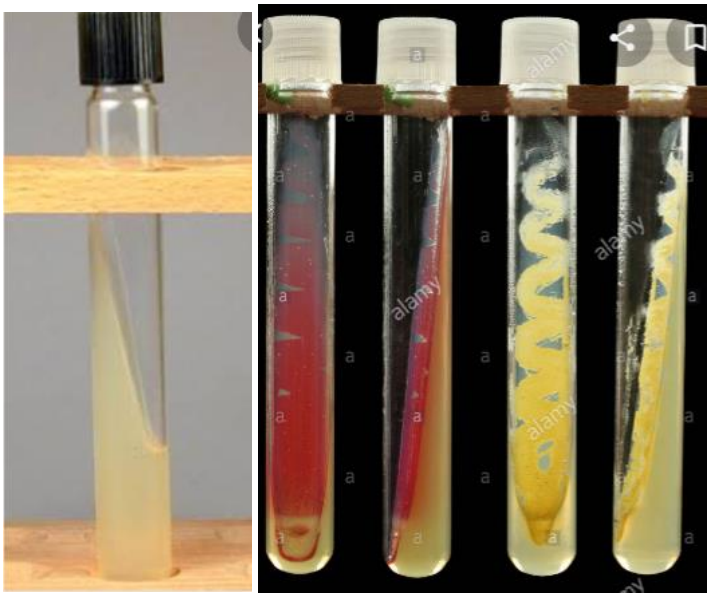
After sterilization, broth and agar stab tubes are allowed to cool in the upright position. Agar slants are prepared by inclining the tubes of melted agar medium on a table top until solidification occurs. To prepare pure cultures, each type of medium is inoculated as follows:

- **Liquid Cultures (Broth):** Using a wire loop, emulsify a small amount of growth on the moist wall of the tube just above the liquid and wash down by tilting the tube. If the inoculum is liquid, a loopful is simply placed in the broth and dispersed by gentle agitation.



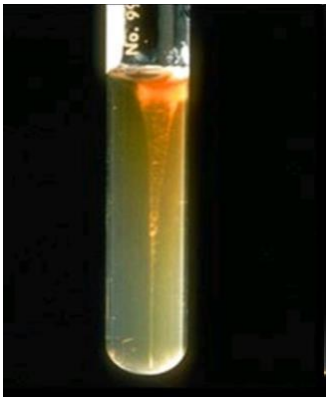
Picture 7: Liquid Cultures (Broth)

- **Slant Cultures:** Slant cultures are prepared by streaking the inoculum over the slant surface from bottom to top. If the slant contains some water of condensation at its base, do not spread the water over the surface of the slant since the resulting growth will not yield characteristic colony appearance.



Picture 8: Slant Cultures

- **Stab Cultures:** Stab cultures are made with a straight needle into tubes of unslanted solid or semisolid medium. The stab line, centered without lateral movement, should extend approximately one-half to two-thirds the depth of the medium.



Picture 9: Stab Cultures

- Topic 5: Identification of colonies (Read, Count colonies, identify)

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell, therefore a colony constitutes a clone of bacteria all genetically alike.

Colony morphology is a method that scientists use to describe the characteristics of an individual colony of bacteria growing on agar in a Petri dish. It can be used to help to identify them.

Colony morphology

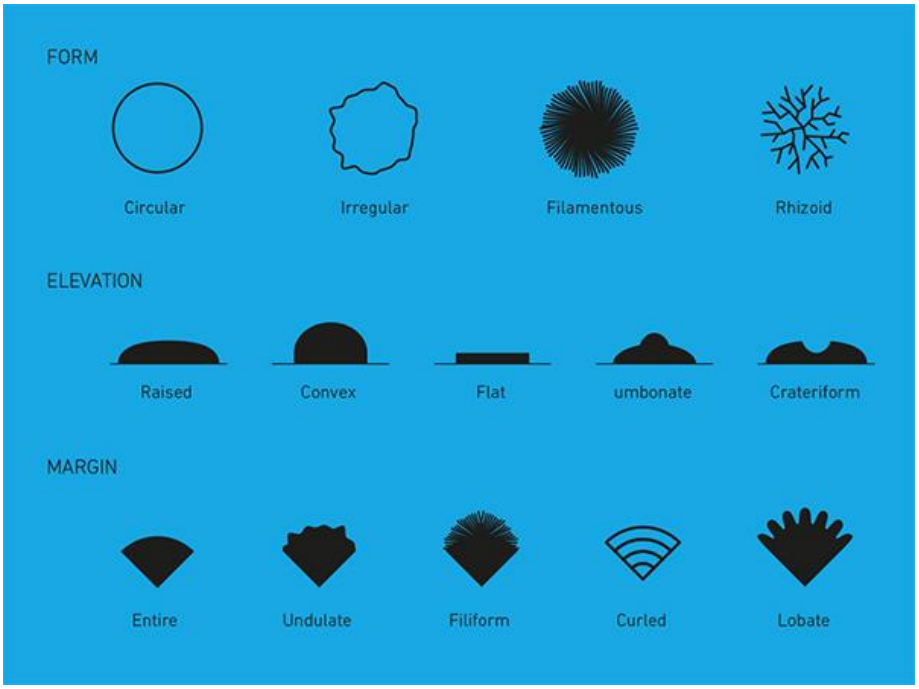


Figure 15: Colony morphology

Colonies differ in their shape, size, colour and texture. Can you count how many different colony types there are? Use the diagrams on colony morphology to help you interpret your plate.

In the identification of bacteria and fungi much weight is placed on how the organism grows in or on media. This exercise will help you identify the cultural characteristics of a bacterium on an agar plate - called colony morphology. Although one might not necessarily see the importance of colonial morphology at first, it really can be important when identifying the bacterium. Features of the colonies may help to pinpoint the identity of the bacterium. Different species of bacteria can produce very different colonies.

Figure 16: Colony morphology in petri dish

In the above picture of a mixed culture, an agar plate that has been exposed to the air and many different colony morphologies can be identified. Nine obviously different colonies are numbered: some colony types recur in various areas of the plate (note # 3 and # 4). Not only are pigment differences seen, but also size, edge, pattern, opacity, and shine. Two circles have been drawn around merging colonies, where the species of the 2 colonies are different. Trying to pick a bit of one of those adjacent colonies increases the chances of picking up another mixed culture, consisting of the 2 species that were merged together. Always pick a well-isolated colony when subculturing.

Whole shape of colony: Varies from round to irregular to filamentous and rhizoid (root-like)

Size of colony: Can vary from large colonies to tiny colonies less than 1mm = punctiform (pin-point). Measure with a millimeter rule.

Edge/margin of colony: Magnified edge shape (use a dissecting microscope to see the margin edge well)

Chromogenesis: Color of colonies, pigmentation: white, buff, red, purple, etc. Some pigments are water-soluble, others are not.

Opacity of colony: Is the colony transparent (clear), opaque (not transparent or clear), translucent (almost clear, but distorted vision–like looking through frosted glass), iridescent (changing colors in reflected light)?

Elevation of colony: How much does the colony rise above the agar (turn the plate on end to determine height)?

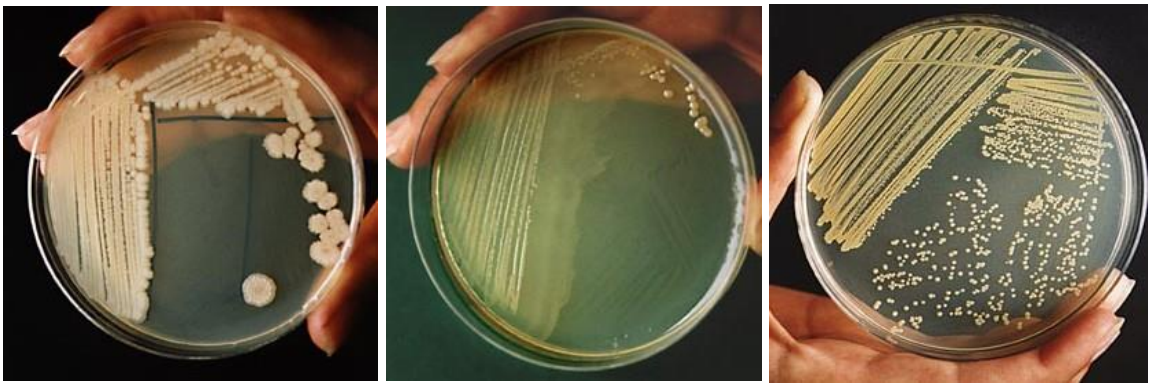
Surface of colony: Smooth, glistening, rough, dull (opposite of glistening), rugose (wrinkled)

Consistency or texture: Butyrous (buttery), viscid (sticks to loop, hard to get off), brittle/friable (dry, breaks apart), mucoid (sticky, mucus-like)

Bacteria

Each distinct circular colony should represent an individual bacterial cell or group that has divided repeatedly. Being kept in one place, the resulting cells have accumulated to form a visible patch. Most bacterial colonies appear white, cream, or yellow in color, and fairly circular in shape.

For example:



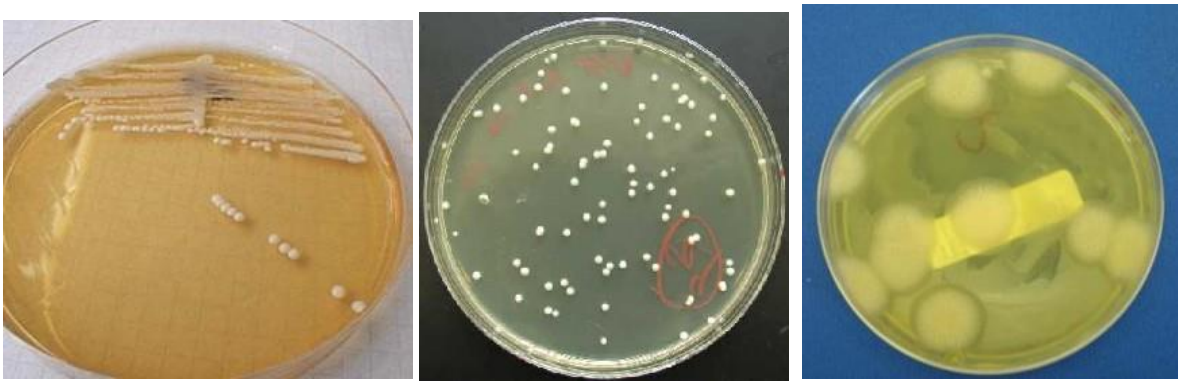
Picture 10: ***Bacillus subtilis, Proteus vulgaris and Staphylococcus aureus***

Picture 11: ***Streptococcus pyogenes***

Yeasts

Yeast, a type of fungi (plural for fungus), is found in many places from nature, to research labs and even everyday kitchens for baking. Yeast colonies generally look similar to bacterial colonies. Some species, such as Candida, can grow as white patches with a glossy surface.

