M.Sc. MEDICAL MICROBIOLOGY
LAB MANUAL
2nd & 3rd Year

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Medical Microbiology

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Microscopy

Introduction: Microscope is a powerful and crucial basic tool in the field of microbiology. The existence of microbial life to the world was introduced first by Antony Van Leeuwenhoek in 1673, with the help of simple, crude, self-made, single-lens microscope having a magnification of about 300. (In the late 16th century several Dutch lens makers designed devices that magnified objects, but in 1609 Galileo Galilei perfected the first device known as a microscope. Dutch spectacle makers Zaccharias Janssen and Hans Lipperhey are noted as the first men to develop the concept of the compound microscope.) Over the years, microscopes have evolved to increase the magnification several thousand-fold. Modern day microscopes are either light microscopes or electron microscopes. Light microscopes use either visible light to illuminate specimens. A compound light microscope is the most common microscope used in microbiology. It consists of two lens systems (combination of lenses) to magnify the image. Each lens has a different magnifying power. A compound light microscope with a single eye-piece is called monocular; one with two eye-pieces is said to be binocular. These are generally used to look at intact cells. Ideally a microscope should be parfocal; that is, the image should remain in focus when objective lenses are changed.

Microscopes that use a beam of electrons (instead of a beam of light) and electromagnets (instead of glass lenses) for focusing are called electron microscopes. These microscopes provide a higher magnification and are used for observing extremely small microorganisms such as viruses. Besides, these are generally used to look at internal structures or details of cell surface.

Common Types of Light Microscopy

Brightfield microscopy: This is the commonly used type of microscope. In brightfield microscopy the field of view is brightly lit so that organisms and other structures are visible against it because of their different densities. It is mainly used with stained preparations. Differential staining may be used depending on the properties of different structures and organisms.

Darkfield microscopy: In darkfield microscopy the field of view is dark and the organisms are illuminated. A special condenser is used which causes light to reflect from the specimen at an angle. It is used for observing bacteria such as treponemes (which cause syphilis) and leptospires (which cause leptospirosis).

Principle: When the light passes from one medium to another, refraction occurs, i.e., the ray is bent at the interface. The direction and the magnitude of bending are determined by the refractive indices of the two media forming the interface. The **refractive index** is a measure of how greatly a substance slows the velocity of light when light passes from air to glass or vice versa.

When the light rays strike the lens, a convex lens will focus these rays at specific point called **focal point**. The distance between the center of the lens and the focal point is called the **focal length**. Convex lens act as a magnifier. It provides a clear magnifying image at a much closer range. Lens strength is related to focal length. A lens with a short focal length has a more magnification power than a lens having a longer focal length.

Magnification means enlargement. In compound microscope, it is carried out by two-lens system – objective lens and ocular lens. **Objective lens** produces the real image of the specimen, which is projected up into focal plane and then magnified by the ocular lens to produce the final image, as illustrated through the light pathway of compound bright field microscope. Though magnification is important, it has limits. Unlimited enlargement by increasing magnifying power of the lenses is not possible because of the limitations of resolving power.

Microscope should not only produce the enlarged image but also the clear image. **Resolution** or **resolving power** of the lens is its ability to separate two close points as separate entities.

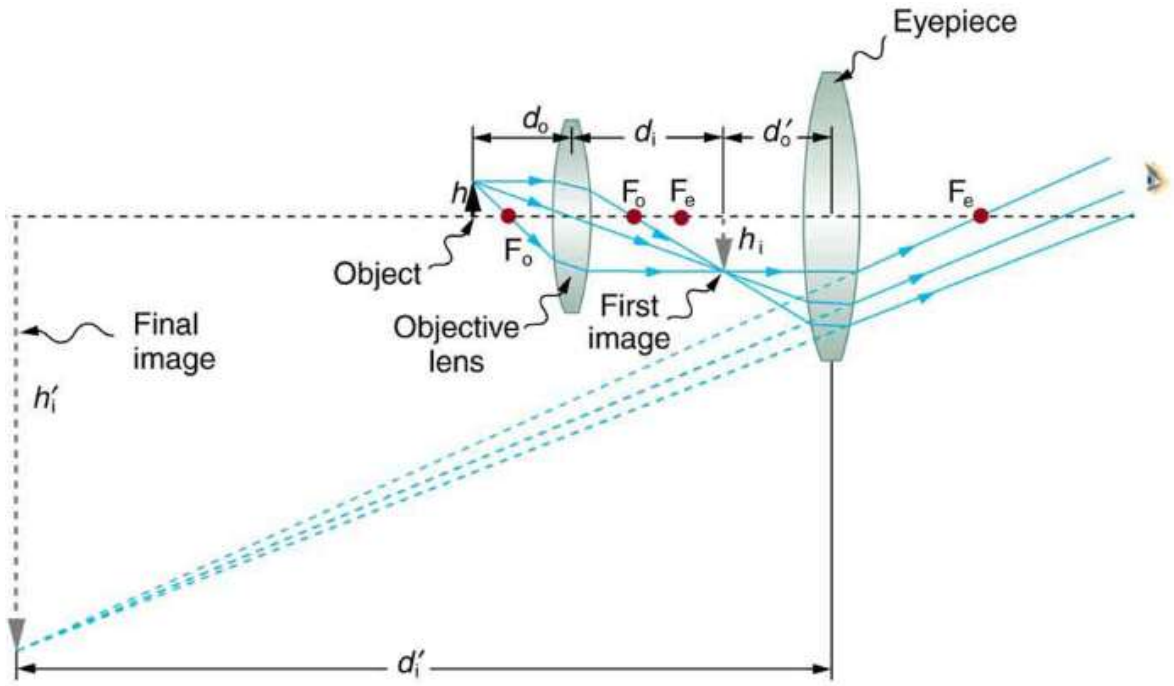
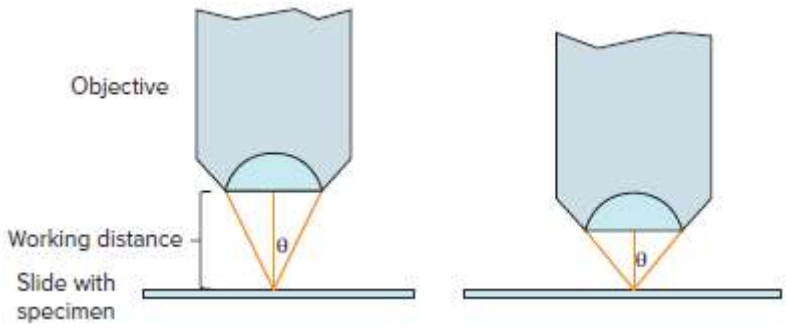


Figure: A compound microscope composed of two lenses, an objective and an eyepiece. The objective forms a case 1 image that is larger than the object. This first image is the object for the eyepiece. The eyepiece forms a case 2 final image that is further magnified.

The minimum distance (d) between two objects that reveals them as separate entities or resolving power of a lens can be calculated by Abbe's equation as:

$$d = \frac{0.5\,\lambda}{\eta sin\phi}$$

Where λ is the wavelength of the light used and η*sin* φ is the numerical aperture (NA).

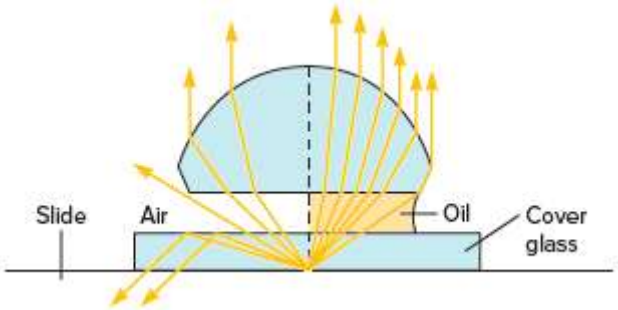


Numerical Aperture in Microscopy: The numerical aperture of a lens is related to a value called the angular aperture (symbolized by θ), which is 1/2 the angle of the cone of light that enters a lens from a specimen. The equation for numerical aperture is n sin θ. In the right-hand illustration, the lens has larger angular and numerical apertures; its resolution is greater and its working distance smaller.

Numerical aperture is a characteristic of each lens and is printed on the lens. It can be defined as a function of the diameter of the objective lens in relation to its focal length. It depends on the refractive index (η) of the medium in which the lens works and also upon the objective itself. Theta (φ) is defined as half of the angle of the cone of light entering an objective. Sin φ cannot be more than 1. Therefore, the lens working in air with refractive index 1 can have N.A.1. η will have to be increased for increasing the resolution. This can be done by using the mineral oil. Wavelength is also an important factor in resolution. With shorter wavelength, the resolving power increases. Just like magnification, resolution also has a limit. The smaller d is, the better the resolution, and finer detail can be discerned in a specimen; d becomes smaller as the wavelength of light used decreases and as the numerical aperture increases. Thus, the greatest resolution is obtained using a lens with the largest possible numerical aperture and light of the shortest wavelength, at the blue end of the visible spectrum (in the range of 450 to 500 nm). By decreasing wavelength, resolving power of the lens cannot be increased indefinitely because –

(a) the visible portion of the electromagnetic spectrum is very narrow and borders of very short wavelengths are found in ultraviolet range of spectrum.

(b) This relationship of Resolving Power with λ is valid only when light rays are parallel. As such 'Resolving Power' is also dependent on another factor, i.e., refractive index. When the light passes from air to glass slide, and from glass slide to air, there is a loss of light due to bending of rays. This reduces the numerical aperture and thus the resolving power of the lens. This loss in light can be compensated by using mineral oil in between glass slide and objective lens. Mineral oil is a colourless liquid having same refractive index as glass. This does decrease the bending of ray, as shown in Figure 1.5, so that more light rays enter the objective lens thus increasing the resolving power. Proper specimen illumination is also important in determining resolution.



Resolving Power of a microscope depends upon the numerical aperture of both condenser and objective, i.e.,

$$d_{\text{microscope}} = \frac{\lambda}{NA_{\text{Objective}} + NA_{\text{Condenser}}}$$

It was found that limit of resolving power of a microscope at best be about 0.2 μm and limit of magnification is about 1000 times the numerical aperture of the objective lens.

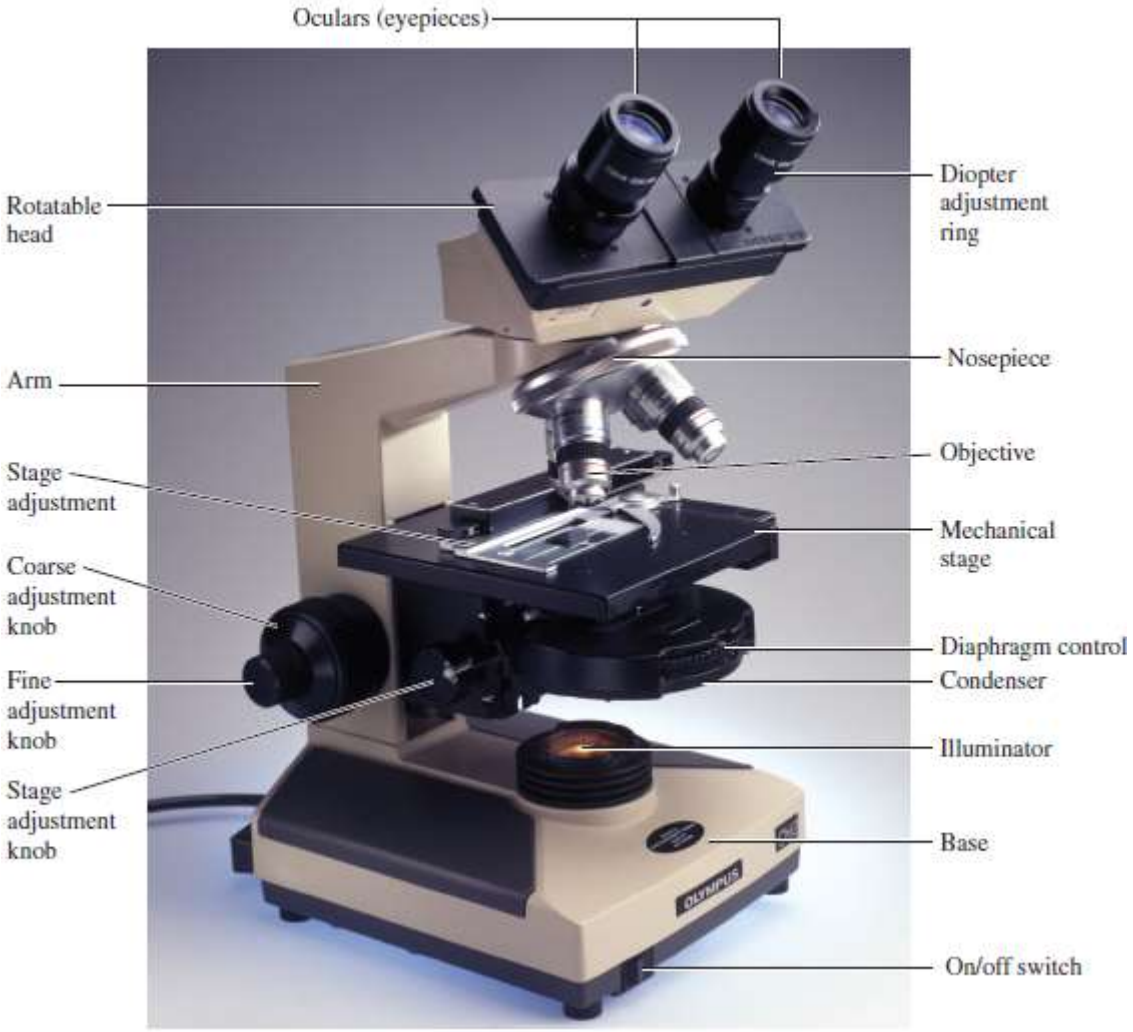


Figure: Compound Microscope

Parts of the Microscope: The components of the microscope are:

1. OCULAR LENS or EYEPIECE — On a binocular scope there are two ocular lenses, one for each eye. These lenses magnify the image at 10× power. The power of the ocular lens multiplied by the objective lens gives the total magnification of the microscope.

2. ARM — A support for the upper portion of the scope. It also serves as a convenient carrying handle.

3. MECHANICAL STAGE CONTROLS — A geared device to move the slide (placed in the slide clamp) precisely.

4. COARSE ADJUSTMENT KNOB — A rapid control which allows for quick focusing by moving the objective lens or stage up and down. It is used for initial focusing.

5. FINE ADJUSTMENT KNOB — A slow but precise control used to fine focus the image when viewing at the higher magnifications.

6. BASE — The part of your microscope that sits on a level, stable support.

7. OCULAR ADJUSTMENT — An adjustment for differences in the focusing abilities of your eyes.

8. DIOPTIC ADJUSTMENT — A horizontal adjustment of the oculars. Adjust for your eyes so you see only one field of view with both eyes open.

9. NOSEPIECE — A circular plate with 4 objective lenses that can be rotated into position for different magnifications.

10. OBJECTIVE LENS — Four separate lenses that magnify the image (10×, 40×, and 100×) depending on the objective in use. The lens is positioned just above the object being viewed.

11. SLIDE CLAMP — A clamp to hold the slide on the stage.

12. STAGE — A platform for placement of the microscope slide.

13. CONDENSER — A lens that concentrates or directs the light onto the slide.

14. IRIS DIAPHRAGM CONTROL — A lever (or rotating disk) that adjusts the amount of light illuminating the slide. Use just enough light to illuminate the object on the slide and give good contrast.