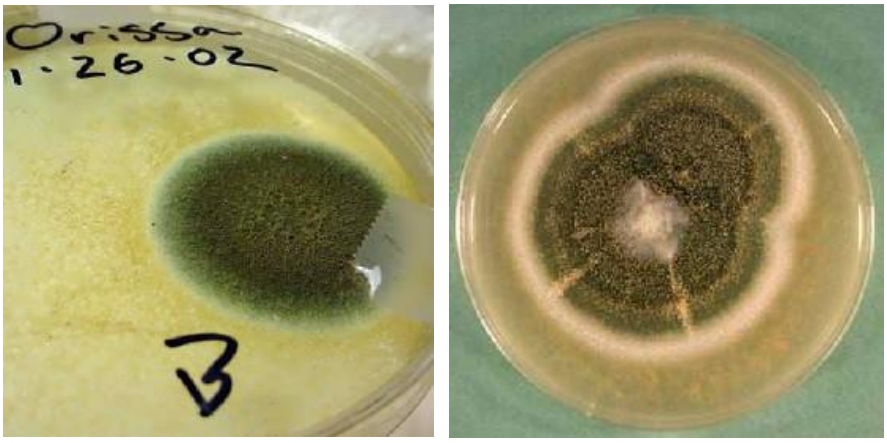
For example:



Picture 12: *Candida Albicans,* Round yeast colonies and Pink yeast colonies

Molds

Molds are actually fungi, and they often appear whitish grey, with fuzzy edges. They usually turn into a different color, from the center outwards. Two examples of molds are shown below:



Picture 13: Green Mold (*Trichoderma harzianum*) and Black Mold (*Aspergillus nidulaus*)

L O 3.3 – Use microscope

- Topic 1: Types of Microscope

The microscope (micron = small, scope = application) is an instrument that is generally used to study the very small organisms (microorganisms) or particles which are not visible by the naked eyes.

A. Light Microscope

Bright-field microscopy

The most overlooked component of a modern microscope is the condenser. It is a lens system that focuses the illuminating beam on the specimen and limits the resolving power of most microscopic applications.

The Kohler method of illumination, described below, enables one to adjust the condenser such that an evenly illuminated field is obtained (no hot or dark spots). A few seconds spent properly adjusting the condenser will greatly improve the resolving power of the microscope and is absolutely necessary for many of the more sophisticated techniques.

Dark-field microscopy (3.2X, 10X only)

Adjust as for bright-field microscopy, then swing in the phase-contrast annulus and remove the auxiliary lens. Light now strikes the specimen as a hollow cone of light with an angle too large to enter the objective. The only light that enters the objective is scattered light from particles in the specimen.

Fluorescence Light Microscope

A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities.

- The conventional microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample.
- A fluorescence microscope, on the other hand, uses a much higher intensity light source which excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source.

Fluorescent microscopy is often used to image specific features of small specimens such as microbes. It is also used to visually enhance 3-D features at small scales. This can be accomplished by attaching fluorescent tags to anti-bodies that in turn attach to targeted features, or by staining in a less specific manner.

When the reflected light and background fluorescence is filtered in this type of microscopy the targeted parts of a given sample can be imaged. This gives an investigator the ability to visualize desired organelles or unique surface features of a sample of interest. Confocal fluorescent microscopy is most often used to accentuate the 3-D nature of samples. This is achieved by using powerful light sources, such as lasers, that can be focused to a pinpoint.

These microscopes are often used for -

- Imaging structural components of small specimens, such as cells
- Conducting viability studies on cell populations (are they alive or dead?)
- Imaging the genetic material within a cell (DNA and RNA)

- Viewing specific cells within a larger population with techniques such as FISH

Phase-contrast microscopy (40X only)

A specimen can be viewed under phase contrast if it is first properly adjusted as in bright-field microscopy at 40X. Rotate the condenser auxiliary lens out of the light path. If the condenser phase annulus is now swung into position and is coupled to a phase-contrast objective (40X, Ph2), the specimen will be viewed in phase contrast.

- Topic 2: Microscope parts and structure

A. Light Microscope parts

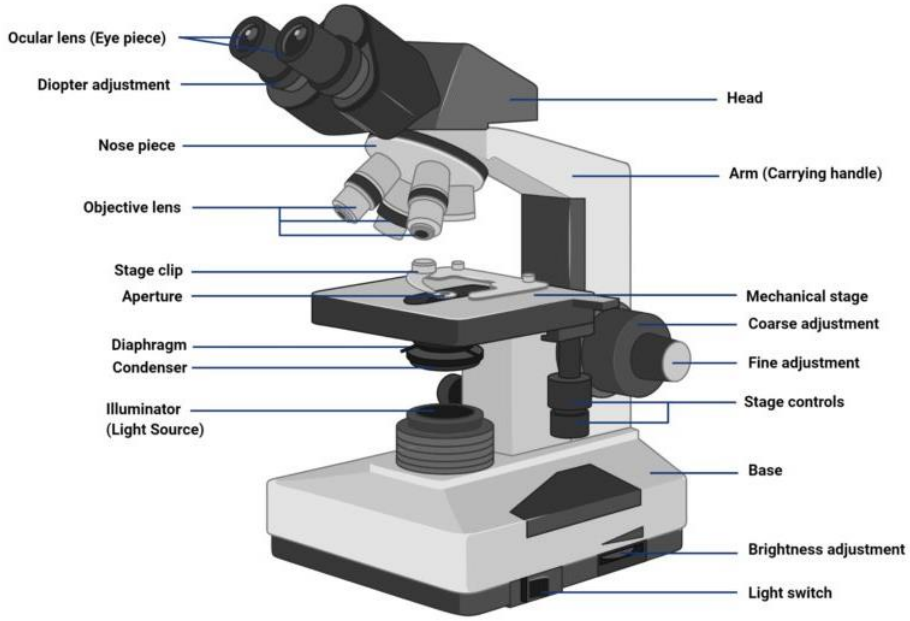


Figure 17: Light Microscope parts

Function of parts of Microscope

There are three structural parts of the microscope i.e. head, base, and arm.

1. **Head** – This is also known as the body, it carries the optical parts in the upper part of the microscope.
2. **Base** – It acts as microscopes support. It also carriers the microscopic illuminators.
3. **Arms** – This is the part connecting the base and to the head and the eyepiece tube to the base of the microscope. It gives support to the head of the microscope and it also used when carrying the microscope. Some high-quality microscopes have an articulated arm with more than one joint allowing more movement of the microscopic head for better viewing.

Optical parts of a microscope and their functions

The optical parts of the microscope are used to view, magnify, and produce an image from a specimen placed on a slide.

These parts include:

1. **Eyepiece** – also known as the ocular. This is the part used to look through the microscope. It's found at the top of the microscope. Its standard magnification is 10x with an optional eyepiece having magnifications from 5X – 30X.
2. **Eyepiece tube** – It's the eyepiece holder. It carries the eyepiece just above the objective lens. In some microscopes such as the binoculars, the eyepiece tube is flexible and can be rotated for maximum visualization, for variance in distance. For monocular microscopes, they are none flexible.
3. **Objective lenses** – These are the major lenses used for specimen visualization. They have a magnification power of 40x-100X. There are about 1- 4 objective lenses placed on one microscope, in that some are rare facing and others face forward. Each lens has its own magnification power.
4. **Nose piece** – also known as the revolving turret. It holds the objective lenses. It is movable hence it call revolve the objective lenses depending on the magnification power of the lens.
5. **The Adjustment knobs** – These are knobs that are used to focus the microscope. There are two types of adjustment knobs i.e fine adjustment knobs and the coarse adjustment knobs.
6. **Stage** – This is the section on which the specimen is placed for viewing. They have stage clips hold the specimen slides in place. The most common stage is a mechanical stage, which allows the control of the slides by moving the slides using the mechanical knobs on the stage instead of moving it manually.
7. **Aperture** – This is a hole on the microscope stage, through which the transmitted light from the source reaches the stage.
8. **Microscopic illuminator** – This is the microscopes light source, located at the base. It is used instead of a mirror. It captures light from an external source of a low voltage of about 100v.
9. **Condenser** – These are lenses that are used to collect and focus light from the illuminator into the specimen. They are found under the stage next to the diaphragm of the microscope. They play a major role in ensuring clear sharp images are produced with a high magnification of 400X and above. The higher the magnification of the condenser, the more the image clarity. More sophisticated microscopes come with an Abbe condenser that has a high magnification of about 1000X.
10. **Diaphragm** – It's also known as the iris. It's found under the stage of the microscope and its primary role is to control the amount of light that reaches the specimen. It's an adjustable apparatus, hence controlling the light intensity and the size of the beam of light that gets to the specimen. For high-quality microscopes, the diaphragm comes attached with an Abbe condenser and combined they are able to control the light focus and light intensity that reaches the specimen.

11. **Condenser focus knob** – this is a knob that moves the condenser up or down thus controlling the focus of light on the specimen.
12. **Abbe Condenser** – this is a condenser specially designed on high-quality microscopes, which makes the condenser to be movable and allows very high magnification of above 400X. The high-quality microscopes normally have a high numerical aperture than that of the objective lenses.
13. **The rack stop** – It controls how far the stages should go preventing the objective lens from getting too close to the specimen slide which may damage the specimen. It is responsible for preventing the specimen slide from coming too far up and hit the objective lens.

- Topic 3: Care and handling of microscope

A microscope is an expensive precision instrument. Proper respect and treatment will maximize its performance.

A. General care and handling

Consider the following:

- Keep the body of the microscope clean. Be especially careful when using fixatives, stains, and salt solutions on the slide. Wipe up spills with a Kimwipe immediately.
- Always use a clean slide and cover slip. This is absolutely necessary if a satisfactory image is to be obtained.
- It is sometimes necessary, in aligning or adjusting the microscope, to remove one or both of the eyepieces. However, do not leave the ocular tubes open for long periods of time or dust may get into the system. Always keep the microscope covered when not in use. Dust leads to dirty optics and a severe decrease in optical resolution. Dirt in the optical system is, without a doubt, the worst enemy of the microscopist.
- Never use anything but lens tissue to clean any of the optical parts of the microscope. Do not confuse lens tissue with filter paper or Kimwipes; there are profound differences among these types of paper. Kimwipes may be used to polish the body of the microscope, but they must never be used on any optical parts.