15. FILTER HOLDER — A blue filter rests in this holder below the substage condenser.

16. CONDENSER HEIGHT CONTROL — A knob that controls the height of the condenser.

17. LAMP — The light source.

18. LAMP SWITCH — Turns the lamp “on” and “off”.

Lens Systems of a Microscope: All compound microscopes have three lens systems: the oculars, the objectives, and the condenser. The **ocular**, or **eyepiece**, is a complex piece, located at the top of the instrument, that consists of two or more internal lenses and usually has a magnification of 10×. Most modern microscopes have two ocular (binocular) lenses. Note that they are attached to a rotatable nosepiece, which makes it possible to move them into position

over a slide. **Objectives** on most laboratory microscopes have magnifications of 10×, 40×, and 100×, designated as low-power, high-dry, and oil immersion, respectively. The total magnification of a compound microscope is determined by multiplying the power of the ocular lens times the power of the objective lens used. Thus, the magnification of a microscope in which the oil immersion lens is being used is: 10×100=1000. The object is now magnified 1000 times its actual size. The third lens system is the condenser, which is located under the stage. It collects and directs the light from the lamp to the slide being studied. Unlike the ocular and objective lenses, the condenser lens does not affect the magnifying power of the compound microscope. The condenser can be moved up and down by a knob under the stage. A diaphragm within the condenser regulates the amount of light that reaches the slide. Microscopes that lack a voltage control on the light source rely entirely on the diaphragm for controlling light intensity. In order to maximize the resolving power from a lens system, the following should be considered:

(i) A blue filter should be placed over the light source because the shorter wavelength of the resulting light will provide maximum resolution.

(ii) The condenser should be kept at the highest position that allows the maximum amount of light to enter the objective lens and therefore limit the amount of light lost due to refraction.

(iii) The diaphragm should not be stopped down too much. While closing the diaphragm improves the contrast, it also reduces the numerical aperture.

(iv) Immersion oil should be used between the slide and the 100× objective lens. This is a special oil that has the same refractive index as glass. When placed between the specimen and objective lens, the oil forms a continuous lens system that limits the loss of light due to refraction. Thus, a greater magnification cannot be achieved simply by adding a stronger ocular lens.

Figure: The light pathway of a microscope.

Use and Care of Microscope:

Proper care and maintenance of microscope is needed. Following points should be kept in mind while handling the microscope:

(i) Instrument should be kept in special cabinets while not in use.

(ii) Microscope should be held firmly by holding the arm with right hand and base with left arm.

(iii) All the lens systems should be cleaned with lens tissue to remove dust, oil, etc., which may decrease the efficiency of the microscope. Blotting paper, cloth or towel should not be used for cleaning.

(iv) If the lens is sticky or oily, the lens can be cleaned with xylol followed by 95% alcohol. The lens is wiped dry with lens paper. This should be performed only if necessary as consistent use of xylol may loosen the lens.

Method of Sterilization

Sterilization describes a process that destroys or eliminates all forms of microbial life and is carried out in health-care facilities by physical or chemical methods.

The various methods of sterilization are:

1. Physical Method

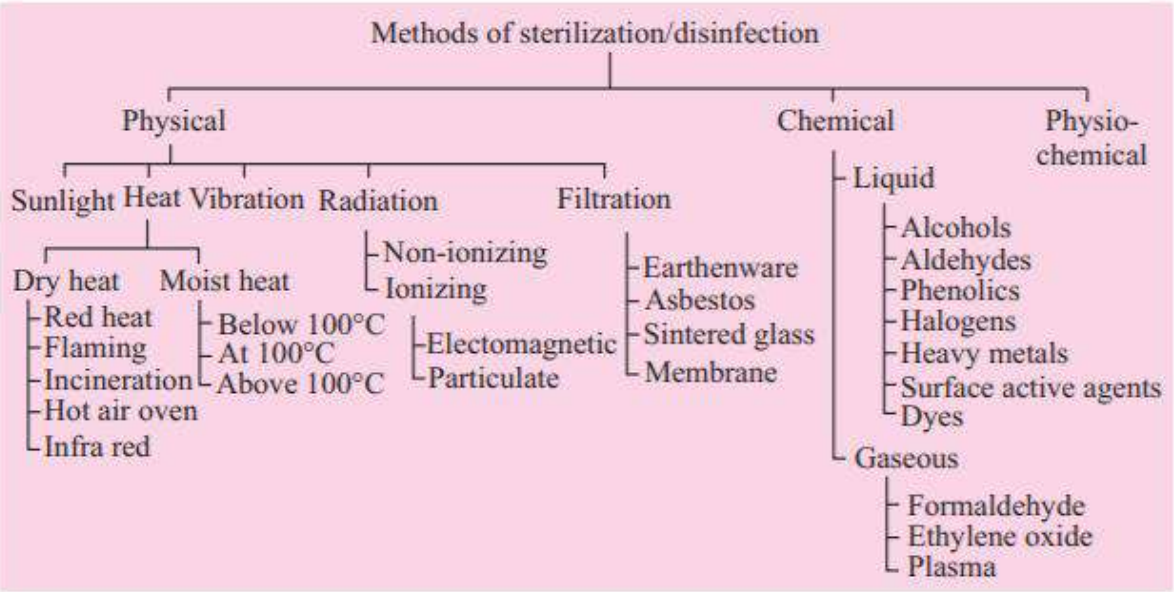
(a) Thermal (Heat) methods

(b) Radiation method

(c) Filtration method

2. Chemical Method

3. Gaseous method



Heat Sterilization: Heat sterilization is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents. The process is more effective in hydrated state where under conditions of high humidity, hydrolysis and denaturation occur, thus lower heat input is required. Under dry state, oxidative changes take place, and higher heat input is required.

This method of sterilization can be applied only to the thermostable products, but it can be used for moisture-sensitive materials for which dry heat (160-180°C) sterilization, and for moisture-resistant materials for which moist heat(121-134°C) sterilization is used.

The efficiency with which heat is able to inactivate microorganisms is dependent upon the degree of heat, the exposure time and the presence of water. The action of heat will be due to induction of lethal chemical events mediated through the action of water and oxygen. In the presence of water much lower temperature time exposures are required to kill microbe than in the absence of water. In these processes, both dry and moist heat are used for sterilization.

Dry Heat Sterilization: Examples of Dry heat sterilization are:

1. Incineration

2. Red heat

3. Flaming

4. Hot air oven

It employs higher temperatures in the range of 160-180°C and requires exposures time up to 2 hours, depending upon the temperature employed. The benefit of dry heat includes good penetrability and non-corrosive nature which makes it applicable for sterilizing glass-wares and metal surgical instruments.

It is also used for sterilizing non-aqueous thermo-stable liquids and thermostable powders. Dry heat destroys bacterial endotoxins (or pyrogens) which are difficult to eliminate by other means and this property makes it applicable for sterilizing glass bottles which are to be filled aseptically.

Hot-Air Oven:

Dry heat sterilization is usually carried out in a hot air oven, which consists of the following:

(i) An insulated chamber surrounded by an outer case containing electric heaters.

(ii) A fan

(iii) Shelves

(iv) Thermocouples

(v) Temperature sensor

(vi) Door locking controls.

Operation:

(i) Articles to be sterilized are first wrapped or enclosed in containers of cardboard, paper or aluminium.

(ii) Then, the materials are arranged to ensure uninterrupted air flow.

(iii) Oven may be pre-heated for materials with poor heat conductivity.

(iv) The temperature is allowed to fall to 40°C, prior to removal of sterilized material.

Autoclave: The autoclave is a steam-pressure sterilizer. Steam is the vapour given off by water when it boils at 100°C. If steam is trapped and compressed, its temperature rises as the pressure on it increases. As pressure is exerted on a vapour or gas to keep it enclosed within a certain area, the energy of the gaseous molecules is concentrated and exerts equal pressure against the opposing force. The energy of pressurized gas generates heat as well as force. Thus, the temperature of steam produced at 100°C rises sharply above this level if the steam is trapped within a chamber that permits it to accumulate but not to escape. A kitchen pressure cooker illustrates this principle because it is, indeed, an ―autoclave. When a pressure cooker containing a little water is placed over a hot burner, the water soon comes to a boil. If the lid of the cooker is then clamped down tightly while heating continues, steam continues to be generated but, having nowhere to go, creates pressure as its temperature climbs steeply. This device may be used in the kitchen to speed cooking of food, because pressurized steam and its high temperature (120 to 125°C) penetrates raw meats and vegetables much more quickly than does boiling water or its dissipating steam. In the process, any microorganisms that may also be present are similarly penetrated by the hot pressurized steam and destroyed.

Essentially, an autoclave is a large, heavy-walled chamber with a steam inlet and an air outlet. It can be sealed to force steam accumulation. Steam (being lighter but hotter than air) is admitted through an inlet pipe in the upper part of the rear wall. As it rushes in, it pushes the cool air in the chamber forward and down through an air discharge line in the floor of the chamber at its front. When all the cool air has been pushed down the line, it is followed by hot steam, the temperature of which triggers a thermostatic valve placed in the discharge pipe. The valve closes off the line and then, as steam continues to enter the sealed chamber, pressure and temperature begin to build up quickly. The barometric pressure of normal atmosphere is about 15 lb to the square inch. Within an autoclave, steam pressure can build to 15 to 30 lb per square inch above atmospheric pressure, bringing the temperature up with it to 121 to 123°C. Steam is wet and penetrative to begin with, even at 100°C (the boiling point of water). When raised to a high temperature and driven by pressure, it penetrates thick substances that would be only superficially bathed by steam at atmospheric pressure. Under autoclave conditions, pressurized steam kills bacterial endospores, vegetative bacilli, and other microbial forms quickly and effectively at temperatures much lower and less destructive to materials than are required in a dry-heat oven (160 to 170°C).

Temperature and time are the two essential factors in heat sterilization. In the autoclave (steam-pressure sterilizer), it is the intensity of steam temperature that sterilizes (pressure only provides the means of creating this intensity), when it is given time measured according to the nature of the load in the chamber. In the dry-heat oven, the temperature of the hot air (which is not very penetrative) also sterilizes, but only after enough time has been allowed to heat the oven load and oxidize vital components of microorganisms without damaging materials.

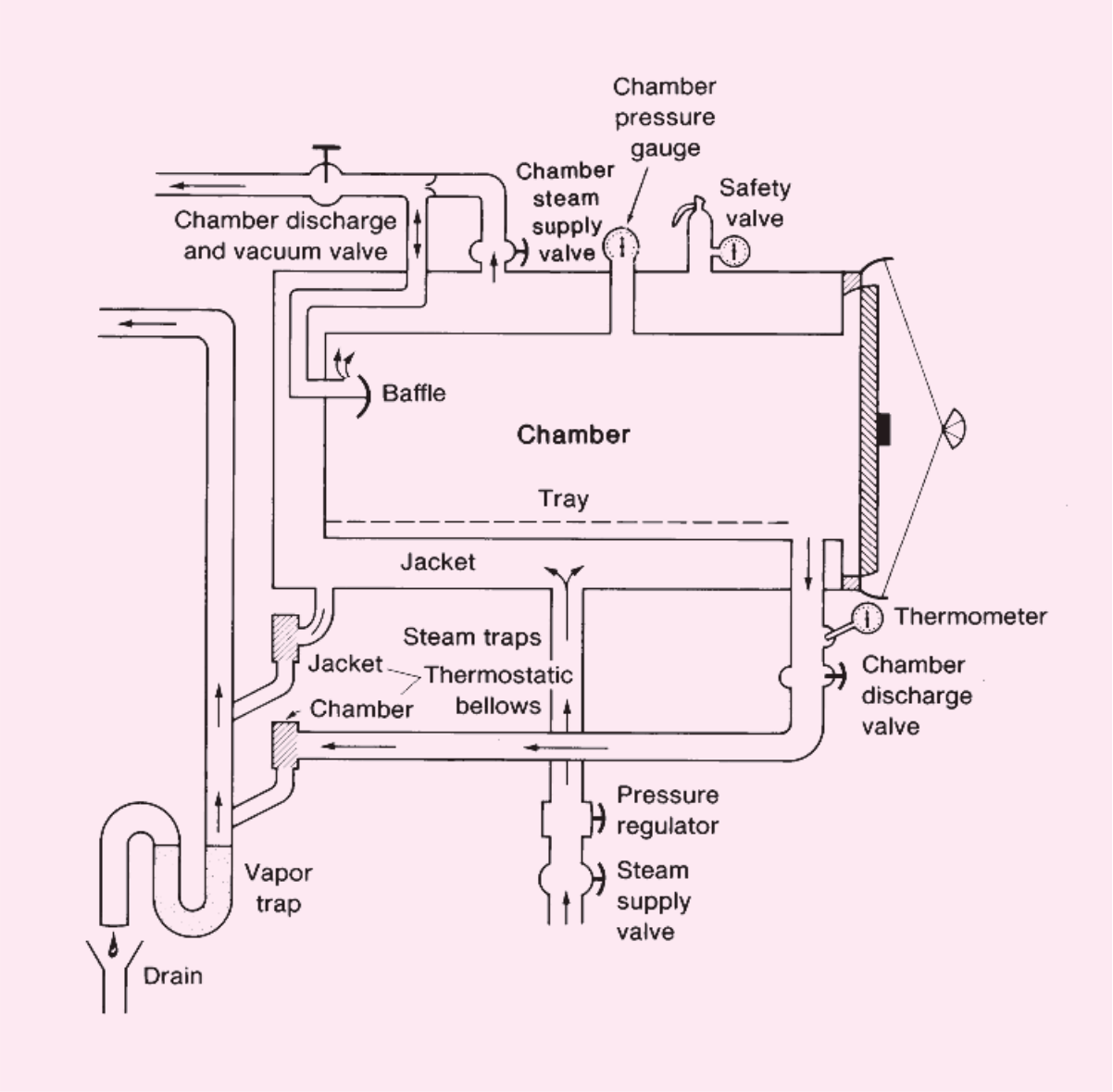


Fig. Autoclave

Table. Pressure-Temperature-Time Relationships in Steam-Pressure Sterilization.

Temperature^{0}C	Steam pressure(lb/sq.inch)	Holding time (minutes)
115-118	10	30
121-124	15	15
126-129	20	10
135-138	30	3

Filtration Sterilization: Filtration process does not destroy but removes the microorganisms. It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non-viable particles. The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material. Sterilizing

grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products and air and other gases for supply to aseptic areas. They are also used in industry as part of the venting systems on fermentors, centrifuges, autoclaves and freeze driers.

Membrane filters are used for sterility testing.

Application of filtration for sterilization of gases: HEPA (High efficiency particulate air) filters can remove up to 99.97% of particles >0.3 micrometer in diameter. Air is first passed through prefilters to remove larger particles and then passed through HEPA filters. The performance of HEPA filter is monitored by pressure differential and airflow rate measurements.

There are two types of filters used in filtration sterilization

(a) **Depth Filters:** Consist of fibrous or granular materials so packed as to form twisted channels of minute dimensions. They are made of diatomaceous earth, unglazed porcelain filter, sintered glass or asbestos.

(b) **Membrane Filters:** These are porous membrane about 0.1 mm thick, made of cellulose acetate, cellulose nitrate, polycarbonate, and polyvinylidene fluoride, or some other synthetic material. The membranes are supported on a frame and held in special holders. Fluids are made to transverse membranes by positive or negative pressure or by centrifugation.

Application of filtration for sterilization of liquids: Membrane filters of 0.22 micrometer nominal pore diameter are generally used, but sintered filters are used for corrosive liquids, viscous fluids and organic solvents. The factors which affects the performance of filter is the titre reduction value, which is the ratio of the number of organisms challenging the filter under defined conditions to the number of organisms penetrating it. The other factors are the depth of the membrane, its charge and the tortuosity of the channels.

Laminar Air Flow: A Laminar flow hood/cabinet is an enclosed workstation that is used to create a contamination-free work environment through filters to capture all the particles entering the cabinet. These cabinets are designed to protect the work from the environment and are most useful for the aseptic distribution of specific media and plate pouring. Laminar flow cabinets are similar to biosafety cabinets with the only difference being that in laminar flow cabinets the effluent air is drawn into the face of the user. In a biosafety cabinet, both the sample and user are protected while in the laminar flow cabinet, only the sample is protected and not the user.