- Objective lenses should be used carefully; they are the most important and most expensive part of the microscope. The following cautions should be routine:

a. Always begin an observation using the 10X objective. Move from lower power to higher power objectives carefully in order to avoid ramming the objective into the slide, cover slip, or mechanical stage.

b. Since the objectives are approximately parfocal, one should move to a higher power objective only when the specimen is in focus with the lower power objective. Constantly observe the objective front lens from the side while rotating the nosepiece. Use the utmost caution to prevent damage to the front lens.

c. To clean the front lens of the objective, use a piece of lens tissue moistened with lens cleaner or distilled water; dry the lens with a clean piece of folded lens tissue. An untrained technician must never use any other cleaning agent.

Note: Never use the coarse focus adjustment while observing the specimen through the "high-dry" (40X) or oil-immersion (100X) objectives.

B. Use of the oil immersion objective:

Consider the following:

- Focus on the specimen with the high-dry (40X) objective. Make sure there is something in focus in the center of the field.
- Move the nosepiece to a position halfway between the high-dry and oil-immersion objectives; place a drop of immersion oil on the cover slip.
- Carefully move the oil-immersion objective into place and focus with the fine focus adjustment. The stage may be moved around under the oil-immersion objective.
- Once the cover slip has been "oiled" for use with the oil-immersion objective, it is absolutely necessary to remove the drop of oil before re-examining the preparation with dry objectives. This may mean, in the case of temporary mounts, preparing another slide.
- You do not need to clean the residual oil from the 100X objective during the lab. However, at the end of the lab, please do wipe the oil off the front lens of the objective using a piece of lens tissue. This will prevent oil from getting on the microscope cover and spreading to other parts of the Microscope.
- Carry the microscope with both hands: one on the arm and the other under the base of the microscope.

- Topic 4: Image focusing

Depth of Focus

The depth of focus is greatest on the lowest power objective. Each time you switch to a higher power, the depth of focus is reduced.

Therefore a smaller part of the specimen is in focus at higher power. Again, this makes it easier to find an object on low power, and then switch to higher power after it is in focus. A common exercise to demonstrate depth of focus involves laying three different colored threads one on top of the other.

As the observer focuses down, first the top thread comes into focus, then the middle one, and finally the bottom one. On higher power objectives one may go out of focus as another comes into focus.

- Topic 5: Interpretation of results

Interpretation of a microscopic image requires consideration of the original three-dimensional nature of the samples represented in this two-dimensional image.

Microscope Drawings

When drawing what you see under the microscope, follow the format shown below. It is important to include a figure label and a subject title above the image. The species name (and common name if there is one) and the magnification at which you were viewing the object should be written below the image. All relevant parts of the drawing should be labelled on the right side of the image using straight lines. Lines should not cross. Drawings should be done in pencil, while labels should be in pen or typed. Remember that total magnification is determined by multiplying the ocular x objective.

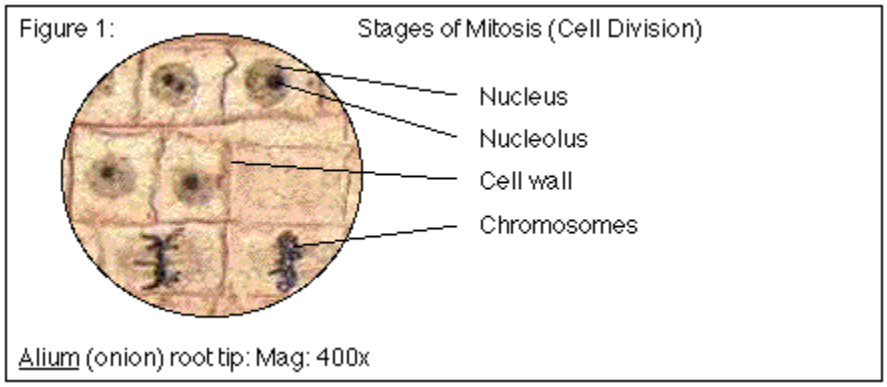


Figure 18: Example of Microscope Drawings

L O 3.4 – Identify bacteria

- Topic 1: Techniques of bacteria identification

Microorganisms are identified by using different methods. Here the main method which is being discussed is the microscopic identification. As described at the beginning, bacteria are small organisms which cannot be seen with a naked eye, the use of microscope is required.

To differentiate bacteria, there is a common used staining procedure called Gram stain that is being used for that purpose.

A. Gram stain: Gram+, Gram –

Gram Stain

The most widely used staining procedure in the bacteriological laboratory is the gram stain. The purpose of the gram stain is to differentiate bacteria on the basis of their gram-staining reaction.

Principle

Gram-positive bacteria, following initial staining with crystal violet, will retain the purple dye upon subsequent treatment with a mordant (iodine) and the application of alcohol or acetone-alcohol decolorizing agents.

Gram-negative bacteria, which lack specific cellular substances responsible for binding crystal violet, fail to retain the dye upon similar treatment. The latter forms are, therefore stained red upon application of safranin counterstain.

Reagents

Crystal violet stain:

- Make SOLUTION A by dissolving 4 g of crystal violet in 20 ml of 95 percent ethyl alcohol.
- Make SOLUTION B by dissolving 0.8 g of ammonium oxalate ((NH4)2C204 H20) in 80 ml of distilled water.
- Mix solutions A and B ordinarily in equal parts.
- This procedure may sometimes cause organisms to retain the basic dye and resist decolorization. To avoid this, solution A may be diluted as much as ten times. Then the diluted solution A is added in equal parts to solution B.

Iodine solution:

- Grind 1 gram of iodine (I2) and 2g of potassium iodide (KI) in a mortar. Dissolve the ground reagents in 5 ml of distilled water and add sufficient distilled water to make 240 ml. Add 60 ml of 5 percent sodium bicarbonate (made by adding enough distilled water to 5 g of sodium bicarbonate (NaHC03) to make 100 ml).
- When a color loss of the iodine solution is noted, prepare a fresh solution. The iodine solution will remain stable longer if stored in a dark bottle.

Decolorizer:

Mix equal volumes of 95 percent ethyl alcohol and acetone.

Safranin counterstain:

Dissolve 0.5 g of safranin in a small amount of distilled water. Add sufficient distilled water to make 100 ml. (All stains may be available as prepared solutions or in powder form stated above).

Technique

Preparation of slide:

- By an inoculating loop 1 drop of water, and put it on clean slide;

- Select an isolated colony to be tested in that drop and spread by using that loop and fix the firm.

Staining:

- Cover the heat-fixed smear with crystal violet solution, allowing the stain to remain for 1 minute.
- Remove all stains with a gentle flow of water.
- Cover the smear with iodine solution (mordant) for at least 1 minute.
- Wash with water.
- Treat with decolorizer solution by flowing the reagent dropwise over the smear while the slide is held at an angle. Decolorization should be stopped as soon as the drippings from the slide become clear.
- Remove excess decolorizer immediately with a gentle flow of water.
- Apply the safranin counterstain for 30 seconds.
- Remove counterstain with gentle flow of water and gently blot dry.
- Examine the smear microscopically using the oil immersion objective.

B. Ziehl-Neelsen stain

It is the differential staining techniques which was first developed by Ziehl and later on modified by Neelsen. So this method is also called ***Ziehl-Neelsen staining*** techniques. Neelsen in 1883 used Ziehl's carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques was developed.

The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups.

This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus *Mycobacterium*, are resistant and can only be visualized by acid-fast staining.

Principle of Acid-Fast Stain

When the smear is stained with carbol fuchsin, it solubilizes the lipoidal material present in the Mycobacterial cell wall but by the application of heat, carbol fuchsin further penetrates through lipoidal wall and enters into cytoplasm. Then after all cell appears red. Then the smear is decolorized with decolorizing agent (3% HCL in 95% alcohol) but the acid fast cells are resistant due to the presence of large amount of lipoidal material in their cell wall which prevents the penetration of decolorizing solution. The non-acid fast organism lack the lipoidal material in their cell wall due to which they are easily decolorized, leaving the cells colorless. Then the smear is stained with counterstain, methylene blue. Only decolorized cells absorb the counter stain and take its color and appears blue while acid-fast cells retain the red color.

Table 2: Summary of Acid-Fast Stain

Application	Reagent	Cell colour	
		Acid fast	Non-acid fast
Primary dye	Carbol fuchsin	Red	Red
Decolorizer	Acid alcohol	Red	Colorless
Counter stain	Methylene blue	Red	Blue

Procedure of Acid-Fast Stain

- Prepare bacterial smear on clean and grease free slide, using sterile technique.
- Allows mearto air dry and then heat fix.
- Alcohol-fixation: This is recommended when the smear has not been prepared from sodium hypochlorite (bleach) treated sputum and will not be stained immediately. *M. tuberculosis* is killed by bleach and during the staining process. Heat-fixation of untreated sputum will not kill M. tuberculosis whereas alcohol-fixation is bactericidal.
- Cover the smear with carbol fuchsin stain.
- Heat the stain until vapour just begins to rise (i.e. about 60 C). Do not overheat. Allow the heated stain to remain on the slide for 5 minutes.

Heating the stain: Great care must be taken when heating the carbol fuchsin especially if staining is carried out over a tray or other container in which highly flammable chemicals have collected from previous staining. Only a small flame should be applied under the slides using an ignited swab previously dampened with a few drops of acid alcohol or 70% v/v ethanol or methanol. Do not use a large ethanol soaked swab because this is a fire risk.

- Wash off the stain with clean water. Note: When the tap water is not clean, wash the smear with filtered water or clean boiled rainwater.
- Cover the smear with 3% v/v acid alcohol for 5 minutes or until the smear is sufficiently decolorized, i.e. pale pink. *Caution:* Acid alcohol is flammable, therefore use it with care well away from an open flame.
- Wash well with clean water.
- Cover the smear with malachite green stain for 1–2 minutes, using the longer time when the smear is thin.
- Wash off the stain with clean water.

- Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry (do not blot dry).
- Examine the smear microscopically, using the 100 X oil immersion objective.