Components/ Parts of Laminar Flow Hood: A laminar flow cabinet consists of the following parts:

1. **Cabinet:** The cabinet is made up of stainless steel with less or no gaps or joints preventing the collection of spores. The cabinet provides insulation to the inner environment created inside the laminar flow and protects it from the outside environment. The front of the cabinet is provided with a glass shield which in some laminar cabinets opens entirely or in some has two openings for the users' hands to enter the cabinet.

2. **Working station:** A flat working station is present inside the cabinet for all the processes to be taken place. Culture plates, burner and loops are all placed on the working station where the operation takes place. The worktop is also made up of stainless steel to prevent rusting.

3. **Filter pad/ Pre-filter:** A filter pad is present on the top of the cabinet through which the air passes into the cabinet. The filter pad traps dust particles and some microbes from entering the working environment within the cabinet.

4. **Fan/ Blower:** A fan is present below the filter pad that sucks in the air and moves it around

in the cabinet. The fan also allows the movement of air towards the HEPA filter sp that the remaining microbes become trapped while passing through the filter.

5. **UV lamp:** Some laminar flow hoods might have a UV germicidal lamp that sterilizes the interior of the cabinet and contents before the operation. The UV lamp is to be turned on 15 minutes before the operation to prevent the exposure of UV to the body surface of the user.

6. **Fluorescent lamp:** Florescent light is placed inside the cabinet to provide proper light during the operation.

7. **HEPA filter:** The High-efficiency particulate air filter is present within the cabinet that makes the environment more sterile for the operation. The pre-filtered air passes through the filter which traps fungi, bacteria and other dust particles. The filter ensures a sterile condition inside the cabinet, thus reducing the chances of contamination.

Principle of Laminar flow hood: The principle of laminar flow cabinet is based on the laminar flow of air through the cabinet. The device works by the use of inwards flow of air through one or more HEPA filters to create a particulate-free environment. The air is taken through a filtration system and then exhausted across the work surface as a part of the laminar flow of the air. The air first passes through the filter pad or pre-filter that allows a streamline flow of air into the cabinet. Next, the blower or fan directs the air towards the HEPA filters. The HEPA filters then trap the bacteria, fungi and other particulate materials so that the air moving out of it is particulate-free air. Some of the effluent air then passes through perforation present at the bottom rear end of the cabinet, but most of it passes over the working bench while coming out of the cabinet towards the face of the operator. The laminar flow hood is enclosed on the sides, and constant positive air pressure is maintained to prevent the intrusion of contaminated external air into the cabinet.

Procedure for running the laminar flow cabinet:

I. The procedure to be followed while operating a laminar flow cabinet is given below:
II. Before running the laminar flow cabinet, the cabinet should be checked to ensure that nothing susceptible to UV rays is present inside the cabinet.
III. The glass shield of the hood is then closed, and the UV light is switched on. The UV light should be kept on for about 15 minutes to ensure the surface sterilization of the working bench.
IV. The UV light is then switched off, and a time period of around 10 minutes is spared before the airflow is switched on.
V. About 5 minutes before the operation begins, the airflow is switched on.
VI. The glass shield is then opened, and the fluorescent light is also switched on during the operation.
VII. To ensure more protection, the working bench of the cabinet can be sterilized with other disinfectants like 70% alcohol.

VIII. Once the work is completed, the airflow and florescent lamp both are closed and the glass
IX. shield is also closed.

Types of Laminar Flow Cabinet: Depending on the direction of movement of air, laminar flow cabinets are divided into two types:

1. **Vertical Laminar Flow Cabinet:** In the vertical flow cabinets, the air moves from the top of the cabinet directly towards the bottom of the cabinet. A vertical airflow working bench does not require as much depth and floor space as a horizontal airflow hood which makes it more manageable and decreases the chances of airflow obstruction or movement of contaminated air downstream. The vertical laminar flow cabinet is also considered safer as it doesn‘t blow the air directly towards the person carrying out the experiments.

2. **Horizontal Laminar Flow Cabinet:** In the horizontal laminar flow cabinets, the surrounding air comes from behind the working bench, which is then projected by the blower towards the HEPA filters. The filtered air is then exhausted in a horizontal direction to the workplace environment. One advantage of this cabinet is that airflow parallel to the workplace cleanses the environment with a constant velocity. The eluent air directly hits the operator, which might reduce the security level of this type of laminar flow cabinets.

Uses of Laminar Flow Hood: The following are some common uses of a laminar flow cabinet in the laboratory:

I. Laminar flow cabinets are used in laboratories for contamination sensitive processes like plant tissue culture.
II. Other laboratories processes like media plate preparation and culture of organisms can be performed inside the cabinet.
III. Operations of particle sensitive electronic devices are performed inside the cabinet.
IV. In the pharmaceutical industries, drug preparation techniques are also performed inside the cabinet to ensure a particulate-free environment during the operations.
V. Laminar flow cabinets can be made tailor-made for some specialized works and can also be used for general lab techniques in the microbiological as well as the industrial sectors.

Precautions: While operating the laminar airflow, the following things should be considered-

I. The laminar flow cabinet should be sterilized with the UV light before and after the operation.
II. The UV light and airflow should not be used at the same time.
III. No operations should be carried out when the UV light is switched on.
IV. The operator should be dressed in lab coats and long gloves.
V. The working bench, glass shield, and other components present inside the cabinet should be sterilized before and after the completion of work.

Biosafety Cabinets: Biosafety Cabinets (BSCs) are enclosed workspaces with a ventilated hood that is designed to contain pathogenic microorganisms during microbiological processes.

The primary purpose of biosafety cabinets is to protect the laboratory personnel and the environment from the pathogenic microorganism as aerosols might be formed during the processing of such microorganisms.

Biosafety cabinets are only used for certain risk group organisms and for processes that might result in aerosol formation.

These cabinets are provided with HEPA-filters that decontaminate the air moving out of the cabinet.

Biosafety cabinets might be confused with the laminar hood as both of these pieces of equipment work as enclosed workspaces. But, laminar hood only provides protection to the sample and not to the personnel and the environment, whereas biosafety cabinets protect all three.

The use of biosafety cabinets or other such physical containment is not required in the biosafety level 1, but depending on the risk assessment, some processes might require such containment.

BSCs are an essential part of biosafety as they minimize the formation of aerosol, protecting the environment, the pathogen, and the laboratory personnel.

Besides, most BSCs also function to sterilize biological materials that are kept inside the cabinets.

Nutritional media and their Preparation

Principle: Different types of media have been formulated for growing bacteria. Media generally contain a carbon source, nitrogen source and some essential minerals and salts. Some media may contain additional nutritional supplements. In addition, solid media contain agar as a solidifying agent. Meat extract and peptone are the commonest sources of carbohydrates and amino acids.

Media are of different types. These are:

1. **Basal Media:** These contain nutrients that support the growth of non-fastidious bacteria. They do not confer any selective advantage, e.g. nutrient agar.

2. **Enriched Medium:** These are solid selective media. These media, in addition to basal nutrients also contain nutritional supplements like blood, serum, etc., which favour the growth of fastidious bacteria. e.g. blood agar, chocolate agar, Löwenstein- Jensen medium, etc.

3. **Enrichment Media:** These are liquid selective media. They favor the growth of some bacteria by extending the lag phase of others e.g. Selenite F broth.

4. **Selective Media:** These media contain ingredients that selectively enable the growth of some species, while inhibiting others e.g. Deoxycholate citrate agar (DCA) medium. This medium is a selective medium for growth of *Salmonella* spp. present in stool which contains a mixed bacteria flora. This medium inhibits *Escherichia coli* and other Gram-negative bacteria.

5. **Differential Media:** These media differentiate between species of bacteria depending on a specific property.

Example: MacConkey agar is a differential medium. This medium is used to demonstrate lactose fermenting properties, and differentiate between lactose and non-lactose fermenting bacteria.

Requirements:

I. Equipment: Bacteriological incubator.

II. Reagents and media: Different kinds of media such as nutrient agar, blood agar, MacConkey agar and Selenite F broth.

III. Specimen: 24-hour broth cultures of *Staphylococcus aureus, E. coli, Proteus mirabilis* and *Salmonella* spp.

Procedure:

1. Inoculate a loopful of the test organism, using a sterile inoculating loop, into appropriately labeled plates and tubes.

2. Incubate the plates and tubes for 18 hours at 37°C.

3. Examine the plate and tubes for growth and record observations.

Quality Control: 1. One un-inoculated set of media as sterility control

2. Nutrient agar: Colonies of non-fastidious bacteria such as *S. aureus*.

3. Blood agar: Haemolytic strain of *S. aureus* streaked on the plate surrounded by a zone of hemolysis.

4. MacConkey agar: Pink, lactose fermenting colonies of *E. coli* and colorless colonies of Proteus spp.

5. Selenite F broth: Growth positive *Salmonella* spp, and growth negative *Proteus* spp.

6. Potato-Dextrose agar: Supporting the growth of fungi.

7. Czapek-Dox agar: Supporting the growth of fungi.

8. Sabouraud agar: Supporting the growth of fungi.

9. Starch casein agar: Supporting the growth of actinomyces.

10. Glycerol asparagine medium: Supporting the growth of actinomyces.

Observations: All the inoculated bacteria (e.g. *S. aureus, E. coli, P. mirabilis* and *Salmonella* spp) produce colonies on the nutrient agar (basal medium) and blood agar (enriched medium). In addition, *S. aureus* may or may not produce haemolysis on the blood agar.

Enumeration of Microbes

Principle: Studies involving the analysis of materials, including food, water, milk, and in some cases—air, require quantitative enumeration of microorganisms in the substances. Many methods have been devised to accomplish this, including direct microscopic counts, use of an electronic cell counter such as the Coulter Counter, chemical methods for estimating cell mass or cellular constituents, turbidimetric measurements for increases in cell mass, and the serial dilution–agar plate method.

Serial Dilution–Agar Plate Analysis: While all these methods (Coulter Counter, chemical methods, turbidimetric measurements) may be used to enumerate the number of cells in a bacterial culture, the major disadvantage common to all is that the total count includes dead as well as living cells. Sanitary and medical microbiology, at times, require determination of

viable cells. To accomplish this, the serial dilution–agar plate technique is used. Briefly, this method involves serial dilution of a bacterial suspension in sterile water blanks, which serve as a diluent of known volume. Once diluted, the suspensions are placed on suitable nutrient media. The pour-plate technique is usually employed. Molten agar, cooled to 45°C, is poured into a Petri dish containing a specified amount of the diluted sample. Following addition of the molten-then-cooled agar, the cover is replaced, and the plate is gently rotated in a circular motion to achieve uniform distribution of microorganisms. This procedure is repeated for all dilutions to be plated. Dilutions should be plated in duplicate for greater accuracy, incubated overnight, and counted on a Quebec colony counter either by hand or by an electronically modified version of this instrument. Plates suitable for counting must contain neither fewer than 30 nor more than 300 colonies. The total count of the suspension is obtained by multiplying the number of cells per plate by the dilution factor, which is the reciprocal of the dilution. Advantages of the serial dilution–agar plate technique are- (i) Only viable cells are counted; and (ii) It allows isolation of discrete colonies that can be sub-cultured into pure cultures, which may then be easily studied and identified.

Materials: 24- to 48-hour nutrient broth culture of *Escherichia coli*, test tubes, nutrient broth, agar, conical flask, autoclave, vortex mixture, non-adsorbent cotton, Petri dish, incubator, pipette, micropipette, colony counter.

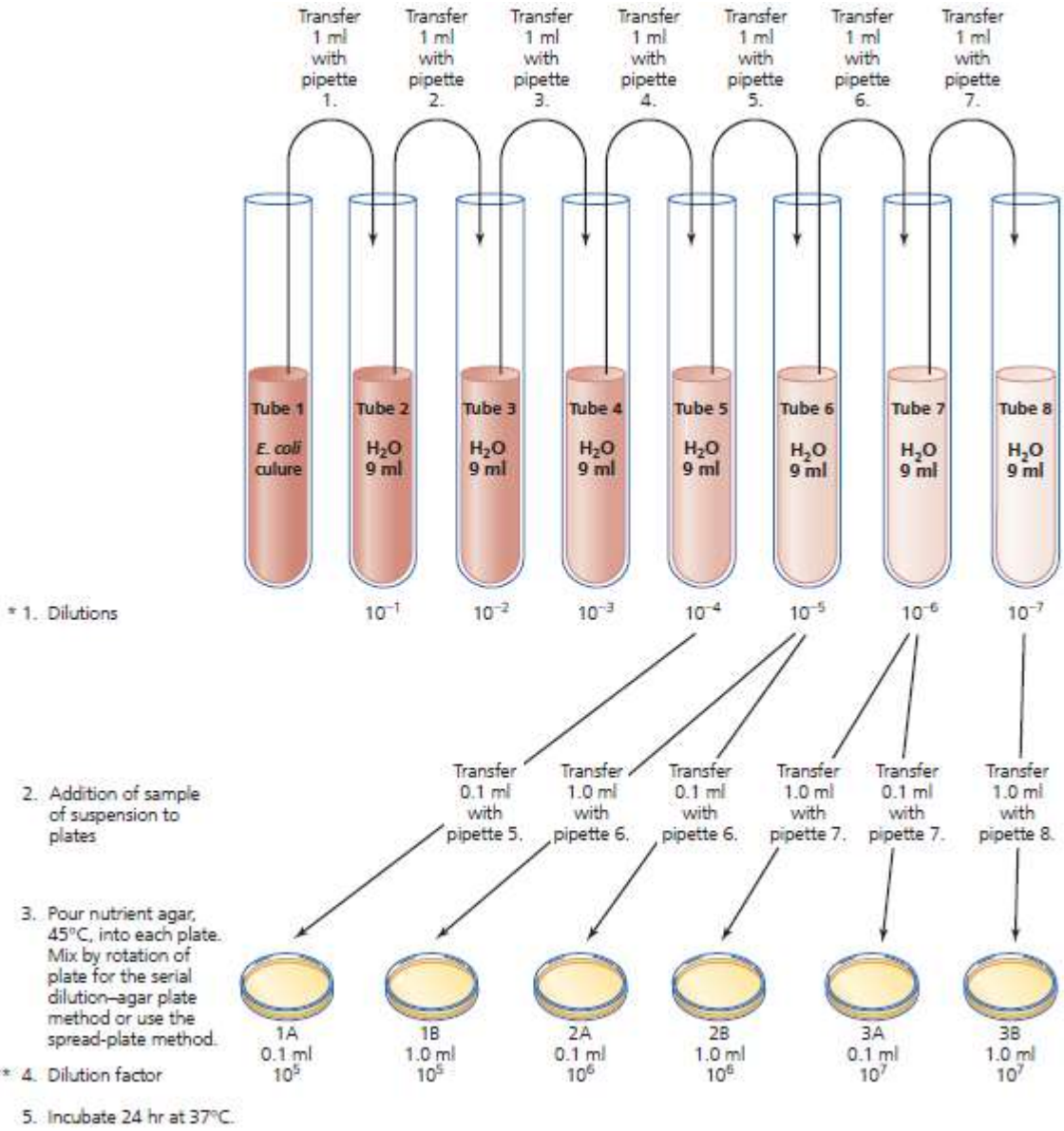
Procedure:

1. Liquefy six agar deep tubes in an autoclave. Cool the molten agar tubes and maintain in a water bath at 45°C.

2. Label the *E. coli* culture tube with the number 1 and the seven 9-ml water blanks as numbers 2 through 8. Place the labelled tubes in a test tube rack. Label the Petri dishes 1A, 1B, 2A, 2B, 3A, and 3B.