C. Microscopic observation

Viewing Prepared Slides

You can only view one at a time, so that's all you should be holding. Return it before getting another, and if you break it, tell your instructor so that it can be properly cleaned up and replaced!

- Start by rotating the objective lens to lowest power.
- Place a slide on the stage, label side up, with the coverslip centered.
- On LOW POWER ONLY, use the coarse focus knob to get the object into focus.
- If you cannot see anything, move the slide slightly while viewing and focusing.
- If nothing appears, reduce the light and repeat step 4.
- Once in focus on low power, center the object of interest by moving the slide.
- Rotate the objective to the medium power and adjust the fine focus only.
- If needed, rotate the objective to the high power and adjust fine focus only.

- Topic 2: Interpretation of results

A. Gram staining

Interpretation

Most bacteria may be placed in one of two groups by their reaction to the gram stain. If an organism retains crystal violet (cells purple or blue), it is referred to as **gram-positive**. Organisms, which lose crystal violet stain under treatment with the decolorizing agent and are stained red upon applying the safranin counterstain, are termed **gram-negative**.

Limitations

Gram-positive organisms may become gram-negative as a result of autolysis, aging, acidity of culture medium, improper temperature of incubation, or the presence of toxic substances (drugs, metabolic wastes, and so forth).

For best results, gram stains should be prepared on cultures 18-24 hours old. A known gram-positive organism may be used as a control.

- If films are prepared unevenly, or excessively thick, dense clumps of growth will be present which retain crystal violet upon decolorization, regardless of the gram reaction. Under these conditions, gram-positive appearing clumps may be present in an otherwise gram-negative smear.

- Smears should be completely dry before being heat-fixed; otherwise, any protein material carried over into the smear from culture media or specimens will be precipitated. As a result, the background of the smear will be difficult to decolorize and may be filled with debris and misleading artifacts.
- False decoloration of gram-positive cells may result from the use of an iodine (mordant) solution which has deteriorated. Gram's iodine solution will remain stable for longer periods of time when protected from light by storage in a dark colored bottle. When iodine (mordant) solution fades from a dark brown to light amber or yellow, it is no longer suitable for use.
- Too drastic a treatment with the decolorizing solution usually results in a false gram-negative reaction. Immediately after the drippings appear clear, when treating the smear with acetone-alcohol mixture, the slide must be washed with water to prevent over decolorization.
- Although most bacteria are either gram-positive or gram-negative, certain species exhibit a definite tendency to display both gram-positive and gram-negative forms ("gram-variable"). Usually whether positive, negative, or variable, the gram reaction is species specific.

B. Ziehl-Neelsen stain (Acid-Fast-Stain)

Interpretation

If definite bacilli are seen, report as AFB (Acid- Fast-Buffer) positive. However, it is better to report the result quantitatively as follows:

- > 10 AFB/high power field –>+++
- 1-10 AFB/high power field –> ++
- 10-100 AFB/100 high power fields –> +
- 1-9 AFB/100 high power fields –> exact number
- However if no AFB is seen, write the result as 'no AFB seen' and never write negative.

Limitation of AFB Microscopy

- Does not distinguish between viable and dead organisms: Follow-up specimens from patients on treatment may be smear positive yet culture negative
- Limited sensitivity: High bacterial load 5,000-10,000 AFB /mL is required for detection (In contrast, 10 to 100 bacilli are needed for a positive culture).
- Limited specificity: All mycobacteria are acid fast. Does not provide species identification

- Topic 3: Drug susceptibility testing (Antibiogram)

A true antibiotic is an antimicrobial chemical produced by microorganisms against other microorganisms. Mankind has made very good use of these antimicrobials in its fight against infectious disease.

Many drugs are now completely synthetic or the natural drug is manipulated to change its structure somewhat, the latter called semi-synthetics. The antimicrobial activity is depending on the type of microorganisms.

The Kirby-Bauer test for antibiotic susceptibility, **called the disc diffusion test**, is a standard that has been used for years. This test is used to determine the resistance or sensitivity of aerobes or facultative anaerobes to specific chemicals, which can then be used by the clinician for treatment of patients (animals) with bacterial infections. The presence or absence of an inhibitory area around the disc identifies the bacterial sensitivity to the drug.

The basics are easy: The bacterium (**pure culture**) is swabbed on the agar and the antibiotic discs are placed on top. The antibiotic diffuses from the disc into the agar in decreasing amounts the further it is away from the disc. If the organism is killed or inhibited by the concentration of the antibiotic, there will be **No growth** in the immediate area around the disc: This is called the **zone of inhibition**. The zone sizes are looked up on a standardized chart to give a result of sensitive, resistant, or intermediate.

The Mueller-Hinton medium being used for the Kirby-Bauer test is very high in protein.

A. Preparation of Müeller-Hinton Agar

Müeller-Hinton agar (MH) preparation includes the following steps:

- Müeller-Hinton agar should be prepared from a commercially available dehydrated base according to the manufacturer's instructions.
- Immediately after autoclaving, allow it to cool in a 45 to 50°C water bath.
- Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.
- The agar medium should be allowed to cool to room temperature and, unless the plate is used the same day, stored in a refrigerator (2 to 8°C).
- Plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize drying of the agar.
- A representative sample of each batch of plates should be examined for sterility by incubating at 30 to 35°C for 24 hours or longer.

B. Preparation of inoculum

Follow the steps below:

- Using a sterile inoculating loop or needle, touch four or five isolated colonies of the organism to be tested.
- Suspend the organism in 2 ml of sterile saline (physiological solution).
- Vortex the saline tube to create a smooth suspension.
- Adjust the turbidity of this suspension by adding more organism if the suspension is too light or diluting with sterile saline if the suspension is too heavy.
- Use this suspension within 15 minutes of preparation.

C. <u>Inoculation of the MH plate</u>

Procedure:

Step1: Dip a sterile swab into the inoculum tube.

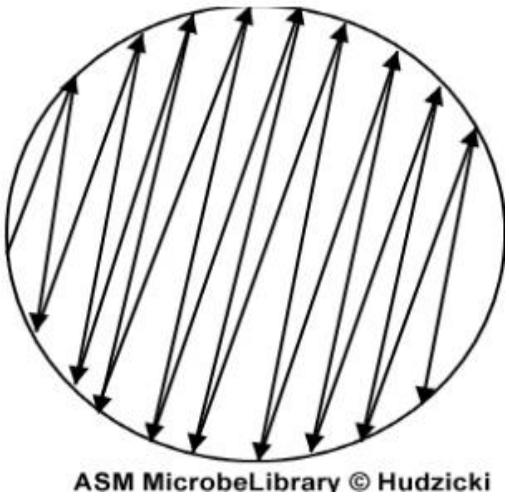
- Rotate the swab against the side of the tube (above the fluid level) using firm pressure, to remove excess fluid. The swab should not be dripping wet
- Inoculate the dried surface of a MH agar plate by streaking the swab three times over the entire agar surface; rotate the plate approximately 60 degrees each time to ensure an even distribution of the inoculum
- Rim the plate with the swab to pick up any excess liquid
- Discard the swab into an appropriate container.

Leaving the lid slightly ajar, allow the plate to sit at room temperature at least 3 to 5 minutes, but no more than 15 minutes, for the surface of the agar plate to dry before proceeding to the next step.

Picture 14: Kirby-Bauer disk diffusion susceptibility test protocol, inoculation of the test plate

Step 2. Rotate the swab against the side of the tube while applying pressure to remove excess liquid from the swab prior to inoculating the plate.

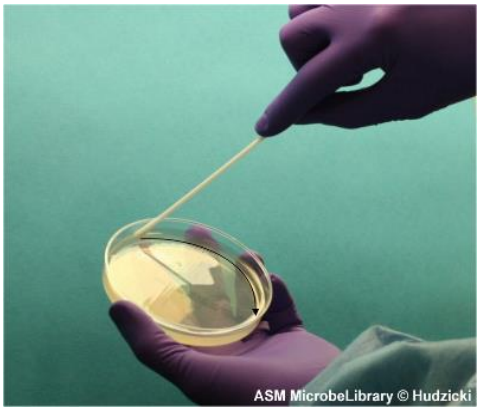
Kirby-Bauer disk diffusion susceptibility test protocol, inoculation of the Mueller-Hinton agar plate.



Step 3.

(A) Inoculate the plate with the test organism by streaking the swab in a back-and-forth motion very close together as you move across and down the plate. Rotate the plate 60° and repeat this action. Rotate the plate once more and repeat the streaking action. This ensures an even distribution of inoculum that will result in a confluent lawn of growth.

(B) Diagram illustrating the pattern the swab should follow as it is drawn across the plate..



Picture 15: Kirby-Bauer disk diffusion susceptibility test protocol, inoculation of the Mueller-Hinton agar plate

Step 4: After streaking the Mueller-Hinton agar plate as described in Step 3, rim the plate with the swab by running the swab around the edge of the entire the plate to pick up any excessive inoculum that may have been splashed near the edge. The arrow indicates the path of the swab.

D. Placement of the antibiotic disks

Place the appropriate antimicrobial-impregnated disks on the surface of the agar, using either forceps to dispense each antimicrobial disk one at a time, or a multidisk dispenser to dispense multiple disks at one time.

To use a multidisk dispenser

- Place the inoculated MH agar plate on a flat surface and remove the lid.

- Place the dispenser over the agar plate and firmly press the plunger once to dispense the disks onto the surface of the plate.
- Lift the dispenser off the plate and using forceps sterilized by either cleaning them with an alcohol pad or flaming them with isopropyl alcohol, touch each disk on the plate to ensure complete contact with the agar surface. This should be done before replacing the petri dish lid as static electricity may cause the disks to relocate themselves on the agar surface or adhere to the lid.
- Do not move a disk once it has contacted the agar surface even if the disk is not in the proper location, because some of the drug begins to diffuse immediately upon contact with the agar.

To add disks one at a time to the agar plate using forceps

- Place the MH plate on the template provided in this procedure. Sterilize the forceps by cleaning them with a sterile alcohol pad and allowing them to air dry or immersing the forceps in alcohol then igniting.
- Using the Forceps carefully remove one disk from the cartridge
- Partially remove the lid of the petri dish. Place the disk on the plate over one of the dark spots on the template and gently press the disk with the forceps to ensure complete contact with the agar surface. Replace the lid to minimize exposure of the agar surface to room air.
- Continue to place one disk at a time onto the agar surface until all disks have been placed as directed in steps f. and g. above.

Once all disks are in place, replace the lid, invert the plates, and place them in a 35°C air incubator for 16 to 18 hours. When testing Staphylococcus against oxacillin or vancomycin, or Enterococcus against vancomycin, incubate for a full 24 hours before reading.

A B C

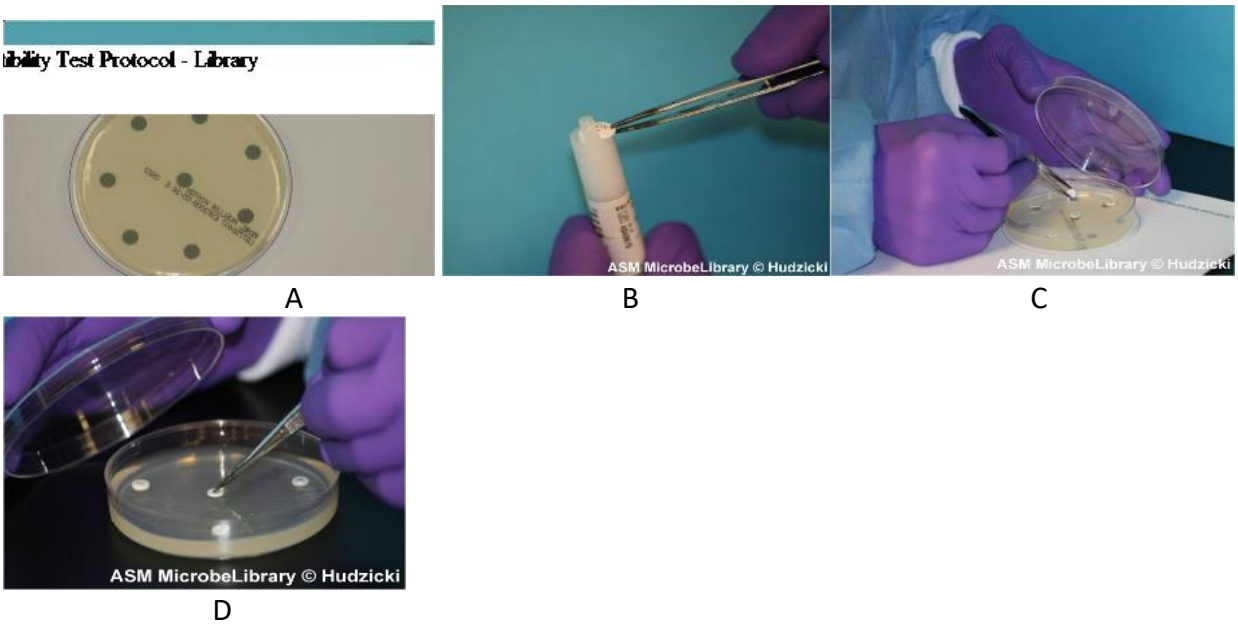
Picture 16: Placement of antibiotic disks using an automated disk dispenser

Three steps: An automatic disk dispenser can be used to place multiple disks simultaneously on a MH agar plate.

(A) Set the dispenser over the plate.

(B) Place the palm of your hand on the top of the handle.

(C) Press down firmly and completely to dispense the disks. The spring loaded handle will return to the original position when pressure is removed.



A B C

D

Picture 17: Placement of antibiotic disks using forceps to manually place the disks.

Four steps: Antibiotic disks can be manually placed on the MH agar plate if desired using a forceps:

(A) Place the Mueller-Hinton agar plate over the disk template.

(B) Remove one disk from the cartridge using forceps that have been sterilized.

(C) Lift the lid of the plate and place the disk over one of the positioning marks.

(D) Press the disk with the forceps to ensure complete contact with the agar surface. Replace the lid of the plate between disks to minimize exposure to air-borne contaminants.

Disk placement precautions

Disks should not be placed closer than 24 mm (center to center) on the MH agar plate. Ordinarily, no more than 12 disks should be placed on a 150-mm plate or more than 5 disks on a 100-mm plate.

However, the semiautomatic disk dispensers hold 16 and 8 disks respectively and may not maintain the recommended 24 mm center to center spacing.

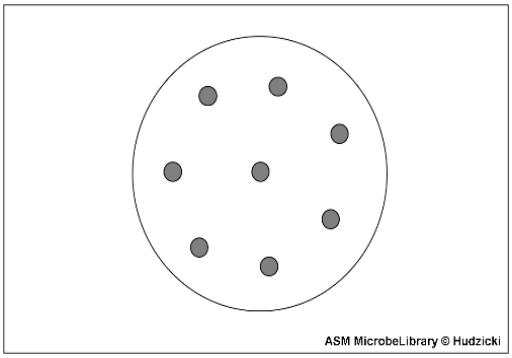


Figure 19: Manual disk placement template for eight disks on a 100-mm plate.

Place the MH agar plate on the figure above so that the edge of the plate lines up with the outer circle. Remove the lid from the plate and place one antibiotic disk on each dark gray circle. If fewer than eight antibiotics are used, adjustments can be made to the spacing of the disks.

Note:

- You should avoid placing disks close to the edge of the plate as the zones will not be fully round and can be difficult to measure.
- Each disk must be pressed down with forceps to ensure complete contact with the agar surface or irregular zone shapes may occur. If the surface of the agar is disrupted in any way (a disk penetrating the surface, visible lines present due to excessive pressure of the swab against the plate during inoculation, etc.) the shape of the zone may be affected.
- When printing the template for use in your microbiology lab, be sure that the diameter of the circle on the template is the same size as the Mueller-Hinton agar plates that you use in lab (100 mm). The "reduce" or "enlarge" function on a photocopier can be used to change the size of the template if needed. You may also make your own template by drawing a circle around a MH agar plate on a sheet of paper. Add the placement marks based on the number of disks you plan to use in your lab session, maintaining the recommended spacing as indicated above.

E. Incubation of the plates

A temperature range of 35°C ± 2°C is required.

Note: The temperatures above 35°C may not allow the detection of methicillin-resistant Staphylococcus. Do not incubate plates in CO_2 as this will decrease the pH of the agar and result in errors due to incorrect pH of the media.

Results can be read after 18 hours of incubation unless you are testing Staphylococcus against oxacillin or vancomycin, or Enterococcus against vancomycin.

Read the results for the other antimicrobial disks then reincubate the plate for a total of 24 hours before reporting vancomycin or oxacillin and others.

F. Measuring zone sizes

Following incubation, measure the zone sizes to the nearest millimeter using a ruler or caliper; include the diameter of the disk in the measurement:

- 2. When measuring zone diameters, always round up to the next millimeter.
- 3. All measurements are made with the unaided eye while viewing the back of the petri dish. Hold the plate a few inches above a black, nonreflecting surface illuminated with reflected light.
- 4. View the plate using a direct, vertical line of sight to avoid any parallax that may result in misreading.
- 5. Record the zone size on the recording sheet.
- If the placement of the disk or the size of the zone does not allow you to read the diameter of the zone, measure from the center of the disk to a point on the circumference of the zone where a distinct edge is present (the radius) and multiply the measurement by 2 to determine the diameter
- Growth up to the edge of the disk can be reported as a zone of 0 mm.
- Organisms such as *Proteus mirabilis*, which swarm, must be measured differently than non-swarming organisms. Ignore the thin veil of swarming and measure the outer margin in an otherwise obvious zone of inhibition.
- Distinct, discrete colonies within an obvious zone of inhibition should not be considered swarming. These colonies are either mutant organisms that are more resistant to the drug being tested, or the culture was not pure and they are a different organism. If it is determined by repeat testing that the phenomenon repeats itself, the organism must be considered resistant to that drug.

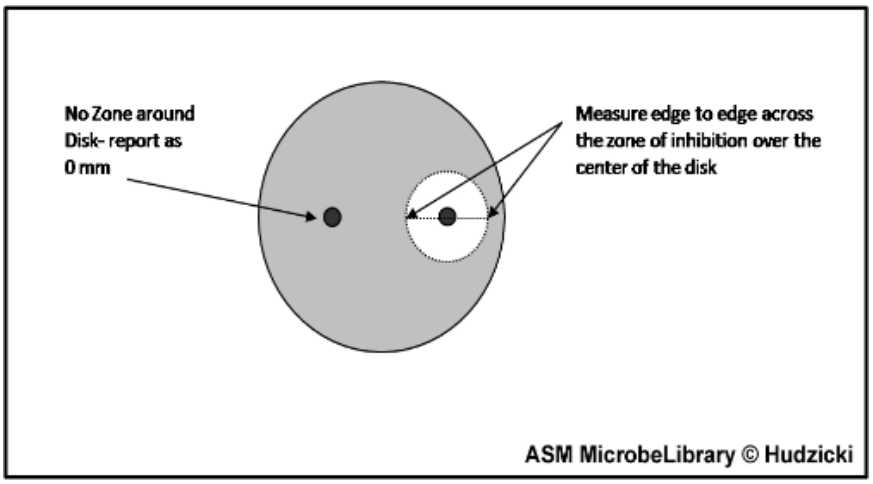
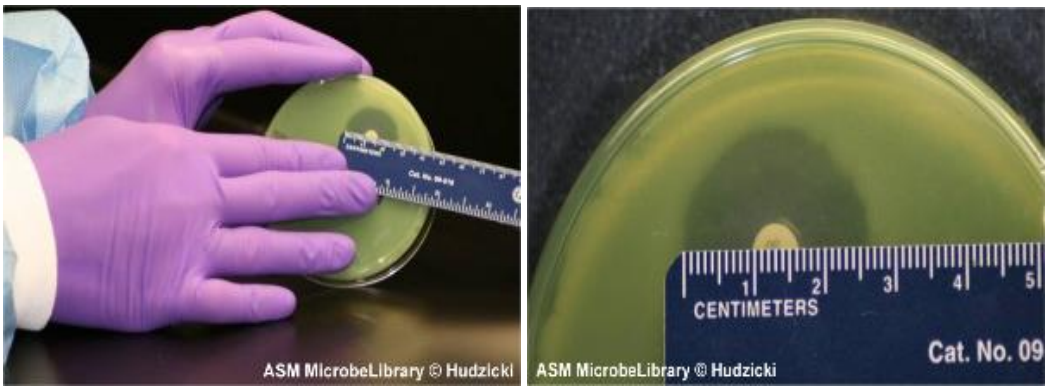


Figure 20: Measuring zones of inhibition

Gray shading represents a confluent lawn of bacterial growth. The white circle represents no growth of the test organism.