3. Mix the *E. coli* culture (Tube 1) by rolling the tube between the palms of hands to ensure even dispersal of cells in the culture.

4. With a sterile pipette, aseptically transfer 1 ml from the bacterial suspension, Tube 1, to water blank Tube 2. The culture had been diluted 10 times to 10^{-1} and follow this procedure for other such tubes.



5. Check the temperature of the molten agar medium to be sure the temperature was 45°C. Remove a tube from the water bath and wipe the outside surface dry with a paper towel. Using the pour-plate technique, pour the agar (100 µl) into Plate 1A and rotate the plate gently to ensure uniform distribution of the cells in the medium.

6. Repeat step 5 for the addition of molten nutrient agar to Plates 1B, 2A, 2B, 3A, and 3B. Once the agar had solidified, incubate the plates in an inverted position for 24 hours at 37°C.

7. After 24 h of incubation, Petri dish were counted for number of colonies grown. Using a Quebec colony counter and a mechanical hand counter, observe all colonies on plates. Statistically valid plate counts are only obtained from bacterial cell dilutions that yield between 30 and 300 colonies. Plates with more than 300 colonies cannot be counted and are designated as too numerous to count—**TNTC**; plates with fewer than 30 colonies are designated as too few to count—**TFTC**. Count only plates containing between 30 and 300 colonies. Remember to count all subsurface as well as surface colonies.

8. The number of organisms per ml of original culture is calculated by multiplying the number of colonies counted by the dilution factor:

number of cells per ml = $\frac{number\ of\ colonies \times dilution\ factor}{volume\ (0.1\ ml)}$

Result:

No. of Colonies	Dilution Factor	CFU/ml
40	10^4	4×10^6
76	10^5	7.6×10^7

Interpretation: At 10^{-4} dilution of a soil sample, 4×10^6 CFU/ml of bacteria was obtained.

Isolation of Pure Culture

Introduction: In nature, microbial populations do not segregate themselves by species, but exist with a mixture of many other cell types. In the laboratory, to separate these populations into pure cultures is performed. These cultures contain only one type of organism and allow us to study their cultural, morphological, and biochemical properties.

Principle: The techniques commonly used for isolation of discrete colonies initially require that the number of organisms in the inoculum be reduced. The resulting diminution of the population size ensures that, following inoculation, individual cells will be sufficiently far apart on the surface of the agar medium to separate the different species.

Materials: Nutrient agar, conical flask, inoculation loop or spreader, Petri dish, natural sample.

Procedure:

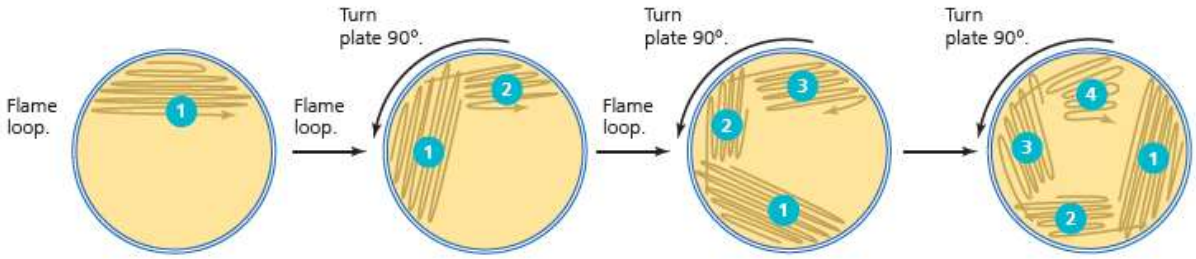
1. The **streak-plate method** is a rapid qualitative isolation method. It is a dilution technique that spreads a loopful of culture over the surface of an agar plate as a means to separate and dilute the microbes and ensure individual colony growth. There are many different procedures for preparing a streak plate-

a. Place a loopful of culture on the agar surface in Area 1. Flame the loop, cool it by touching it to an unused part of the agar surface close to the periphery of the plate, and then drag it rapidly several times across the surface of Area 1.

b. Reflame and cool the loop, and turn the Petri dish 90°. Then touch the loop to a corner of the culture in Area 1 and drag it several times across the agar in Area 2. The loop should never enter Area 1 again.

c. Reflame and cool the loop and again; turn the dish 90°. Streak Area 3 in the same manner as Area 2.

d. Without reflaming the loop, again turn the dish 90° and then drag the culture from a corner of Area 3 across Area 4, using a wider streak. Don't let the loop touch any of the previously streaked areas. The purpose of flaming of the loop at the points indicated is to dilute the culture so that fewer organisms are streaked in each area, resulting in the final desired separation.



Four-way streak-plate technique

2. The **spread-plate technique** requires also the diluted mixture of microorganisms. During inoculation, the cells were spread over the surface of a solid agar medium with a sterile, L-shaped bent glass rod while the Petri dish is spun on a turntable. The step-by-step procedure for this technique was as follows:

a. Place the bent glass rod into a beaker and add a sufficient amount of 95% ethyl alcohol to cover the lower, bent portion.

b. Place an appropriately labeled nutrient agar plate on the turntable. With a sterile pipette, place one drop of sterile water on the center of the plate, followed by a sterile loopful of Micrococcus luteus. Mix gently with the loop and replace the cover.

c. Remove the glass rod from the beaker, and pass it through the Bunsen burner flame with the bent portion of the rod pointing downward to prevent the burning alcohol from running down your arm. Allow the alcohol to burn off the rod completely. Cool the rod for 10 to 15 seconds.

d. Remove the Petri dish cover and spin the turntable. e. While the turntable is spinning, lightly touch the sterile bent rod to the surface of the agar and move it back and forth. This was spread the culture over the agar surface.

f. When the turntable comes to a stop, replace the cover. Immerse the rod in alcohol and reflame.

g. In the absence of a turntable, turn the Petri dish manually and spread the culture with the sterile bent glass rod.

3. The **pour-plate technique** requires a serial dilution of the mixed culture by means of a loop or pipette. The diluted inoculum is then added to a molten agar medium in a Petri dish, mixed, and allowed to solidify.

4. Incubate all plates in an inverted position for 48 to 72 hours at 25°C.

5. The colonies of microorganisms showed characteristics pattern on the Petri dish, which were documented. Particularly for bacteria, the well-isolated colonies were evaluated as follows-

1. **Size:** pinpoint, small, moderate, or large

2. **Pigmentation:** color of colony

3. **Form:** The shape of the colony is described as follows:

a. **Circular:** unbroken, peripheral edge

b. **Irregular:** indented, peripheral edge

c. **Rhizoid:** rootlike, spreading growth

4. **Margin:** The appearance of the outer edge of the colony is described as follows:

a. **Entire:** sharply defined, even

b. **Lobate:** marked indentations

c. **Undulate:** wavy indentations

d. **Serrate:** toothlike appearance

e. **Filamentous:** threadlike, spreading edge

5. **Elevation:** The degree to which colony growth is raised on the agar surface is described as:

a. **Flat:** elevation not discernible

b. **Raised:** slightly elevated

c. **Convex:** dome-shaped elevation

d. **Umbonate:** raised, with elevated convex central region

Result: Three types of colonies were clearly identified- (i) small size, yellow color, circular form with serrate elevation; (ii) small size, milky appearance, entire margin with convex elevation; and (iii) large size, off-white color, lobate margin with undulate margin.

Gram Staining

Principle: The most important differential stain used in bacteriology is the Gram stain, named after Dr. Hans Christian Gram. It divides bacterial cells into two major groups, gram positive and gram negative, which makes it an essential tool for classification and differentiation of microorganisms. Differential staining requires the use of at least four chemical reagents that are applied sequentially to a heat-fixed smear. The first reagent is called the primary stain. Its function is to impart its color to all cells. The second stain is a mordant used to intensify the color of the primary stain. In order to establish a color contrast, the third reagent used is the decolorizing agent. Based on the chemical composition of cellular components, the decolorizing agent may remove the primary stain from the entire cell or only from certain cell structures. The final reagent, the counterstain, has a contrasting color to that of the primary stain. Following decolorization, if the primary stain is not washed out, the counterstain cannot be absorbed, and the cell or its components will retain the color of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting color of the counterstain. In this way, cell types or their structures can be distinguished from each other on the basis of the stain that is retained.

The Gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram-positive cells have a thick peptidoglycan layer, whereas the peptidoglycan layer in gram-negative cells is much thinner and surrounded by outer lipid-containing layers. Peptidoglycan is a polysaccharide composed of two chemical subunits found only in the bacterial cell wall. These subunits are N-acetylglucosamine and N-acetylmuramic acid. With some organisms, as the adjacent layers of peptidoglycan are formed, they are cross-linked by short chains of peptides by means of a transpeptidase enzyme, resulting in the shape

and rigidity of the cell wall. In the case of gram-negative bacteria and several of the gram-positive, such as the Bacillus, the cross-linking of the peptidoglycan layer is direct because the bacteria do not have short peptide tails. The Gram stain uses four different reagents.

Procedure:

I. A clean glass slide was obtained.

II. The smear was prepared by placing a drop of culture by using sterile inoculating loop.

III. The smear was allowed to air dry and then heat fixed by using Bunsen-burner.

IV. The smear was covered with several drops of crystal violet and incubated for 30 seconds to 1 minute.

V. The slide was gently washed with drops of tap water.

VI. The smear was then flooded with the Gram's iodine and incubated for one minute.

VII. The slide was gently washed with drops of tap water.

VIII. The slide was then decolourized with 90% ethyl alcohol.

IX. The slide was air dried followed by counter staining with safranin for 45 seconds.

X. The slide was gently washed with drops of tap water.

XI. The slide was air dried and observed under microscope.

Result: Under microscope, the supplied sample bacterium seen as rod shaped and violet color; hence, it was a Gram-positive bacterium.

Negative Staining

Principle: Negative staining requires the use of an acidic stain such as India ink or nigrosin. The acidic stain, with its negatively charged chromogen, will not penetrate the cells, because of the negative charge on the surface of bacteria. Therefore, the unstained cells are easily discernible against the colored background. The practical application of negative staining is twofold. First, since heat fixation is not required and the cells are not subjected to the distorting effects of chemicals and heat, we can see their natural size and shape. Second, we can observe bacteria that are difficult to stain, such as some spirilla. Because heat fixation is not done during the staining process, keep in mind that the organisms are not killed and slides should be handled with care.

The principle application of negative staining is to determine if an organism possesses a capsule (a gelatinous outer layer that makes the microorganism more virulent), although it can also be used to demonstrate spore formation. The technique is frequently used in the identification of fungi such as Cryptococcus neoformans, an important infectious agent found in bird droppings that is linked to meningeal and lung infections in humans.

Materials: Nigrosin, 48 h old culture, glass slide, cover slip.

Procedure:

1. Place a small drop of nigrosin close to one end of a clean slide.

2. Using aseptic technique, place a loopful of inoculum from the supplied culture in the drop of nigrosin and mix.

3. Place a slide against the drop of suspended organisms at a 45° angle and allow the drop to spread along the edge of the applied slide.

4. Push the slide away from the drop of suspended organisms to form a thin smear and air dried.

Observations: Colorless cells were observed against the dark field background.

Spore Stain (Schaeffer-Fulton Method)

Principle: Members of the anaerobic genera *Clostridium* and *Desulfotomaculum* and the aerobic genus *Bacillus* are examples of organisms that have the capacity to exist either as metabolically active vegetative cells or as highly resistant, metabolically inactive cell types called spores. When environmental conditions become unfavourable for continuing vegetative cellular activities, particularly with the exhaustion of a nutritional carbon source, these cells have the capacity to undergo sporogenesis and give rise to a new intracellular structure called the endospore, which is surrounded by impervious layers called spore coats. As conditions continue to worsen, the endospore is released from the degenerating vegetative cell and becomes an independent cell called a free spore. Because of the chemical composition of spore layers, the spore is resistant to the damaging effects of excessive heat, freezing, radiation, desiccation, and chemical agents, as well as to the commonly employed microbiological stains. With the return of favourable environmental conditions, the free spore may revert to a metabolically active and less resistant vegetative cell through germination. Note that sporogenesis and germination are not meaning of reproduction but merely mechanisms that ensure cell survival under all environmental conditions.

Malachite green (Primary Stain): Unlike most vegetative cell types that stain by common procedures, the free spore, because of its impervious coats, will not accept the primary stain easily. For further penetration, we must apply heat. After we apply the primary stain and heat the smear, both the vegetative cell and spore appear green.

Water (Decolorizing Agent): Once the spore accepts the malachite green, it cannot be decolorized by tap water, which removes only the excess primary stain. The spore remains green. On the other hand, the stain does not demonstrate a strong affinity for vegetative cell components; the water removes it, and these cells will be colorless.

Safranin (Counterstain): This contrasting red stain is used as the second reagent to color the decolorized vegetative cells, which will absorb the counterstain and appear red. The spores retain the green of the primary stain.

Materials: 24 h grown culture, malachite green, glass slide.

Procedure:

1. Obtain two clean glass slides.

2. Make individual smears in the usual manner using aseptic technique.

3. Allow smear to air-dry, and heat fix in the usual manner.

4. Flood the smears with malachite green and place on top of a water bath, allowing the preparation to steam for 2 to 3 minutes.

Note: Do not allow stain to evaporate; replenish stain as needed. Prevent the stain from boiling by adjusting the hot plate temperature.

5. Remove the slides from the hot plate, cool, and wash under running tap water.

6. Counterstain with safranin for 30 seconds.

7. Wash with tap water.

8. Blot dry with bibulous paper and examine under oil immersion.

Observations: Only pink color rod cells appeared under the microscope, therefore, the supplied sample is endospore negative.

Albert Staining

Principle: Albert staining technique aims at detecting the presence of metachromatic granulated bodies of *Corynebacterium diphtheriae*. *Corynebacteria* are gram-positive, non-spore forming, non-motile bacilli that contain metachromatic (Volutin) granules which are intracellular inclusion bodies, found in the cytoplasmic membrane of some bacterial cells for storage of complexed inorganic polyphosphate (poly-P) and enzymes. *Corynebacterium diphtheriae* causes diphtheria, a nasopharyngeal infection (affecting the nasal, throat) that can also affect the skin, after bacterial colonization and infection.

Materials: Albert stain is made up of two staining solutions; designated as Albert Solution 1 (toluidine blue, malachite green, glacial acetic acid, and alcohol) and Albert Solution 2 (iodine and potassium iodide in water). Besides, glass slide, coverslip, cedarwood oil, inoculating loop, alcohol, cotton, tissue papers were required.

Procedure:

A. Stain Preparation:

1. **Albert Stain 1:** Preparation of 100 ml Albert stain 1.

i. Into 100 ml of water, add 0.1 ml of glacial acetic acid.

ii. Add 2 ml of 95% ethanol into the solution.

iii. Then, dissolve 0.15 g of toluidine blue into the solution.

iv. Lastly, dissolve 0.2g of malachite green into the solution.

2. Albert Stain 2: Preparation of 300 ml of Albert stain 2.

i. Dissolve 2 g of iodine in 50 ml of distilled water.

ii. Add 250 ml of water to the solution.

iii. Dissolve 3 g of potassium iodide into the solution in the solution.

B. Staining:

i. Aseptically, take a loopful culture of *Corneybacterium diphtheriae*.

ii. Make a smear at the centre of a clean sterile glass slide.

iii. Heat fix the smear, gently.

iv. On a staining rack, place the smeared glass slide.

v. Add Albert staining Solution 1 into the smear and leave it for 3-5 minutes

vi. Wash the smeared slide with gently flowing tap water

C. Mordanting:

i. Add Albert staining solution 2 and leave it for 1 minute

ii. Wash the slide with gently flowing tap water.

iii. Blot to dry the smeared glass slide

iv. Add cedarwood oil on the smear and observed under a microscope by oil immersion at 1000x.