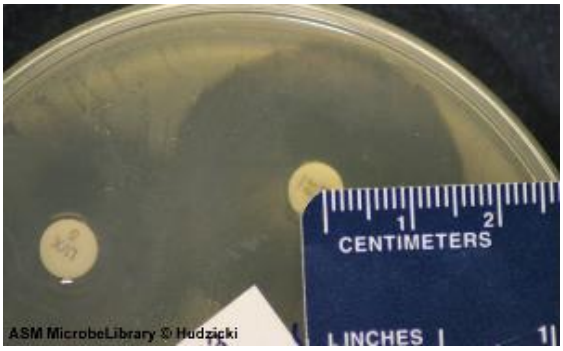
A B

Picture 18: Kirby-Bauer disk diffusion susceptibility test protocol, measuring zone sizes

(A) Using a ruler or caliper measure each zone with the unaided eye while viewing the back of the petri dish. Hold the plate a few inches above a black, nonreflecting surface illuminated with reflected light.

(B) The size of the zone for this organism-antibiotic combination is 26 mm.

Kirby-Bauer disk diffusion susceptibility test protocol, measuring zone sizes; an alternate method for measuring zones.



Picture 19: Alternate method for measuring inhibition zones.

If the zones of adjacent antibiotic disks overlap, the zone diameter can be determined by measuring the radius of the zone. Measure from the center of the antibiotic disk to a point on the circumference of the zone where a distinct edge is present.

Multiply this measurement by 2 to determine the diameter of the zone of inhibition. In this example, the radius of the zone is 16 mm. Multiply this measurement by 2 to determine the zone size of 32 mm for this organism-antibiotic combination.

If the plate was properly inoculated and all other conditions were correct, the zones of inhibition should be uniformly circular and there will be a confluent lawn of growth.

If individual colonies are apparent across the plate, the inoculum was too light and the test must be repeated.

The zone margin should be considered the area showing no obvious, visible growth that can be detected with the unaided eye. Do not use a magnification device to observe zone edges.

G. Interpretation and Reporting of the Results

- Using the guidelines, determine the susceptibility or resistance of the organism to each drug tested. Note that there are different charts for different organisms
- For each drug, indicate on the recording sheet whether the zone size is susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart.
- The results of the Kirby-Bauer disk diffusion susceptibility test are reported only as susceptible, intermediate, or resistant.

Table 3: Zones of clearing for various antibiotics

Antibiotic (and disc identifier)	**Disk potency**	**Inhibition zone diameter to nearest mm**		
		Resistant	Intermediate	Susceptible
Ampicillin (AM10) Gram-negative rods and enterococci	10 µg	11	12-13	14
Ampicillin (AM10) Staphylococci and highly penicillin-sensitive organisms	10 µg	20	21-28	29
Bacitracin (B)	10 units	8 or less	9 to 12	13 or more
Chloramphenicol (C)	30 µg	12 or less	13 to 17	18 or more
Ciprofloxacin (CIP)	5 µg	15 or less	16 to 20	21 or more
Colistin (CL)	10 µg	8 or less	09 to 10	11 or more
Doxycycline (D)	30 µg	12	13-15	16
Erythromycin (E)	15 µg	13 or less	14 to 17	18 or more
Gentimycin (G)	10 µg	12	13-14	18
Kanamycin (K30)	30 µg	13	14-17	18
Methicillin (ME5)	5 µg	9	10-13	14
Nalidixic Acid (NA)	30 µg	13 or less	14 to 18	19 or more
Oxacillin	1 µg	17	18-24	25
Penicillin G (P)	10 units			
staphylococci		28 or less	-	29 or more
most others		11 or less	12 to 21	22 or more
Polymyxin B (PB)	300 units	8 or less	9 to 11	12 or more
Streptomycin (S)	10 µg	11 or less	12 to 14	15 or more
Sulfadiazine (SD) *	300 µg	12 or less	13 to 16	17 or more
Sulfaoxizole (G300)	300 µg	12 or less	13-16	17 or more
Sulfisoxazole (G25) *	25 µg	12 or less	13 to 16	17 or more
Tetracycline (TE)	30 µg	14 or less	15 to 18	19 or more

References

1. Bauer, M. Kirby, J. C. Sherris, and M. Turck. (1966). *Antibiotic susceptibility testing by a standardized single disk method.* Am. J. Clin. Pathol. 36:493-496.
2. Clinical Laboratory Standards Institute. (2006). *Performance standards for antimicrobial disk susceptibility tests;* approved standard—9th Ed.
3. Difco. (1984). *Difco manual*, 10th ed. Difco Laboratories, Detroit, MI.
4. Winn, Jr., W., et al. (2006). *Konemann's color atlas and diagnostic text of microbiology,* 6th ed., p. 945–1021. Lippencott Williams & Wilkins Publishers, Philadelphia, PA.
5. Jorgensen, J. H., and J. D. Turnidge. (2007). *Susceptibility test methods: dilution and disk diffusion methods,* p. 1152–1172. In P. R. Murray, E. J.
6. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), (2004), *Manual of clinical microbiology,* 9th ed. ASM Press, Washington, D.C.
7. D. S. Weiss. (2004). *Molecular Microbiology- Bacterial cell division and the septal ring.* vol. 54, pp. 588-597.
8. Alberts B, Johnson A, Lewis J, et al. *Molecular Biology of the Cell.* 4th edition. (2002)
9. Jalpa P. Tewari, (2009), *Veterinary Mycology,* University of Alberta, Canada
10. Lansing M. Prescott, (2002), *Microbiology* (5th Edition)
11. Scott Mc Vey D. Melissa Kennedy and M. M. Chengappa, (2013), *Veterinary Microbiology* (3rd Edition)
12. Murphy FA. Gibbs EPJ, Horzinek MC, Studdert MJ. (1999), *Veterinary Virology*, 3rd Edition. San Diego: Academic Press, Inc.;
13. Gelderblom HR. (1996). *Structures and Classification of Viruses*. Medical Microbiology. Baron S, editor.
14. National Committee for Clinical Laboratory Standards (NCCLS) (2000), .*The Manual of Clinical Microbiology*, 8th Ed. Taken by HardyDisk® Antimicrobial Sensitivity Test (AST) Disks, 2001
15. Glen Sonder J & Karen W Post (2005). *Veterinary Microbiology*: Bacterial & Fungal Agents of Animal Diseases. Cold Spring
16. Harbor Lab. Press. Prescot LM, Harley JP & Klen DA. (2005). *Microbiology*. W. C. Brown Publ.
17. Tortora GJ, Funke BR & Case CL. (2004). *Microbiology*: An Introduction. Benjamin/Cummins Publ.
18. Acheson NH. (2006). Fundamentals of Molecular Virology. Wiley.
19. Carter J & Saunders V. (2007). *Virology*: Principles and Applications. 1st Ed. Wiley.

20. Knipe DM, Howley PM, Griffin DE. (2006). *Fields Virology*. 5th Ed. Vols. I, II. Lippincott, Williams & Wilkins.
21. Mahy BWJ & Kangaroo HO. (1996). *Virology Methods Manual*. Academic Press.
22. Murphy FA, Gibbs, EPJ, Holzmek MK & Studdert MJ. (1999). *Veterinary Virology*. 3th Ed. Academic Press.
23. Quinn PJ, Carter ME, Markey B & Carter GR. (1994). *Clinical Veterinary Microbiology*. Wolfe Publ.
24. Dwight C. Hirsh, N. James MacLachlan and Richard L. Walker, (2004), *Veterinary Microbiology*, 2nd Edition.
25. http://www.gsbs.utmb.edu/microbook/toc.htm (retrieved on 5th September, 2020)
26. http://www.virology.com (retrieved on 5th September, 2020)
27. http://www.elsevier.com/locate/vetmic/Veterinary Microbiology/ (retrieved on 5th September, 2020)
28. http://www.jb.asm.org/Journal of Bacteriology/ (retrieved on 5th September, 2020)
29. http://www.jac.oxford.journals.org/Clinical Bacteriology/ (retrieved on 8th September, 2020)
30. http://www.benthem.org/open/tomycj/The Open Mycology Journal/ (retrieved on 8th September, 2020)
31. http://www.nature.com/nrmicro/Nature Review of Microbiology/ (retrieved on 8th September, 2020)
32. http://www.trends.com/tim/Trends in Microbiology/ (retrieved on 8th September, 2020)
33. http://www.arjournals.annualreviews.org/loi/micro (retrieved on 9th September, 2020)
34. http://www.gsbs.utmb.edu/microbook/ch041.htm/Structure and Classification of viruses/ (retrieved on 9th September, 2020)
35. http://www.edusanjalmicro.blogspot.com/2010/07/types-of-bacteriological-culture-medium.html (retrieved on 9th September, 2020)
36. http://www.oie.int/en/scientificexpertise/reference-laboratories/list-of-laboratories/ (retrieved on 14th September, 2020)
37. https://veteriankey.com/sample-collection-and-preparation/ (retrieved on 14th September, 2020)

Appendices

Appendix 1: General Phenotypic Classification of Bacteria

Gram Positive Bacteria					
Name	**Morphology**	**O_2 Require-ments**	**Commens-al**	**Reservoirs / Sites of colonization, Transmission**	**Types of Infections**

Staphylococci	Cocci in grape-like clusters	facultative anaerobe	Yes	Skin, nares / endogenous, direct contact, aerosol	Soft tissue, bone, joint, endocarditis, food poisoning
Streptococci	Cocci in pairs, chains	facultative anaerobe	Some species	Oropharynx, skin / endogenous, direct contact, aerosol	Skin, pharyngitis, endocarditis, toxic shock
Pneumococci	Diplococci, lancet shaped	facultative anaerobe	±	Oropharynx, sinus / aerosol	Pneumonia, otitis, sinusitis, meningitis
Enterococci	Cocci in pairs, chains	facultative anaerobe	Yes	GI tract / endogenous, direct contact	UTI, GI, catheter-related infections
Bacilli	Rods, spore-forming	aerobic	±	Soil, air, water, animals / aerosol, contact	Anthrax, food poisoning, catheter-related infections
Clostridia	Rods, spore formers	anaerobic	Some species	GI tract, soil / Breach of skin, endogenous, ingestion	Tetanus, diarrhea, gas gangrene, botulism
Corynebac - terium	Rods, nonspore forming	facultative anaerobe	Some species	Skin	Catheter-related infections, diphtheria
Listeria	Rods, nonspore formers	facultative anaerobe	No	Animals, food products / Ingestion	Meningitis
Actinomyces	Irregular, filamentous, form sulfur granules	anaerobic	Yes	GI tract / endogenous	Skin, soft tissue