Result: The metachromatic granules stain bluish black while the rest of the microbial cell stains green.

Interpretation: *Corynebacterium diphtheriae* cytoplasmic membrane contains volutin granules, also known as metachromatic granules, which are a characteristic feature of this bacteria. The staining by Albert solutions, granules were appearing as round-shaped blue-black dots at the bottom of L-shaped or V-shaped green bacilli.

Acid-Fast Staining

Principle: While the majority of bacterial organisms are stainable by either simple or Gram staining procedures, a few genera, particularly the members of the genus Mycobacterium, are visualized more clearly by the acid-fast method. Since Mycobacterium tuberculosis and Mycobacterium leprae represent bacteria that are pathogenic to humans, the stain is of diagnostic value in identifying these organisms. The characteristic difference between mycobacteria and other microorganisms is the presence of a thick, waxy (lipoidal) wall that makes penetration by stains extremely difficult. Mycobacteria tend to clump together, and it is difficult to identify individual cells in stained preparations if this clumping effect occurs. Avoiding or minimizing this phenomenon requires careful preparation of the smear. Place a small drop of water on the slide, suspend the culture in the water, and mix the suspension thoroughly to dislodge and disperse some of the cells. Once the stain has penetrated, however, it cannot be readily removed even with the vigorous use of acid-alcohol as a decolorizing agent (unlike the 95% ethyl alcohol used in the Gram stain). Because of this property, these organisms are called acid-fast, while all other microorganisms, which are easily decolorized by acid-alcohol, are non–acid-fast. The acid-fast stain uses the three different reagents listed below along with a description of their purpose.

Primary Stain (Carbol fuchsin): Unlike cells that are easily stained by ordinary aqueous stains, most species of mycobacteria are not stainable with common dyes such as methylene blue and crystal violet. Carbol fuchsin, a dark red stain in 5% phenol that is soluble in the

lipoidal materials that constitute most of the mycobacterial cell wall, does penetrate these bacteria, and is retained. Applying heat enhances penetration further, which drives the carbol fuchsin through the lipoidal wall and into the cytoplasm. This application of heat is used in the Ziehl-Neelsen method. Following application of the primary stain, all cells appear red.

Decolorizing Agent [Acid-alcohol (3% HCl+95% ethanol)]: Prior to decolorization, the smear is cooled, which allows the waxy cell substances to harden. On application of acid-alcohol, acid-fast cells are resistant to decolorization, since the primary stain is more soluble in the cellular waxes than in the decolorizing agent. In this event, the primary stain is retained and the mycobacteria will stay red. This is not the case with non–acid-fast organisms, which lack cellular waxes. The primary stain is more easily removed during decolorization, leaving these cells colorless or unstained.

Counterstain (Methylene blue): This is used as the final reagent to stain previously decolorized cells. As only non–acid-fast cells undergo decolorization, they may now absorb the counterstain and take on its blue color, while acid-fast cells retain the red of the primary stain.

Procedure:

1. Flood smears with carbol fuchsin and place over a beaker of water on a warm hot plate, allowing the preparation to steam for 5 minutes. Note: Do not allow stain to evaporate; replenish stain as needed. Also, prevent stain from boiling by adjusting the hot-plate temperature.

2. Wash with tap water. Heated slides must be cooled prior to washing.

3. Decolorize with acid-alcohol, adding the reagent drop by drop until the alcohol runs almost clear with a slight red tinge.

4. Wash with tap water.

5. Counterstain with methylene blue for 2 minutes.

6. Wash the smear with tap water, air-dried, and observed under microscope.

Interpretation: Blue color rod shaped bacteria appeared under microscope, hence, the supplied sample was non-acid fast bacteria.

Antibiotic Sensitivity Assay (Kirby-Bauer Method)

Principle: The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum of activity, effective against only one group of microorganisms. Others exhibit broad-spectrum activity against a range of microorganisms. The drug susceptibilities of many pathogenic microorganisms are known, but it is sometimes necessary to test several agents to determine the drug of choice.

A standardized diffusion procedure with filter-paper discs on agar, known as the Kirby-Bauer method, is frequently used to determine the drug susceptibility of microorganisms isolated from infectious processes. This method allows the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc. In this procedure, filter-paper discs of uniform

size are impregnated with specified concentrations of different antibiotics and then placed on the surface of an agar plate that has been seeded with the organism to be tested. The medium of choice is Mueller-Hinton agar, with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5 mm. The plates are then heavily inoculated with a standardized inoculum by means of a spreader to ensure the confluent growth of the organism. The discs are aseptically applied to the surface of the agar plate at well-spaced intervals. Once applied, each disc is gently touched with a sterile applicator stick to ensure its firm contact with the agar surface. Following incubation, the plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. The susceptibility of an organism to a drug is assessed by the size of this zone, which is affected by other variables such as the following:

1. The ability and rate of diffusion of the antibiotic into the medium and its interaction with the test organism.

2. The number of organisms inoculated.

3. The growth rate of the organism.

Requirements: Muller-Hinton Agar, Antibiotics disc, Forceps, Inoculating loop, Cultures, Spreader.

Procedure:

1. Label the bottom of each of the agar plates with the name of the test organism to be inoculated.

2. Using aseptic technique, inoculate all agar plate with the test organism and properly spread it over the plates.

3. Allow all culture plates to dry for about 5 minutes.

4. Using sterilized forceps the antibiotic discs were placed over the agar surface and pressing the disc slightly.

5. Examine all plate cultures for the presence or absence of a zone of inhibition surrounding each disc and carefully measure each zone of inhibition in millimetre scale.

Result and Observation: A measurement of the diameter of the zone of inhibition in millimeters is made, and its size is compared with that contained in a standardized chart. Based on this comparison, the test organism is determined to be resistant, intermediate, or susceptible to the antibiotic.

Table 42.2 Zone Diameter Interpretive Standards for Organisms Other Than *Haemophilus* and *Neisseria gonorrhoeae*

		ZONE DIAMETER, NEAREST WHOLE MM		
ANTIMICROBIAL AGENT	DISC CONCENTRATION	RESISTANT	INTERMEDIATE	SUSCEPTIBLE
Ampicillin				
when testing gram-negative bacteria	10 μg	≤13	14–16	≥17
when testing gram-positive bacteria	10 μg	≤28	—	≥29
Carbenicillin				
when testing *Pseudomonas*	100 μg	≤13	14–16	≥17
when testing other gram-negative organisms	100 μg	≤19	20–22	≥23
Cefoxitin	30 μg	≤14	15–17	≥18
Cephalothin	30 μg	≤14	16–17	≥18
Chloramphenicol	30 μg	≤12	13–17	≥18
Clindamycin	2 μg	≤14	15–20	≥21
Erythromycin	15 μg	≤13	14–22	≥23
Gentamicin	10 μg	≤12	13–14	≥15
Kanamycin	30 μg	≤13	14–17	≥18
Methicillin when testing staphylococci	5 μg	≤9	10–13	≥14
Novobiocin	30 μg	≤17	18–21	≥22
Penicillin G				
when testing staphylococci	10 units	≤28	—	≥29
when testing other bacteria	10 units	≤14	—	≥15
Rifampin	5 μg	≤16	17–19	≥20
Streptomycin	10 μg	≤11	12–14	≥15
Tetracycline	30 μg	≤14	15–18	≥19
Tobramycin	10 μg	≤12	13–14	≥15
Trimethoprim/sulfamethoxazole	1.25/23.75 μg	≤10	11–15	≥16
Vancomycin				
when testing enterococci	30 μg	≤14	15–16	≥17
when testing *Staphylococcus* spp.	30 μg	—	—	≥15
Sulfonamides	250 or 300 μg	≤12	—	≥17
Trimethoprim	5 μg	≤10	—	≥16

Source: Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disk Susceptibility Tests*, Tenth Edition, 2008.

Cultivation of Anaerobes

Principle: Microorganisms differ in their abilities to use oxygen for cellular respiration. Respiration involves the oxidation of substrates for energy necessary to life. A substrate is oxidized when it loses a hydrogen ion and its electron (H^+e^-). Since the H^+e^- cannot remain free in the cell, it must immediately be picked up by an electron acceptor, which becomes reduced. Therefore, reduction means gaining the H^+e^-. These reactions are termed oxidation-reduction (redox) reactions. Some microorganisms have enzyme systems in which oxygen can serve as an electron acceptor, thereby being reduced to water. These cells have high oxidation-reduction potentials; others have low potentials and must use other substances as electron acceptors.

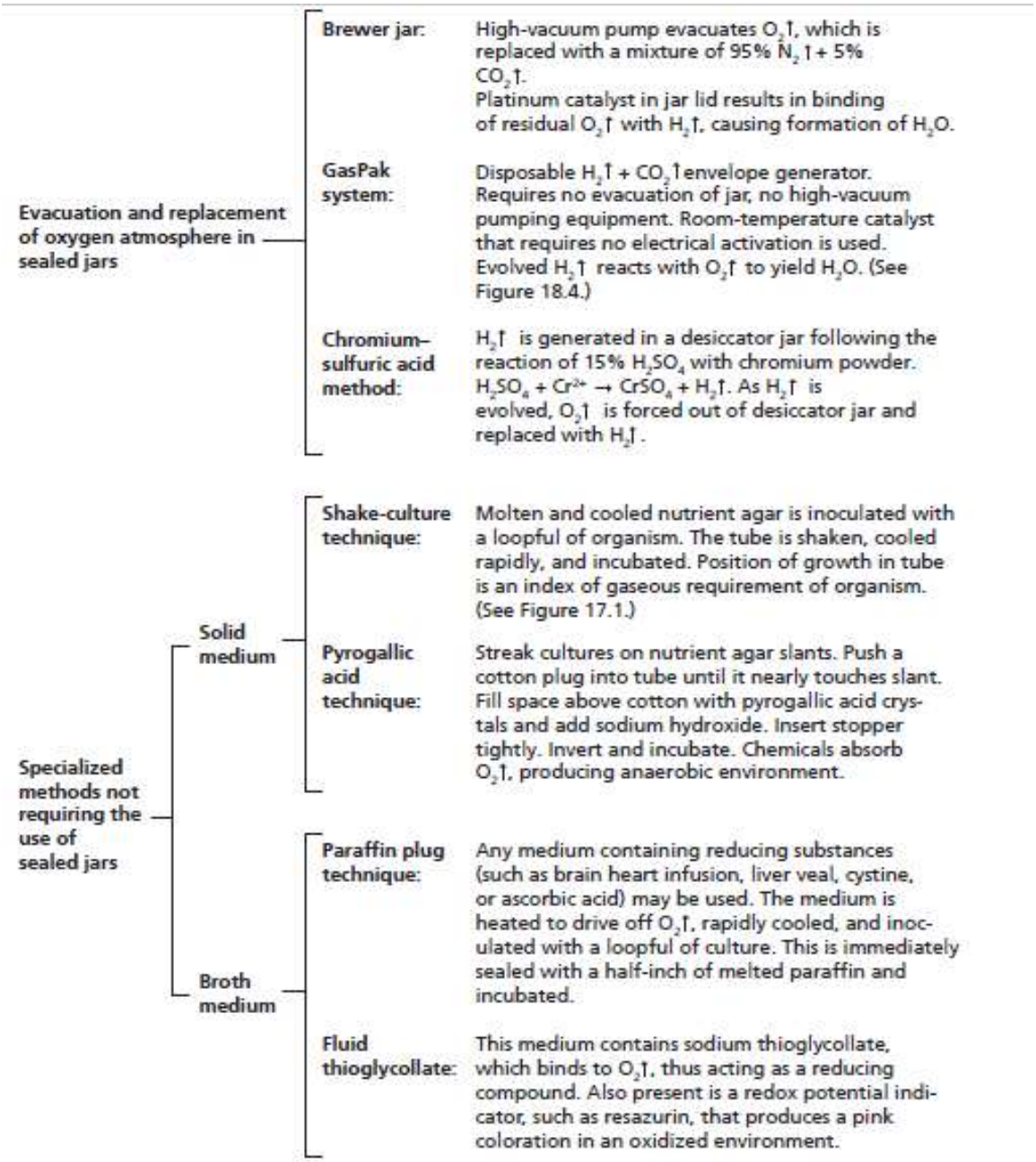


Figure 18.2 **Methods for the cultivation of anaerobic microorganisms**

Note: The causative agent of gas gangrene, *Clostridium perfringens*, is an anaerobic bacterium that thrives in wounds deprived of circulation and oxygen and that can cause limb loss and death. Treatment may involve amputation or surgical removal of infected tissue. Doctors may also prescribe therapy using enriched oxygen delivered to the patient in a hyperbaric chamber. This allows the blood to carry more oxygen to the wounds, slowing the growth of anaerobic microbes. Patients typically undergo five 90-minute sessions lying in a chamber pressurized to 2.5 atmospheres, possibly alleviating the need for surgery.

Requirement: Thioglycolate, Test tubes, Cultures.

Procedure:

1. For the performance of this procedure, the fluid thioglycollate medium must be fresh. Freshness is indicated by the absence of a pink color in the upper one-third of the medium. If this coloration is present, loosen the screw caps and place the tubes in a boiling water bath for 10 minutes to drive off the dissolved O_2 from the medium. Cool the tubes to 45°C before inoculation.

2. Aseptically inoculate the appropriately labelled tubes of thioglycollate with their respective test organisms by means of loop inoculations to the depths of the media.

3. Incubate the cultures for 24 to 48 hours at 37°C.

Observations and Result: The appearance of the growth of organisms according to their gaseous requirements in thioglycolate medium.

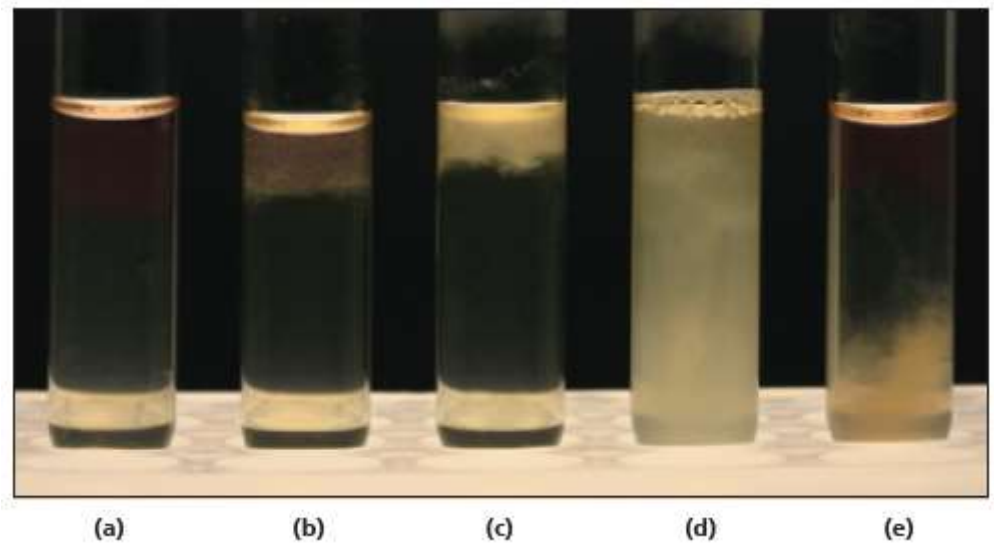
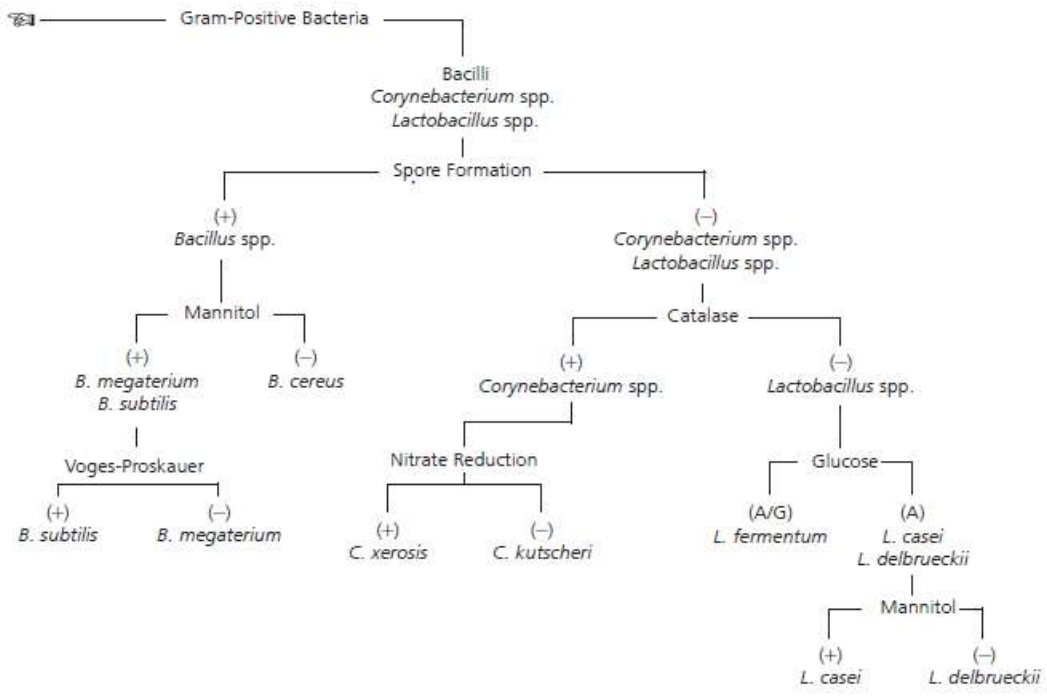


Fig.- Bacterial growth patterns in thioglycollate broth tubes. (a) Uninoculated control. (b, c) Uniform growth indicates facultative anaerobic bacteria. (d) Bubbles indicate gas-producing bacteria. (e) Bottom growth indicates anaerobic bacteria.

Identification of Bacteria up to Species Level

Principle: Identifying unknown bacterial cultures is one of the major responsibilities of a microbiologist. Samples of blood, tissue, food, water, and cosmetics are examined daily in laboratories throughout the world for the presence of contaminants. Additionally, industrial organizations are constantly screening materials to isolate new antibiotic-producing organisms or organisms that will increase the yield of marketable products, such as vitamins, solvents, and enzymes. Once isolated, these unknown organisms must be identified and classified. The science of classification is called taxonomy and deals with the separation of living organisms into interrelated groups. Bergey's Manual has been the official, internationally accepted reference for bacterial classification since 1923. The current edition, Bergey's Manual of Systematic Bacteriology, arranges related bacteria into 33 groups called sections rather than into the classical taxonomic groupings of phylum, class, order, and family. The interrelationship of the organisms in each section are based on characteristics such as morphology, staining reactions, nutrition, cultural characteristics, physiology, cellular chemistry, and biochemical test results for specific metabolic end products.

Requirements: Trypticase soy broth, MacConkey broth, Agar, Phenylethyl broth, Crystal violet, 95% ethanol, Safranin, Glass slides, Petridishes, Cover slip, and Cultures of Gram-positive and Gram-negative bacteria.



NG= No growth; G= Growth; A/G= Acid and gas; A= Acid only

Figure 68.1 (continued) Schema for the identification of gram-positive bacteria

Procedure I (Separation of the Bacteria in Mixed Unknown Culture):

1. Prepare a Trypticase soy agar broth subculture of the unknown bacterial species and refrigerate following incubation.

2. Prepare a Gram-stained smear of the original unknown culture.

3. Prepare four-way streak inoculations on the following media for the separation of the microorganisms in the mixed cultures:

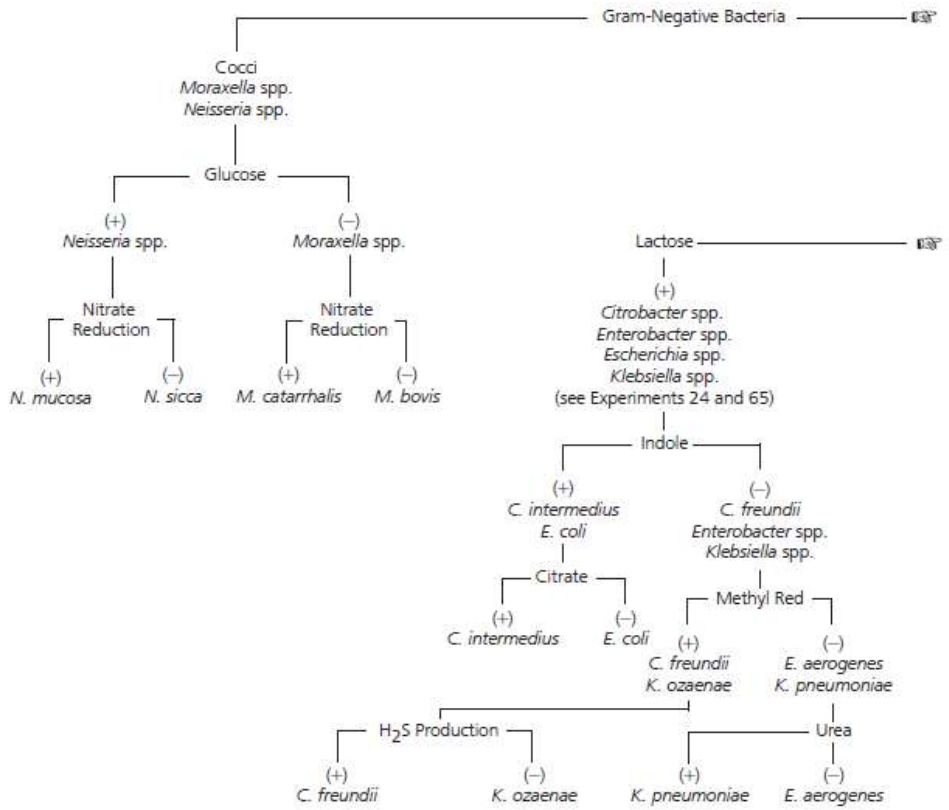
- Trypticase soy agar for observation of colonial characteristics.
- Phenylethyl alcohol agar for isolation of gram-positive bacteria.
- MacConkey agar for isolation of gram-negative bacteria.

4. Incubate all the plates in an inverted position and then subculture for 24 to 48 hours at 37°C.

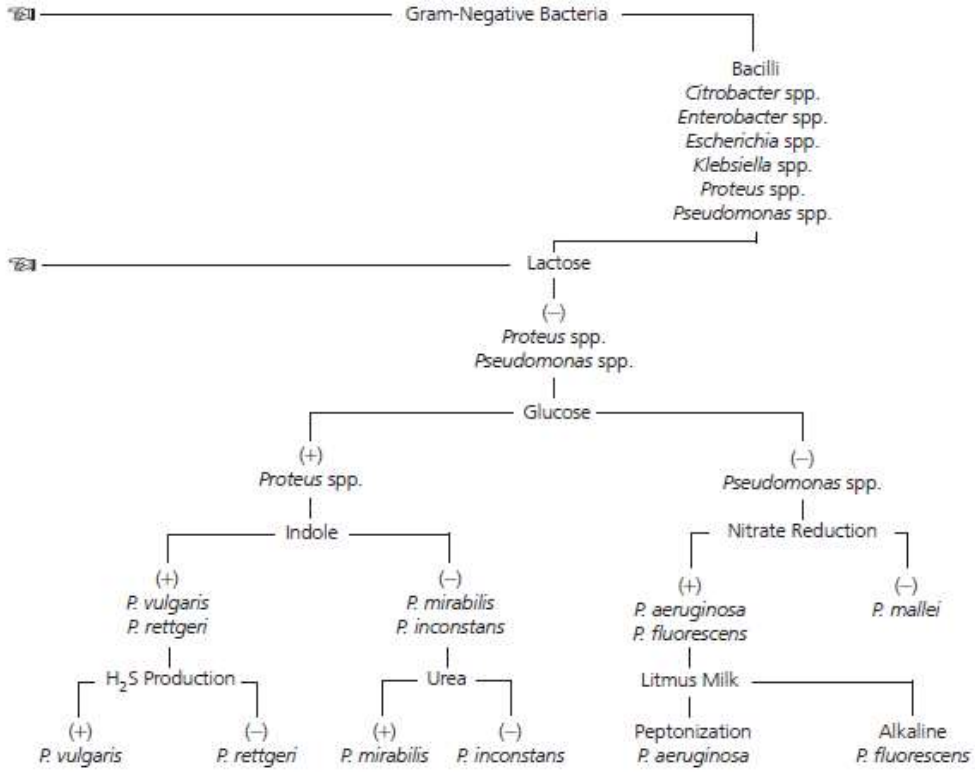
Procedure II (Preparation of Pure Cultures):

1. Isolate a discrete colony on both the phenylethyl alcohol agar plate and the MacConkey agar plate, and aseptically transfer each onto a Trypticase soy agar slant.

2. Incubate the Trypticase soy agar slants for 24 to 48 hours at 37°C.



NG=No growth; G=Growth; A/G=Acid and gas; A=Acid only



NG= No growth; G= Growth; A/G= Acid and gas; A= Acid only

Figure 68.2 (continued) Schema for the identification of gram-negative bacteria

Procedure III (Preparation of Pure Cultures):

1. Examine the Trypticase soy agar plate for the appearance of discrete colonies. Select two colonies that differ in appearance and determine their colonial morphologies.

2. Examine the phenylethyl alcohol and MacConkey agar plates.

Identification of Unknown Bacterial Species

1. Prepare a Gram-stained smear from each of the Trypticase soy agar slant cultures to verify its purity by means of the Gram reaction and cellular morphology.

2. If each Gram-stained preparation is not solely gram-positive or gram-negative, repeat the steps in Labs One and Two, using the refrigerated Trypticase soy agar subculture as the test culture.

3. If the isolates are deemed to be pure on the basis of their cultural and cellular morphologies, continue with the identification procedure. During this period perform the necessary biochemical tests on each of isolates for identification of its species. Incubate all cultures for 24 to 48 hours at 37°C.

Observations and Result: Following the aforementioned biochemical tests and staining procedures, the unknown bacterium was matched with figure and identify the species of the unknown bacterium.

Aseptic Practices in Laboratory and Safety Precautions

1. Never uses any pipette substance by mouth. In laboratory always avoid contact hand to mouth or hand to eye. In the laboratory, never drink, eat, apply cosmetics, lip balm, handle contact lenses and taken medication.
2. Aseptic techniques properly use. Right way washes your hand after removing gloves, other personnel protective equipment (PPE), handling potentially infectious agents or materials and exiting of laboratory.
3. Centers for Disease Control and Prevention (CDC)/National Institutes of Health (NIH) Biosafety in Microbiological and Biomedical Laboratories (BMBL) have recommends that laboratory personnel must be protected their streetwear clothing from contamination by wearing appropriate attire (e.g., gloves, lab shoes or use shoe covers) when conducting works in BSL-2 laboratory. Streetwear clothing and street shoes are not permitted for use in BSL-3 laboratory; in the lab always preferred to change of streetwear clothes and shoe covers or shoes keen for uses. In BSL-4 is mandatory changed from streetwear clothes/shoes to use the permitted laboratory attires and footwear.
4. Once removing the hazardous material in the laboratory, personnel must be followed Occupational Safety and Health Act (OSHA). When infectious agents are handled in laboratories; syringes, and needles or other sharp instruments should be restricted. A used needle never repeatedly used. Dispose of syringe–needle assemblies in properly labeled, lesion resistant, autoclavable sharps containers.
5. Knob infectious materials as determined by a risk assessment. Airborne infectious agents should be controlled by a certified biosafety cabinet (BSC) appropriate to the BSLs and risks for the agent.

6. Make sure that engineering controls (e.g., eyewash, BSCs, safety showers, and sinks) are properly maintained and inspected.
7. The contaminated lab ware or materials never leave open to the outside of BSC. All biohazardous materials steadily stored with proper labeled, and sealed containers.
8. Must be displayed all laboratories doors with the recognized biohazard symbol, a list of the infectious agents, access requirements (e.g., PPE) and emergency contact information.
9. Avoid the use of aerosol-producing techniques when working with infectious materials. Pipette mixing, needle clipping, centrifugation, and sonication can generate considerable aerosols.
10. Antiseptic traps and inline filters on vacuum lines are used to defend vacuum lines from contamination.
11. Follow the laboratory biosafety design for the infectious materials that are conducting with the accurate decontamination methods to decontaminate the infectious materials. Always keep spill kits available in the lab for managing an accidental spill of pathogenic materials.
12. Clean the worktable area with an appropriate disinfectant after conducting work with infectious materials in the laboratory. M ust not be cluttered the laboratory containment in order to authorize suitable floor and disinfect work area.
13. Without decontaminated or sterilized or autoclaved (temperature (121 °C), pressure (15 psi), and time (15–20 min)), never allow to leave unwanted infectious materials or contaminated agents in the laboratory or to be put in the sanitary sewer.
14. When transferring or shipping infectious materials to other laboratory, always use Postal or Department of Transportation (DOT) approved, leak-proof sealed and properly packed containers (primary and secondary containers). Be sure the lid is on tight and avoiding the container contamination from the outside. Before transporting infectious materials, make sure that the outside of container is decontaminated. Ship infectious materials in accordance with Federal and local necessities.
15. All accidents, occurrences, and unexplained illnesses must be reported to lab technician/supervisor and the Occupational Health Physician.
16. Always think safety first priority during laboratory operations. Evoke, if you are not clearly apprehending the proper handling and safety procedures or how properly use safety equipment; avoid conducting work with the infectious materials until you get proper instruction.

Disposal of Contaminated Materials

Chemical Decontamination:

- Use of an intermediate decontamination step during the storage or transport of waste, e.g., the addition of liquid chlorine bleach, iodophors, or phenolic disinfectants to pipette discard pans at work stations. The concentration of decontaminant for this use should be such that the addition of liquid waste will not interfere with its effectiveness.
- Gaseous decontamination of HEPA filters in biological safety cabinets. This procedure should be carried out prior to removal of the filter for replacement or prior to repairing the cabinet. Decontamination is usually carried out with formaldehyde sublimed by heat from paraformaldehyde flakes in the presence of high humidity. The cabinet must be sealed with plastic sheets and tape prior to initiating decontamination. Human contact

with the formaldehyde should be prevented because of the highly irritating, toxic, and possibly carcinogenic properties of the gas (the OSHA limit for permissible exposure is 2 ppm).

- Decontamination of large items of equipment that are to be removed from the laboratory for repair or discard. Care should be taken to avoid corrosion of sensitive parts if the equipment is to be reused rather than discarded. A disinfectant that has low corrosive properties and has been proven to be effective against the specific microorganism should be used for this purpose.
- Treatment of mixed hazardous waste such as combinations of infectious agents and radioisotopes. After an appropriate assessment of the waste, it may be prudent to use chemically compatible decontaminants to avoid the release of potentially hazardous emissions.

Steam Autoclaving:

- Steam autoclaving usually is considered to be the method of choice for decontaminating cultures, laboratory glassware, pipettes, syringes, or other small items known to be contaminated with infectious agents. Location of the autoclave within the laboratory minimizes storage and transport problems. It provides a technically proved treatment method for rendering infectious material safe. Autoclaved waste can be disposed of as general waste.