Gram Negative Bacteria

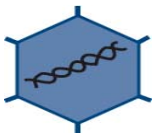
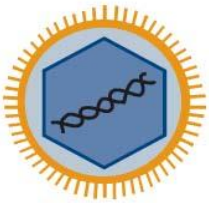
Name	**Morphology**	**O_2 Require-ments**	**Commens-al**	**Reservoirs / Sites of colonization, Transmission**	**Types of Infections**
Enterobact - eriaceae (*E. coli*, klebsiella, salmonella , shigella)	Rods	facultative anaerobe	Some species	GI tract, animals / Endogenous, fecal-oral	Diarrhea, urinary tract, food poisoning, sepsis
Bacteroides	Rods	anaerobic	Yes	GI tract / Endogenous	Abscesses, intraabdominal infections
Pseudomonas	Rods	aerobic	No	Water, soil / Endogenous, breach of skin barrier	Infections in immunocompro mised hosts, Cystic Fibrosis
Vibrio (cholera)	Rods, curved shape	microaerophil ic	No	Water / Contaminated food, water	Diarrhea
Campylobac ter	Rods, curved shape	microaerophil ic	No	Food / Ingestion of contaminated food	Diarrhea, Bacteremia
Legionella	Rods, poorly stained	microaerophil ic	No	Water / Inhalation of aerosol	Pneumonia, febrile illness
Neisseria	Cocci, kidney-bean shaped	Microaero-philic	No (*N. meningitidis* sometimes)	Humans / Sexual , aerosol	Meningitis, pelvic inflammatory disease

Hemophilus	Coccobacillary - pleomorphic	facultative anaerobe	Some species	Respiratory tract / Endogenous, aerosol	Respiratory, sinusitis, otitis meningitis
Bartonella	Small, pleomorphic rods	aerobic / microaero-philic	No	Cats, fleas, lice / cat bites, lice or fleas	Cat scratch disease, endocarditis, bacillary angiomatosis

Miscellaneous Bacteria					
Name	**Morphology**	**O_2 Require-ments**	**Commensal**	**Reservoirs / Sites of Colonization, Transmission**	**Types of Infections**
Helicobacter	GN, but not visible on Gram stain - helical (corkscrew) shaped	microaero-philic	Yes	Stomach / Endogenous, Fecal-oral	peptic ulcer disease, gastric ulcer
Mycobacteria	Rods, Weakly Gram positive, Acid fast stain positive	aerobic	No	Lungs / Fomites	Tuberculosis
Treponemes	Not visible on Gram stain, spiral shaped on dark field exam	nonculturable on routine media	No	Humans / Sexual transmission	Syphilis
Borrelia	Not visible on Gram stain, spiral shaped on dark field exam	nonculturable on routine media	No	Rodents, Ticks / Tick bites	Lyme, Relapsing fever
Mycoplasma	Not visible on Gram stain, no cell wall, pleomorphic	Non-culturable on routine media	Some species	Humans / aerosol	Respiratory tract infections
Rickettsia/ Ehrlichia	Obligate intracellular (Gram negative but not visible on Gram Stain)	Non-culturable on routine media	No	Ticks, Mites/ transmitted from the feces of infected lice, fleas, ticks	Cause a variety of illnesses including systemic vasculitis (e.g. Rocky Mountain Spotted Fever), rash, pneumonia

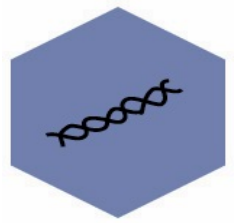
Appendix 2: Some viral families, viruses and animal species affected

	E: Enveloped NE: Nonenveloped	**Virus Family** (relative size) SS = single stranded DS = double stranded	Foreign Animal Disease (for US)	Zoonotic (Z)	**Vir**	Humans Affected	**Animal Species Affect**
DNA Virus Families							
D	NE	Adenoviridae			Bovine adenoviruses A, B, C		B

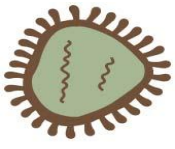
DNA		 80 – 100 nm DS linear			Canine adenovirus (infectious canine hepatitis)		C
					Caprine adenovirus		Cp
					Equine adenoviruses A, B		Eq
					Fowl adenoviruses A – E		A
					Human adenoviruses A – F (respiratory and/or ocular	H	NHP
					Ovine adenoviruses A, B, C		O
					Porcine adenoviruses A, B, C		P
DNA	E	Asfarviridae  175 – 215 nm DS linear	Φ		African swine fever		P
DNA	NE	Circoviridae  17 – 22 nm SS circular			Chicken anemia virus		A
					Porcine circovirus		P
					Psittacine beak and feather disease virus		A
DNA	E	Hepadnaviridae  42 nm partial DS circular		Z	Hepatitis B virus	H	NHP
DNA	E	Herpesviridae  150 - 200 nm DS linear	Φ		Alcelaphine herpesvirus-1 (malignant catarrhal fever)		B, Cv
					Avian herpesvirus 1 (infectious laryngotracheitis)		A
					Bovine herpesvirus 1 (infectious bovine rhinotracheitis)		B
					Bovine herpesvirus 2 (pseudo-lumpy skin disease, bovine		B
					Bovine herpesvirus 3/ bovine cytomegalovirus		B
					Canine herpesvirus 1, 2 (hemorrhagic disease of pups)		C
					Caprine herpesviruses 1, 2		Cp
					Equine herpesvirus 1 (equine viral rhinopneumonitis;		Eq
					Equine herpesvirus 2		Eq
					Equine herpesvirus 3 (equine coital exanthema)		Eq
					Equine herpesvirus 4 (equine viral rhinopneumonitis)		Eq
					Feline viral rhinotracheitis virus		F
					Human herpes simplex virus 1	H	NHP
					Human herpes simplex virus 2	H	
					Human herpesvirus 3/ varicella-zoster virus (chicken pox,	H	
					Human herpesvirus 4/ Epstein Barr virus	H	
					Human herpesvirus 5/ human cytomegalovirus	H	
					Human herpesviruses 6, 7 (roseola infantum)	H	

A=avian; B=bovine; Bt=bat; C=canine; Cp=caprine; Cv=cervine; Eq=equine; F=feline; Fr=ferret; H=human; L=lagomorph; R=rodent; NHP=non-human primate; O=ovine; P=porcine

	E: Enveloped NE: Nonenveloped	**Virus Family** (relative size) SS = single stranded DS = double stranded	Foreign Animal Disease (for US)	Zoonotic (Z)	**Virus**	Humans Affected	**Animal Species Affected**
		Herpesviridae (continued)			Ictalurid herpesvirus 1 (channel catfish virus disease)		Fish
					Koi herpesvirus disease		Fish
					Marek's disease virus		A
			Φ		Oncorhynchus masou virus disease (or salmonid herpesvirus type 2 disease)		Fish
					Ovine herpesvirus-1		O

					Ovine herpesvirus-2 (malignant catarrhal fever)		B, Cp, Cv, O, P
					Porcine herpesvirus 2/ porcine cytomegalovirus		P
					Pseudorabies virus (Aujeszky's disease)		B, C, Cp, F, O, P
DNA	NE	Iridoviridae  125 – 300 nm DS linear	Φ		Epizootic haemotopoietic necrosis (EHN)		Fish
					Largemouth bass disease		Fish
DNA	NE	Papovaviridae  45 - 55 nm DS circular			Bovine papillomavirus		B
					Equine papillomavirus		Eq
					Human papillomavirus	H	
DNA	NE	Parvoviridae  18 - 26 nm SS linear			Adeno-associated viruses 1-6	H	
					B19 virus	H	
					Canine minute virus/ canine parvovirus 1		C
					Canine parvovirus 2 ("parvo")		C
					Feline panleukopenia virus (Feline parvovirus)		F
					Porcine parvovirus		P
DNA	E	Poxviridae  250 X 200 X 200 nm DS linear		Z	Bovine papular stomatitis virus	H	B
				Z	Contagious ecthyma/contagious pustular dermatitis/orf virus	H	C, Cp, Cv
			Φ	Z	Cowpox virus	H	B, F, R
					Feline pox virus		F
					Fowlpox virus		A
			Φ		Lumpy skin disease virus		B, Bf
			Φ	Z	Monkeypox virus	H	NHP, R
				Z	Pseudocowpox virus (milker's nodules)	H	B
			Φ		Sheep and goat pox viruses		Cp, O
					Smallpox virus (Variola)	H	
					Swinepox virus		P
				Z	Vaccinia virus	H	B, L, P

A=avian; B=bovine; Bt=bat; C=canine; Cp=caprine; Cv=cervine; Eq=equine; F=feline; Fr=ferret; H=human; L=lagomorph; R=rodent; NHP=non-human primate; O=ovine; P=porcine

	E: Enveloped NE: Nonenveloped	**Virus Family** (relative size) SS = single stranded DS = double stranded	Foreign Animal Disease (for US)	Zoonotic (Z)	**Virus (Disease)**	Humans Affected	**Animal Species Affected**
RNA Virus Families							
RNA	E	Arenaviridae  110 - 300 nm SS linear segments	Φ	Z	Lassa virus	H	NHP, R
				Z	Lymphocytic choriomeningitis virus	H	C, NHP, P, R
			Φ	Z	Machupo virus (Bolivian hemorrhagic fever)	H	NHP, R