Incineration:

- Incineration is the method of choice for treating large volumes of infectious waste, animal carcasses, and contaminated bedding materials. Because incinerators usually are located some distance from the laboratory, additional precautions for handling and packaging of infectious waste are necessary.
- Incinerators require approval and permits from local and state pollution control authorities. Although the initial capital costs and maintenance costs are high, incineration offers many advantages as a method for the treatment of infectious waste. Incineration significantly reduces waste volume and produces an unobjectionable end-product, ash. Proper design and operation can provide for energy (heat) recovery, making the operation more economical.
- Many modern incinerators achieve the proper conditions for complete and effective combustion by providing secondary combustion chambers or zones with burners to ensure that adequate conditions for time, temperature, and mixing are achieved. Primary combustion temperatures of at least 1600°F with good mixing and a gaseous retention time of about 2 seconds should provide for good burnout for the waste described in this chapter. All pathogens and proteinaceous materials are denatured at temperatures well below the mentioned temperature.

Cultivation of Fungi

Principle: Because the structural components of molds are very delicate, even simple handling with an inoculating loop may result in mechanical disruption of their components. The following slide culture technique is used to avoid such disruption. A deep concave slide containing a suitable nutrient medium with an acidic pH, such as Potato dextrose agar (PDA),

is covered by a removable coverslip. Mold spores are deposited in the surface of the agar and incubated in a moist chamber at room temperature. Direct microscopic observation is then possible without fear of disruption or damage to anatomical components. Molds can be identified as to spore type and shape, type of sporangia, and type of mycelium.

Materials: Cultures, Potato dextrose agar (PDA), Petridish, Lactophenol cotton blue, slide, cover slip.

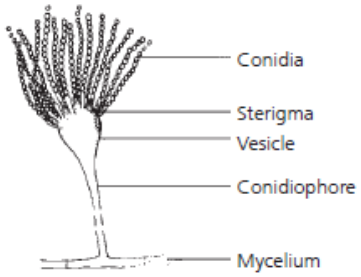
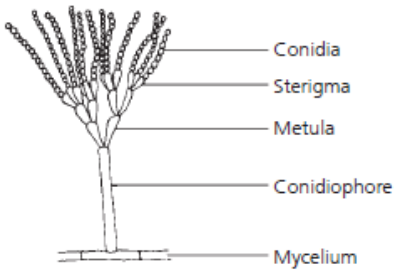
Procedure:

1. Following aseptic technique and with the help of a sterile inoculating loop, inoculated PDA plates with the supplied samples.

2. Incubate all plates at room temperature, 30°C, for 2 to 5 days. Note: Do not invert the plates.

3. 1. Examine each mold plate under the low and high power of a dissecting microscope by following LCB mount.

Result:

DIAGRAM	COLONIAL MORPHOLOGY	MICROSCOPIC APPEARANCE
 *Aspergillus:* Plant and animal pathogens; some species used industrially	White colonies become greenish-blue, black, or brown as culture matures	Single-celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bulb of the conidiophore, the vesicle; long conidiophores arise from a septate mycelium
 *Penicillium:* Antibiotic-producing citrus fruit contaminant; soil inhabitant	Mature cultures usually greenish or blue-green	Single-celled spores (conidia) in chains develop at the end of the sterigma arising from the metula of the conidiophore; branching conidiophores arise from a septate mycelium

KOH Mount

Principle: A KOH test is a simple, non-invasive procedure for diagnosing fungal infections of the skin or nails. KOH is a strong alkali. When specimen such as skin, hair, nails or sputum is mixed with 10% w/v KOH, it softens, digests and clears the tissues (e.g., keratin present in

skins) surrounding the fungi so that the hyphae and conidia (spores) of fungi can be seen under a microscope. Microscopic examination of KOH preparation reveals the presence of fungal structure and aids in diagnosing mycoses.

Materials: Microscope slide and cover glass, 20% potassium hydroxide (KOH), and Microscope.

Procedure:

➢ The affected skin or nail is gently scraped with a small scalpel or the edge of a glass slide.

➢ The scrapings from the skin are placed on a microscope slide and a few drops of a potassium hydroxide (KOH) solution are added.

➢ The slide is heated for a short time and then examined under the microscope using lactophenol cotton blue.

Results and Interpretation: Potassium hydroxide (KOH) solution is alkaline and has the ability to dissolve keratin that is scraped from the outer layer of the skin. As the KOH dissolves the material binding the skin cells together, any fungus present is released. This allows for the identification of organisms such as dermatophytes.

Fig.- Fungal hyphae in a (KOH) preparation of skin scales as seen with the 10x objective.

LCB Mount

Principle: The lactophenol cotton blue (LCB) wet mount preparation is the most widely used method of staining and observing fungi. It has the following constituents: 1) Phenol kills fungus; 2) Lactic acid acts as a clearing agent and helps preserve the fungal structures, 3) Cotton blue is an aniline dye that stains the chitin in the fungal cell walls which adds colour to

the fungal preparation thereby enhancing and contrasting the structures; and 4) Glycerol is a viscous substance that prevents drying of the prepared slide specimen.

Materials: Microscope slide and cover glass, and Microscope.

Procedure:

1) After grease free of the glass slide, a drop of LCB was placed on the slide.

2) Aseptically add the supplied sample mixed to the dye and placed a cover glass on it.

3) Observed under microscope.

Results and Interpretation: Lactophenol cotton blue solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal spores, hyphae, and fruiting structures stain blue while the background stains pale blue.

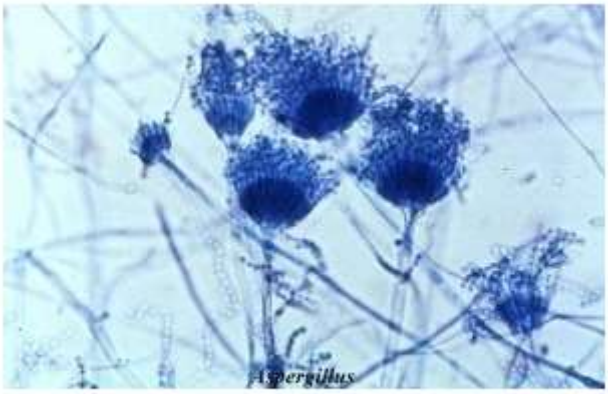
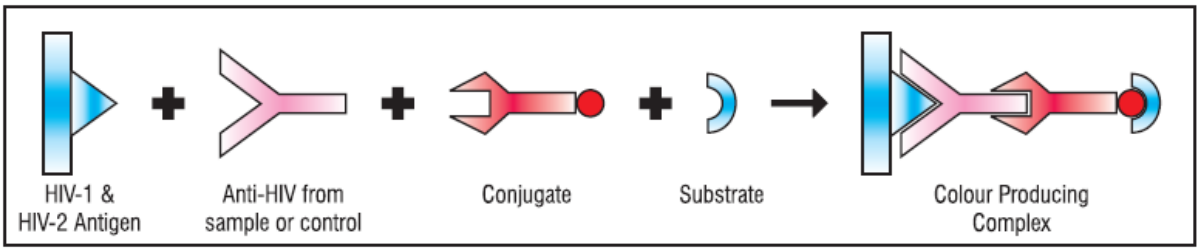


Fig: *Aspergillus* spp in LPCB Mount

Serological Test Related to Viral Diagnosis

HIV-ELISA [Microwell ELISA Test for the Detection of Antibodies to HIV-1 and HIV-2 in Human Serum/ Plasma]

Principle: HIV envelope proteins proteins gp41, C terminus of gp 120 for HIV-1, and gp 36 for HIV-2 representing immunodominant epitopes are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen-antibody complex present. Finally, substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of HIV-1 and / or HIV-2 antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.



Procedure:

A. Preparation of Reagents: Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator at 37ºC.

Microlisa-HIV Strip: Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, considering that two negative & three positive controls should be included in the run while opening the fresh kit. However, for one or two strips, one negative and two positive controls and for more strips at least two negative and three positive controls should be included in each subsequent run.

b. Unused wells should be stored at 2-8ºC, with desiccant in an aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

B. Sample Preparation:

I. Microwell Dilution:

a) Pipette 100 µl of sample diluent in to the microwell.

b) Add 10 µl of serum sample to be tested.

c) Ensure thorough mixing of the sample to be tested.

II. Preparation of Wash Buffer:

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37ºC until all crystals dissolve.

b) Prepare at least 50 ml. (2ml. concentrated buffer with 48 ml. water) of buffer for each microlisa strip used. Mix well before use.

c) Mix 20 ml. 25 X wash buffer concentrate with 480 ml. of distilled or deionized water.

Working Wash Buffer is stable for 2 months when stored at 2-8ºC.

III. Preparation of Working Conjugate: Dilute conjugate concentrates 1:100 in conjugate diluent. Do not store working conjugate. Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from table given below. Mix solution thoroughly before use.

IV. Preparation of working substrate solution: Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate. Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use.

V. Wash Procedure:

1. Incomplete washing will adversely affect the test outcome.

2. Aspirate the well contents completely into a waste container. Then fill the wells completely with wash buffer avoiding overflow of buffer from one well to another and allow to soak (approx. 30 seconds). Aspirate completely and repeat the wash and soak procedure 4 additional times for a total of 5 washes.

3. Automated washer if used should be well adjusted to fill each well completely without over filling

4. Tap upside down on absorbent sheet till no droplets appear on the sheet, taking care not to dislodge the wells.

VI. Test Procedure: Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell. Fit the strip-holder with the required number of Microlisa-HIV strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells so that well A-1 is the reagent blank. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl sample diluent to A-1 well as blank.

2. Add 100µl Negative Control in each well no. B-1 & C-1 respectively. Negative Control is ready to use and hence no dilution is required.

3. Add 100µl Positive Control in D-1, E-1 & F-1 wells. Positive Control is ready to use and hence no dilution is required.

4. Add 100 µl sample diluent in each well star ting from G-1 followed by addition of 10µl sample. (Refer Microwell Dilution)

5. Apply cover seal.

6. Incubate at 37ºC + 2ºC for 30 min. + 2 min.

7. While the samples are incubating, prepare Working Wash Solution and Working Conjugate as specified in Preparation of Reagents.

8. Take out the plate form the incubator after the incubation time is over and, wash the wells 5 times with Working Wash solution according to the wash procedure given in the previous section (wash procedure).

9. Add 100 µl of Working Conjugate Solution in each well including A-1.

10. Apply cover seal.

11. Incubate at 37ºC + 2ºC for 30 min. + 2 min.

12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well including A-1.

14. Incubate at room temperature (20 - 30ºC) for 30 min. in dark.

15. Add 100 µl of stop solution.

16. Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well. (Bichromatic absorbance measurement with a reference wavelength 600 - 650 nm is recommended when available).

Result: Calculation

Abbreviations

NC -	Absorbance of the Negative Control
$\overline{NC}$ -	Mean Negative Control
PC -	Absorbance of the Positive Control
$\overline{PC}$ -	Mean Positive Control

TEST VALIDITY:

Blank acceptance Criteria

Blank must be <0.100 in case of differential filter being used. In case differential filter is not available in the reader the blank value may go higher.

Negative Control Acceptance Criteria:

NC must be ≤ 0.150. If it is not so, the run is invalid and must be repeated.

Positive Control Acceptance Criteria:

1. PC must be ≥ 0.50
2. Determine the mean ($\overline{PC}$) value If one of three positive control values is outside of these limits, recalculate $\overline{PC}$ based upon the two acceptable positive control values.
3. If two of the three positive control values are outside the limits, the assay is invalid and the test must be repeated.

CUT OFF VALUE

Absorbance (O.D.)

NC	-	0.042	B1 Well	PC	-	1.412	D1 Well
	-	0.040	C1 Well		-	1.392	E1 Well
Total:		0.082	2 Wells		-	1.407	F1 Well
				Total :		4.211	3 Wells

$\overline{NC} = 0.082/2 = 0.041$ $\overline{PC} = 4.211/3 = 1.403$

The cut off value is calculated by adding Mean Negative Control ($\overline{NC}$) and Mean Positive Control ($\overline{PC}$) as calculated above and the sum is divided by 6.

$$\text{Cut off Value} = \frac{\overline{NC} + \overline{PC}}{6}$$

e.g. $\overline{NC} = 0.041$, $\overline{PC} = 1.403$

$$\text{Cut off Value} = \frac{0.041 + 1.403}{6} = \frac{1.444}{6} = 0.240$$

Interpretation:

1. Test specimens with absorbance value less than the cut off value are non-reactive and may be considered as negative for anti-HIV.

2. Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti-HIV by Microlisa-HIV.

Note: Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antibodies and should be retested in duplicate.

3. Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Microlisa-HIV. Original specimen should be retested in duplicate.

4. If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non-reactive.

5. If any one of the duplicates retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Microlisa HIV. Further confirmation by other EIA assays or confirmation assays including Western Blot or PCR is recommended.

GAC- ELISA Test for the Detection of Dengue IgG Antibodies in Human Serum/Plasma

Principle: Antibodies to Dengue if present in the specimen, will bind to the Anti-human IgG antibodies adsorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRPO) conjugated dengue antigen (DEN1-4) is added to each well. This dengue antigen conjugate will bind to Dengue specific IgG antibodies which is complexed with anti- human IgG antibodies. Finally, substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of Dengue antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain Dengue IgG antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

Preparation of Reagents:

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator to 37ºC.

i) Anti human IgG antibodies coated strips: Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that one each of negative & positive control & three calibrators should be included in the run while opening the fresh kit. However, for one or two strips, one each of negative, positive control & two calibrator

and for more strips one each of negative and positive control & three calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8oC, with dessicant in aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the

wells.

ii) Sample Preparation:

TUBE DILUTION: Mark the tubes carefully for the proper identification of the samples. Dilute the serum samples to be tested, with sample diluent 1:100 in separate tubes (1 ml. sample diluent + 10 µl serum samples). Use a separate tip for each sample and then discard as biohazardous waste.

iii) Preparation of Working Wash Buffer:

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.

b) Prepare at least 25ml. (1ml. concentrated buffer with 24 ml. water) of buffer for each strip used. Mix well before use.

c) Mix 20 ml. of 25X wash buffer concentrate with 480 ml. of distilled or deionized water. Working wash buffer is stable for 2 months when stored at 2-8°C.

iv) Preparation of working substrate solution: Mix TMB substrate and TMB Diluent in 1:1 to prepare working substrate.

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

Test Procedure:

Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the strip-holder with the required number of Anti-human IgM coated strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl Negative Control in A-1well.

2. Add 100 µl calibrator in B-1, C-1 & D-1 wells.

3. Add 100 µl Positive Control in E-1 well.

4. Add 100 µl of each sample diluted in sample diluent (1:100), in each well star ting from F-1-well. (Refer TUBE DILUTION).

5. Apply cover seal.

6. Incubate at 37ºC ± 1ºC for 60 min. ± 1min.

7. While the samples are incubating, prepare working Wash Solution as specified in preparation of reagents.

8. Take out the plate from the incubator after the incubation time is over and, wash the wells 5 times with working Wash Solution.

9. Add 100 µl of Enzyme Conjugate Solution in each well.

10. Apply cover seal.

11. Incubate at 37ºC ± 1ºC for 60 min ± 1min.

12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well.

14. Incubate at room temperature (20-30ºC) for 30 min. in dark.

15. Add 50 µl of stop solution.

16. Read absorbance at 450 nm and 630 nm (reference filter) within 30 minutes in ELISA Reader.

Calculation of Results:

a. Cut off value = mean O.D. of calibrator × calibration factor

b. Calculation of sample O.D. ratio: Calculate sample O.D. ratio as follows:

Sample O.D.

Sample O.D. ratio $=\frac{Sample\ O.D.}{Cut\ off\ Value}$

c. Calculation of Dengue IgG units: Calculate by multiplying the sample O.D. ratio by 10.

Dengue IgG units = sample O.D. ratio × 10.

e.g.: Mean O.D. of calibrator = 0.75

Calibration factor = 0.7

Cut off value = 0.75 × 0.7 = 0.525

e.g.: sample absorbance (O.D.) = 0.925

Cut off value = 0.525

Sample O.D. ratio = 0.925 / 0.525 = 1.761

Dengue IgG units = 1.761 × 10 = 17.61

Interpretation of Results:

a. If the Dengue IgG Units is < 9 then interpret the sample as Negative for Dengue IgG antibodies.

b. If the Dengue IgG Units is between 9 - 11 then interpret the sample as Equivocal for Dengue IgG antibodies. Equivocal samples should be repeated in duplicate and calculate the average dengue units. Sample that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.

c. If the Dengue IgG Units is > 11 then interpret the sample as Positive for Dengue IgG antibodies.

Virology Specimen Collection Guidelines

Specimen Type	Guidelines	Device and minimum vol.	Transport time and temp	Replica Limits	Comments
For virus specimen selection guidelines, refer to Table 4.	In general, specimens for virus isolation should be collected within 4 days after onset of illness as virus shedding decreases rapidly after that time. With only a rare exception, virus cultures are not worthwhile for specimens collected more than 7 days after the onset of illness.	Except for body fluids (BAL, CSF, urine, blood), place all viral specimens in UTM[a]	Most viruses remain stable at 4°C for 2-3 days and almost indefinitely at –70°C. *Do not freeze at –20°C.*		To ensure proper evaluation, the following information should accompany the specimen: (1) date of illness onset, (2) date and time specimen was collected, (3) admitting diagnosis, (4) source of specimen. Collection of acute and convalescent phase sera should always be considered.
Blood	1. Cleanse venipuncture site with 70% isopropyl alcohol. 2. Starting at the site, swab concentrically with 2% iodine tincture. 3. Allow the iodine to dry (≈ 1 min). 4. *Do not palpate the vein at this point.* 5. Collect 8-10 ml in an anticoagulant tube (viral transport is *not* required). 6. After venipuncture, remove iodine from the skin with alcohol.	Heparin tube, 8-10 ml/tube. You may need to draw 2 or more tubes from patients who are leukopenic.	Submit at RT[a]	None	Commonly ordered for: CMV. Collect blood during the early, acute phase of infection. Maintain at RT[a]. *Do not refrigerate.*

CSF	1. Disinfect site with 2% iodine tincture. 2. Insert a needle with stylet at L3-L4, L4-L5, or L5-S1 interspace. 3. On reaching the subarachnoid space, remove the stylet and collect 2-5 ml in a sterile leakproof tube. (UTM[a] not required).	Sterile screw-cap tube, 1.0 ml	Submit immediately at 4°C.	None	Frequently isolated: coxsackievirus (some), echovirus, enterovirus, mumps virus. Less frequently isolated: arboviruses, HSV, LCMV, rabies virus.
Cervical or vaginal swab[b]	1. If lesions are present, swab vigorously. Place swab in UTM[a]. 2. If lesions are not present, remove mucus from the cervix with a swab and discard the swab. 3. Firmly sample the endocervix (≈ 1 cm.	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day/ source	Frequently isolated: HSV, CMV Noncultivable: papillomavirus, molluscum contagiosum virus.
	into the cervical canal) with a fresh swab by rotating the swab for 5 seconds. 4. Place swab in UTM[a]. 5. Carry out a vulvar sweep using a second swab; place both swabs in the same transport tube.				Although a cervical swab sample is the specimen of choice in the monitoring of pregnant women with a history of genital HSV infection, recovery of HSV may be increased by also sampling the vulva.
Conjunctiva swab[b]	1. Collect material from the lower conjunctiva with a flexible, fine-shafted swab moistened with sterile saline. 2. Place swab in UTM[a].	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	None	Frequently isolated: adenovirus; coxsackievirus A (some), CMV, HSV, enterovirus (including type 70), Newcastle disease virus.
Feces	1. Pass directly into a clean, dry container. 2. 2-4 g of stool to sterile, leakproof container and transport immediately to lab.	Sterile, leakproof, wide-mouth container, at least 2 g or more.	Submit at 4°C.	1/day	Frequently isolated: adenoviruses; enteroviruses. Less frequently isolated: rotavirus Rotavirus antigen is detected by EIA.
Nasal swab[b]	1. Pass a flexible, fine shafted swab into the nostril. 2. Rotate slowly for 5 seconds to absorb secretions. 3. Remove swab and place in UTM[a]. 4. Repeat for other nostril using a fresh swab. Place both swabs in the same transport tube.	Swab[b]	Immediately place swab in UTM. Submit at 4°C.	1/day	Frequently isolated; influenza virus, parainfluenza virus, rhinovirus (limited), RSV (nasopharyngeal preferred) Influenza A virus and RSV are detected by antigen assay (EIA).

Nasopharynx aspirate or wash	1. Pass appropriate size tubing or catheter into the nasopharynx. 2. Aspirate material with a small syringe. 3. If material cannot be aspirated, tilt patient's head back about 70° and instill 4 ml. of sterile PBS until it occludes the nostril. 4. Reaspirate. Place in sterile container. 5. Place specimen at 4°C immediately.	Sterile container.	Submit at 4°C.	1/day	Frequently isolated: influenza virus, parainfluenza virus, rhinovirus (limited), RSV. Influenza A virus and RSV can be detected by antigen assay.
Nasopharynx swab[b]	1. Pass a flexible, fine shafted swab into the nasopharynx. 2. Allow secretions to absorb for 5 seconds; then carefully remove swab and place it in UTM[a]. 3. Repeat for other nostril using a fresh swab. Place both swabs in the same transport tube.	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day	Frequently isolated: influenza virus, parainfluenza virus, rhinovirus (limited), RSV.
Oral swab[b]	1. Firmly sample base of lesion(s) with a swab. 2. Place swab in UTM.	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day	Frequently isolated: enterovirus (some), HSV.
Rash Maculopapular	1. Gently cleanse area with sterile saline. 2. Disrupt the surface of the lesion and firmly sample its base with a swab moistened with sterile saline. 3. Place swab in UTM[a].	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day/ source	Frequently isolated: adenovirus, enterovirus, rubella virus, measles virus (rubeola virus) Less frequently isolated: pox viruses Noncultivable: parvovirus B19
Vesicular	1. Sample only fresh vesicles because older crusted vesicles may not contain viable virus. 2. Cleanse area with sterile saline. 3. Carefully open the vesicle with needle or scalpel blade. 4. Using a swab, collect fluid and cellular material by vigorously sampling the base of the lesion. 5. Place in UTM[a].	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day/ source	Frequently isolated: enterovirus (some), echovirus, HSV, VZV Less frequently isolated: poxviruses The preferred specimen for VZV is a vesicle aspirate placed in 1 ml. UTM[a].
Throat swab[b]	1. Using a tongue depressor, depress the tongue to prevent contamination with saliva. 2. Firmly sample the posterior pharynx, tonsils, and inflamed areas with a sterile swab. 3. Place swab in UTM[a].	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day	Frequently isolated: adenovirus, CMV, enterovirus, HSV, influenza A and B viruses, measles virus, mumps virus, parainfluenza virus. Less frequently isolated: RSV.

Tissue	1. Obtain samples from areas directly adjacent to affected tissue. 2. Place specimen in a sterile vial containing UTM[a].	UTM[a]	Submit at 4°C.	None	Always submit as much tissue as possible. Never submit a swab that has simply been rubbed over the surface.
Urethral swab[b]	Patient should not have urinated within 1 h prior to collection. 1. Express and discard any exudate. 2. Carefully insert flexible, fine-shafted swab 4 cm. into urethra. 3. Rotate swab 2-3 times to obtain an adequate number of cells. 4. Remove swab and place in UTM[a].	Swab[b]	Immediately place swab in UTM[a]. Submit at 4°C.	1/day	Frequently isolated: CMV, HSV
Urine	Refer to specific guidelines for urine collection. Collect 5 ml. of midstream clean, voided urine in a sterile container (UTM[a] not required).	Sterile container, 5 ml	Submit at 4°C	1/day	Frequently isolated: adenovirus, CMV, HSV, mumps virus Less frequently isolated: polyomavirus (JC virus), rubella virus Two or three specimens on successive days maximize recovery of CMV.

[a] BAL, bronchoalveolar lavage; CSF, cerebral spinal fluid; UTM, virus, chlamydia, mycoplasma, ureaplasma, transport medium; PBS, phosphate buffered saline, RT, room temperature; CMV, cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein-Barr virus; HIV-1, human immunodeficiency virus type 1: LCMV, lymphocytic choriomeningitis virus: EIA, enzyme immunoassay; RSV, respiratory syncytial virus; ELISA, enzyme-linked immunosorbent assay; VZV, varicella-zoster virus.

[b] Dacron or rayon with plastic or aluminum shafts are acceptable; calcium alginate swabs or swabs with wooden shafts are not acceptable.

Slide Culture

Principle: Slide culture is a very useful technique in identification of the type of fungi. The fungal element that is to be identified will produce characteristic hyphae and spores, when incubated on a suitable growth medium. This can be visualized undisturbed using this technique. The mould that is to be cultured is inoculated onto a small piece of an agar below a cover slip. The whole setup is kept in a Petri dish with moisture. The cover slip after incubation is lifted, stained and observed under a microscope for identification of the fungi.

Requirements: Petridish having potato dextrose agar (PDA) containing fungal culture, V-shaped glass rod, Slide, cover slip, Inoculating loop, Lactophenol cotton blue (LPCB).

Procedure:

- From the Petri dish containing PDB agar cut out one square cm block of agar for each slide culture to be inoculated.
- With the flat side of a sterile bacteriological loop, or with a spatula, place an agar block in the centre of the slide in the slide culture set up.
- With a probe, inoculate around the periphery of the agar block, three to four fragments of the mold to be cultured.

- With forceps, the tips of which have been flamed, place the cover slip on the agar block.
- With a pipette, thoroughly moisten, but not to excess, the filter paper with sterile distilled water.
- Incubate the slide culture at room temperature.
- Remove the slide culture from the Petri dish and dry the bottom of the slide with a tissue.
- When growth appears beneath the cover slip. Take a slide place a drop of LPCB, place the cover slip removed from the block on the LPCB.
- Place the slide on the microscope stage and examine. The aerial hyphae including the conidiophores will be seen to grow along the under surface of the cover slip.

Observation and Result: Usually a minimum of 48 hr. is needed before a slide culture shows growth of aerial hyphae. Thus, the culture may be examined after 48 hours incubation and as frequently thereafter as necessary.

Small spore bearing fungi make beautiful permanent mounts. Some large spore bearing organisms like *Microsporum gypsum* do not stain as well. With the type of hyphae, arrangement of conidiophores, staining characters etc., the final interpretation of the fungal type can be made.

Fig.-Slide Culture set.

Minimum Inhibitory Concentration Determination

Principle: Due to emergence of many antibiotic resistant strains of bacteria, antimicrobial susceptibility testing is done in order to determine which antimicrobial agent to use against a specific strain of bacteria. The available chemotherapeutic agents vary in their scope of antimicrobial activity. Some have a limited spectrum while others have a wide spectrum of activities against bacteria. The bacterial strains isolated from clinical samples should be tested for antimicrobial sensitivity because it gives the clinician an idea as to what antimicrobial therapy should be started to the patients. The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of the test microorganism. Quantitative data of this nature may be used by a clinician to establish effective antimicrobial regimens for the treatment of a bacterial infection in a host. These data are of particular significance when the toxicity of the antibiotic is known to produce major adverse effects in host tissues. Agar dilution is a quantitative method for determining the minimum inhibitory concentration of the antibiotics against bacteria to be tested.

Requirements:

I. Reagents and lab wares: 0.5 McFarland standard, sterile Mueller Hinton agar (pH 7.2-7.4), sterile Mueller Hinton broth, antibiotic powder, sterile test tubes, pipettes, screw

capped flat bottomed bottles (25 ml capacity) and Petri dishes (90 mm diameter). These also include sterile saline (0.85 %) and stock solution of antibiotic.

II. Preparation of stock solutions of antibiotics: The required dilutions of the antibiotics are made as per the table 33-1. Prepare a stock solution containing 2000 µg / ml of the antibiotic to be tested. For example, weigh 200 mg of the antibiotic powder and dissolve in 5 ml of distilled water / appropriate solvent. Mix 0.5 ml of this solution with 9.5 ml distilled water (working solution contains antibiotics at a strength of 200 µg / ml-solution A)

III. Specimens: Preparation of suspension of bacteria: Approximately, 4-5 well isolated colonies of the bacterial strain to be tested are transferred to Tryptic soy broth or BHI broth. The turbidity of the suspension is adjusted to match 0.5 McFarland standards (10^6 organisms/ml).

Procedure:

Preparation of the agar plate with different concentration of the antibiotics

1. Dispense 2 ml of the diluted antibiotic solution into each of the marked sterile screw capped bottle.

Note: It is advisable to start with the highest dilution so that single pipette can be used to dispense all the dilutions prepared.

2. Sterile Muller-Hinton agar is cooled and maintained at 50°C – 55°C in a water bath.

3. Pour this medium (18 ml) into the screw capped bottle containing the different concentration of antibiotic, shake well and pour into sterile petri dish.

Note: By this method, exact volume of medium (22.6 ml) is delivered into the screw capped bottles without the danger of the molten agar jellifying during transfer into dilution of the antibiotic

4. Keep the poured plates at 4°C for setting.

5. After the plates have set, dry the plates well in an incubator at 37°C for 30-60 mins. The plates must be dry before performing the test.

Test procedure

1. A grid is marked on the bottom of the plates containing antibiotics.

Note: 20 – 25 strains can be tested in plate including the control.

2. A loopful of inoculating loop is calibrated to deliver 0.001 –0.002 ml (1-2 µl) of the culture.

3. Inoculate the culture on the surface of the medium, indicated by the square marked below. In each case 104 bacteria is delivered to a spot 5 – 8 mm in diameter.

Note: Inoculation is done starting with the plates containing highest dilution of the antibiotic.

4. Inoculate a control plate without antibiotics simultaneously as control.

5. Allow the drops to dry and incubate the plates without inverting at 37°C for 16 –18 hours.

Observations and Result: Read the plates for presence or absence of growth. Check the control plate for growth. Control plate must show confluent or near confluent growth. Read the test plate. The concentration at which growth is completely inhibited is considered as the minimum inhibitory concentration (MIC). The organisms are reported sensitive, intermediate or resistant by comparing the test MIC values with that given in the NCCLS table. The highest dilution of antibiotic showing more than 99% inhibition of growth of bacteria is considered as the minimum inhibitory concentration (MIC) of the bacteria.

Table 33.1 System for preparing dilutions for agar dilution method

Antibiotic Solution	+ Sterile Water (Vol)	= Intermediate conc (µg / ml) in tubes	Final conc at 1:10 in agar plates (µg / ml)
Volume	µg/ml		
6.4	2000 - A (Stock)	3.6	1280 - B 128
2	1280 - B	2	640 - C 64
1	1280 - B	3	320 - D 32
1	1280 - B	7	160 - E 16
2	160 - E	2	80 - F 8
1	160 - E	3	40 - G 4
1	160 - E	7	20 - H 2
2	20 - H	2	10 - I 1
1	20 - H	3	5 - J 0.5
1	20 - H	7	2.5 - K 0.25

	Antimicrobial agent	concentration (µg)	Zone of inhibition (mm)		
			resistant	intermediate	susceptible
1.	Ciprofloxacin	5	≤15	16-20	21≥
2.	Erythromycin	15	≤13	14-22	23≥
3.	Gentamicin	10	≤12	13-14	15≥
4.	Tetracycline	30	≤14	15-18	19≥
5.	Streptomycin	10	≤12	13-14	15≥