RNA	E	Arteriviridae  50 – 70 nm SS linear			Equine arteritis virus (equine viral arteritis)		Eq
					Lactate dehydrogenase elevating virus		R
					Porcine respiratory and reproductive syndrome virus		P
					Simian hemorrhagic fever virus		NHP
RNA	NE	Astroviridae  28 – 30 nm SS linear			Avian nephritis viruses 1, 2		A
					Bovine astrovirus		B
					Feline astrovirus (gastroenteritis)		F
					Human astroviruses 1-8 (gastroenteritis)	H	
					Ovine astrovirus (gastroenteritis)		O
					Porcine astrovirus (porcine acute gastroenteritis)		P
					Turkey astrovirus (poultry enteritis and mortality syndrome)		A
RNA	NE	Birnaviridae  60 nm DS linear segments			Infectious bursal disease virus		A
					Infectious pancreatic necrosis (IPN) (hemorrhagic kidney syndrome)		Fish
RNA	E	Bunyaviridae  80 – 120 nm SS linear segments	Φ		Akabane virus (Akabane/congenital arthrogryposis-hydranencephaly)		B, Cp, O
					Cache Valley virus	H	B, O
				Z	California encephalitis virus	H	R
			Φ	Z	Crimean-Congo hemorrhagic fever virus	H	A, B, C, L, O
			Φ*	Z	Hantaviruses (various serotypes)*	H	R
				Z	Jamestown Canyon virus	H	Cv
				Z	La Crosse virus (La Crosse encephalitis)	H	Cp, Cv, R
			Φ	Z	Nairobi sheep disease virus	H	Cp, O, R
			Φ	Z	Rift Valley fever virus	H	B, C, Cp, F, O
RNA	NE	Caliciviridae  30 -38 nm SS linear			Bovine enteric calicivirus		B
					Canine calicivirus		B
					Feline caliciviruses (upper respiratory disease)		F
					Fowl calicivirus		A
				Z	Hepatitis E virus	H	P
					Noroviruses (Norwalk and Norwalk-like viruses)	H	
					Porcine enteric calicivirus		P
			Φ		Rabbit hemorrhagic disease virus		L
					San Miguel sea lion virus		Other, P
			Φ	Z	Vesicular exanthema of swine virus (vesicular exanthema)	H	B, Eq, NHP, P

A=avian; B=bovine; Bt=bat; C=canine; Cp=caprine; Cv=cervine; Eq=equine; F=feline; Fr=ferret; H=human; L=lagomorph; R=rodent; NHP=non-human primate; O=ovine; P=porcine

Appendix 3: List of culture media and their uses

Media	Uses
· Alkaline Peptone Water	Enrichment media for *Vibrio cholera*
· Alkaline Salt Transport Medium · Taurocholate Peptone Transport Medium	Transport media for diarrheal diseases suspected of being caused by *V. cholerae*
· Anaerobic Media	Liquid media by addition of · Glucose (0.5 % to 1 %) · Ascorbic Acid (0.1 %)

	· Cysteine (0.1 %) · Sodium Merceptoacetate (0.1 %) · Thioglycollate (0.1 %) · Particles of cooked meat broth
· Bile Salt Agar · Thiosulphate Citrate Bile Salts-Sucrose Agar (TCBS) · Monsur's Tellurite Taurocholate Gelatin Agar	Selective media for *V. cholera*
· Bile Esculin Agar (Contains 40% Bile)	Selective media for Enterococcus species (Black coloration of the medium)
· Blood Agar	· Enriched media (Supports the growth of fastidious organisms, e.g. *Streptococcus*) · Indicator media to show hemolytic properties of certain organisms (*Staphylococcus aureus*: β-Hemolytic; *Streptococcus pneumonia* and *S viridans*: α-Hemolytic; *Enterococcus*: Non-Hemolytic)
· Bordet-Gengou Agar · Charcoal Blood Agar · Regan-Lowe Medium (Charcoal Agar with blood, cephalexin and Amphotericin B)	Isolation of *Bordetella pertussis*
· Brain Heart Infusion Broth	Used in blood culture bottles (both adult and pediatric patients)
· Buffered Charcoal Yeast Agar (BCYA) · Feeley Gorman Agar	Specialized media for isolation of *Legionella*
· *Campylobacter* Thioglycollate Broth	Selective holding media for recovery of *Campylobacter* species
· Castaneda Medium	Biphasic medium for the isolation of *Brucella*
· Cefoxitin Cycloserine Fructose Agar (CCFA) · Cefoxitin Cycloserine Egg-Yolk Agar (CCEY)	Selective media for isolation of Clostridium difficile form suspected cases of pseudomembranous colitis / antibiotic - associated diarrhea
· Cefsulodin-Irgasan-Novobiocin medium (CIN Medium)	Selective media for *Yersinia* (and may be used for *Aeromonas* also)
· Columbia-Colistin Nalidixic Acid agar (CNA Agar)	Selective media for the isolation of Gram-positive cocci

· Cooked meat broth · Nutrient agar slopes · Semisolid nutrient agar stabs · Heated blood agar slopes	In general, used for preservation and storage of bacterial cultures
· Crystal violet blood agar	Selective media for *Streptococcus pyogenes*
· Cysteine Lactose Electrolyte Deficient Media (CLED Media)	Most commonly used media for culturing urine samples
· Egg Saline Medium	Preservation of cultures of Gram-negative bacilli
· Egg Yolk Agar	Detection of lipase and lecithinase activity of *Clostridium* species
· Ellner's Medium · Medium of Duncan and Strong · Medium of Phillips · Alkaline Egg Medium	Specialized media to induce sporulation in *Clostridium*
· Fildes Blood-Digest Agar and Broth · Levinthal's Agar	Enriched media for recovery of *Haemophilus influenzae*
· Firm Agar (4% to 6% Agar)	Prevents swarming of *Proteus mirabilis, P. vulgaris* and *Clostridium tetani*
· Fletcher's Agar · Ellinghausen and McCullough Medium · Ellinghausen-McCullough-Johnson-Harris (EMJH) Media	Solid media for isolation of *Leptospira*
· Glycerol Saline Transport Medium	Transport stool specimen for typhoid bacilli
· Heated Blood Agar/Chocolate Agar	Growth of fastidious organisms (E.g. *Hemophilus influenzae*, *Neisseria gonorrhoeae* and *S. pneumoniae*
· Hoyle's Tellurite Lysed Blood Agar · Tinsdale Medium	Selective media for isolation of *Corynebacterium* from throat swabs
· Loeffler Serum Slope	Stimulation of metachromatic granules in *Corynebacterium diphtheriae*
· Lowenstein-Jensen Medium · Middlebrook Media	Selective media for isolation of *Mycobacterium tuberculosis* from sputum and other samples
· MacConkey Agar	Differential media for Enterobacteriacaeae (i.e., lactose fermenting and non-lactose fermenting)

· MacConkey Bile Salt Lactose Agar · Brilliant MacConkey Agar · Leifson's Deoxycholate-Citrate Agar (DCA) · Wilson and Blair's Brilliant Green Bismuth-Sulphite Agar (BBSA) · Taylor's Xylose Lysine Deoxycholate Agar (XLD) · Hektoen Enteric Agar · *Salmonella–Shigella* Agar	Differential and media for isolation of *Salmonella* and *Shigella* from stool specimens
· Mannitol Salt Agar	Selective and indicator media for *S. aureus*
· Modified Barbour Stoenner Kelly medium (BSK)	Specialized media for *Borrelia burgdorferi*
· Modified Korthoff's Medium	Liquid media for isolation of *Leptospira*
· Modified New York City Medium (contains colistin, lincomycin, trimethoprim, amphotericin B)	Selective media for *Neisseria gonorrhoeae*
· Mueller-Hinton Agar	Performing antimicrobial susceptibility for bacteria
· Nutrient Agar (1% to 2% Agar)	Basal media in microbiology Supports the growth of all non-fastidious organisms
· Non-Nutrient Agar	Cultivation of parasites (e.g., *Acanthamoeba*)
· Peptone Water	· Basal media for preparation for carbohydrate fermentation media · To ascertain whether a bacteria is motile or non-motile · Basis for Indole test
· Phenol-Red Egg Yolk Polymyxin Agar	Selective media for isolation of *Bacillus cereus* from food, feces, and vomitus
· Pike's Media	Preservation of *S. pyogenes*, pneumococci, and *Hemophilus influenzae* in nose and throat swabs
· Polymyxin B-lysozyme-EDTA-Thallous Acetate (PLET)	Selective media for isolation of *Bacillus anthracis* from soil and other medium materials containing numerous spore formers of other species

· Polymyxin B, Neomycin, Fusidic Acid Media (PNF)	Selective media for *S. pyogenes* (or β-hemolytic *Streptococcus*)
· PPLO Medium (Contains Sterol)	Specialized media for *Mycoplasma pneumoniae*
· Pre-Reduced Anaerobically Sterilized (PRAS) Media	Commercially available media for anaerobic organisms
· Proteose Peptone-Yeast Extraction Broth	Media for carrying out biochemical tests for anaerobes
· Requirements of X and V Factors	Isolation of *Haemophilus influenzae*
· Roswell Park Memorial Institute (RPMI) 1640 Medium	Cultivation of malarial parasites (i.e., *Plasmodium*)
· Robertson Cooked Meat Broth (RCMB)	· Growth of anaerobes (e.g., *Clostridium*) · Maintaining stock cultures of anaerobic organisms
· Salt-Cooked Meat Broth (Cooked Meat Broth with 10% NaCl)	Enrichment media for isolation of *S. aureus* from heavily contaminated materials
· Semisolid Agar (0.05% to 0.1% Agar)	Prevents convection current and allows the growth of anaerobic and micro-aerophilic organisms
· Skirrow's Campylobacter Medium (contains polymixin B, trimethoprim, vancomycin) · Preston Campylobacter Medium (contains polymixin B, rifampicin, trimethoprim) · Campy Blood Agar · CVA Medium (contains cefoperazone, vancomycin, amphotericin)	Selective media for *Campylobacter jejuni*
· Smith-Noguchi Medium	Cultivation of nonpathogenic treponemes (e.g., Reiter strain of *Treponema phagedenis*)
· Sorbitol MacConkey Agar	Isolation of verocytotoxin-producing (enterohemorrhagic) *E. coli* of 0157 type (as it fails to ferment D-sorbitol)
· Stuart Transport Media · Amies Transport Media	Maintaining the viability of gonococci on swabs during transportation
· Tetrathionate Broth · Gram-Negative Broth · Selenite-F Broth	Enrichment media for isolation of *Shigella* and *Salmonella* from stool samples
· Thayer-Martin Medium (contains vancomycin, colistin, nystatin)	Selective media for *Neisseria gonorrhoeae*
· Thioglycollate Broth	All purpose enrichment broth for anaerobes,

· Trypticase Soy Broth	aerobes, micro-aerophilic, and fastidious organisms
· Todd Hewitt Broth with Antibiotics	Selective and enrichment for *Streptococccus agalactiae* in female genital specimens
· Triple Sugar Iron Agar (TSI) Medium	Differentiation of various members of Enterobacteriaceae
· Wilkins-Chalgren Agar	Performing antimicrobial susceptibility of anaerobic bacteria