Pharmaceutical Microbiology Laboratory Manual

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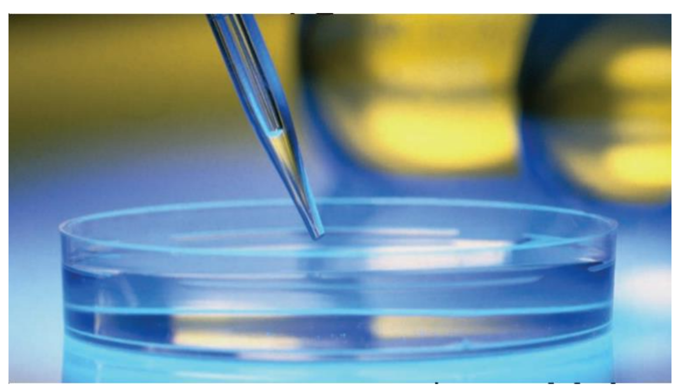

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<u>ORIENTATION TO THE PHARMACEUTICAL MICROBIOLOGY LABORATORY</u>

Introduction

Safety in microbiology laboratory is important in the prevention of infection that may be caused by the microorganisms being studied. In addition many of the reagents and procedures are potentially hazardous.

There will be no highly virulent human or plant **pathogens**. However, some of the organisms used are considered pathogenic. This mean that, although they may not cause disease in normal healthy host, they might if the host can be compromised in number of different ways: wounds and cuts, lowered resistance due to another disease, surgery, stress (including stress examination), or immune –system disability (including autoimmune disease or the use of immunosuppressive drugs).in addition infection can occur, albeit rarely, by relatively nonpathogenic organisms even in healthy hosts.

In addition to microorganism, there are some **chemicals** used in the laboratory which are potentially harmful. Safety is a very important requirement for work in chemical laboratories.

- Sometimes you'll be working with toxic materials, flammable liquid and explosive compounds.
- If all important precautions are not taken into consideration, then hazardous accidents will occur.

The following rules are very important for your safety as well as the safety of other students.
<u>Please</u> read them carefully and follow them each laboratory.

Warning

Some of the laboratory experiments included in this text may be hazardous if you handle materials improperly or carry out procedures incorrectly. Safety precautions are necessary when you work with any microorganism, and with chemicals, glass test tubes, hot water baths, sharp instruments, and similar materials. If you have any problems with materials or procedures, please ask your instructor for help.

Safety procedures and precautions

The microbiology laboratory, whether in a classroom or a working diagnostic laboratory, is a place where cultures of microorganisms are handled and examined. This type of activity must be carried out with good aseptic technique in a thoroughly clean, well-organized workplace. In aseptic technique, all materials that are used have been sterilized to kill any microorganisms contained in or on them, and extreme care is taken not to introduce new organisms from the environment. Even if the microorganisms you are studying are not usually considered **<u>pathogenic</u>** (disease producing), any culture of any organism should be handled as if it were a potential pathogen. With current medical practices and procedures, many patients with lowered immune defenses survive longer than they did before. As a result, almost any microorganism can cause disease in them under the appropriate circumstances. Each student must quickly learn and continuously practice aseptic laboratory technique. It is important to prevent contamination of your hands, hair, and clothing with culture material and also to protect your neighbors from such contamination. In addition, you must not contaminate your work with microorganisms from the environment. The importance of asepsis and proper disinfection is stressed throughout this manual and demonstrated by the experiments. Once these techniques are learned in the laboratory, they apply to almost every phase of patient care, especially to the collection and handling of specimens that are critical if the laboratory is to make a diagnosis of infectious disease. These specimens should be handled as carefully as cultures so that they do not become sources of infection to others.

In general, all safety procedures and precautions followed in the microbiology laboratory are designed to:

1. Restrict microorganisms present in specimens or cultures to the containers in which they are collected, grown, or studied.
2. Prevent environmental microorganisms (normally present on hands, hair, clothing, laboratory benches, or in the air) from entering specimens or cultures and interfering with results of studies.

Microbiology Lab Practices and Safety Rules

1. Careful attention to the principles of safety is required throughout any laboratory course in microbiology.
2. Laboratory coats are worn, long hair is tied back
3. Avoid loose fitting items of clothing. Wear appropriate shoes (sandals are not allowed) in the laboratory
4. Wash your hands with disinfectant soap when you arrive at the lab and again before you leave.
5. Absolutely no food, drinks, chewing gum, or smoking is allowed in the laboratory. Do not put anything in your mouth such as pencils, pens, labels, or fingers. Do not store food in areas where microorganisms are stored.
6. Personal conduct in a microbiology laboratory should always be quiet and orderly.
7. Any student with a fresh, unhealed cut, scratch, burn, or other injury on either hand should notify the instructor before beginning or continuing with the laboratory work. If you have a personal health problem and are in doubt about participating in the laboratory session, check with your instructor before beginning the work.
8. Keep your workspace free of all unnecessary materials. Backpacks, purses, and coats should be placed in the cubbyholes by the front door of the lab. Place needed items on the floor near your feet, but not in the aisle.
9. Disinfect work areas before and after use with 70% ethanol or fresh 10% bleach. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant on a routine basis, and especially after spills, splashes, or other contamination.

10. Familiarize yourself with the location of safety equipment in the lab (e.g., eye-wash station, shower, sinks, fire extinguisher, biological safety cabinet, first aid kit, emergency gas valve).
11. Containers used for specimen collection or culture material are presterilized and capped to prevent entry by unsterile air, and sterile tools are used for transferring specimens or cultures.
12. Treat all microorganisms as potential pathogens. Use appropriate care and do not take cultures out of the laboratory.
13. Wear disposable gloves when working with potentially infectious microbes or samples (e.g., sewage). If you are working with a sample that may contain a pathogen, then be extremely careful to use good bacteriological technique.
14. Never pipette by mouth. Use a pipetting aid or adjustable volume pipettors. [In the distant past, some lab personnel were taught to mouth pipette. This practice has been known to result in many laboratory-acquired infections. With the availability of mechanical pipetting devices, mouth pipetting is strictly prohibited]
15. Consider everything a biohazard. Do not pour anything down the sink. Autoclave liquids and broth cultures to sterilize them before discarding.
16. Dispose of all solid waste material in a biohazard bag and autoclave it before discarding in the regular trash.
17. Replace caps on reagents, solution bottles, and bacterial cultures. Do not open Petri dishes in the lab unless absolutely necessary.
18. Inoculating loops and needles should be flame sterilized in a Bunsen burner before you lay them down.
19. Turn off Bunsen burners when not in use.
20. When you flame or sterilize with alcohol, be sure that you do not have any papers under you.
21. Dispose of broken glass in the broken glass container.
22. Dispose of razor blades, syringe needles, and sharp metal objects in the “sharps” container.
23. Report spills and accidents immediately to your instructor. Clean small spills with care (see instructions below). Seek help for large spills.
24. Report all injuries or accidents immediately to the instructor, no matter how small they seem.
25. Label everything clearly.

<u>Tools in Microbiology Laboratory</u>

The most common equipment are inoculation needle, inoculation/transfer loop, Bunsen burner, autoclave (or pressure cooker), incubator, hot air oven, refrigerator, centrifuge, spectrophotometer, magnetic stirrer, orbital shaker, hot plate, Distillation water still, UV- lamp, water-bath, carbon dioxide cylinder, single-pan balance with weights (for rough use), chemical balance, pH meter, colony counter, laminar air flow, electrophoretic apparatus, microscopes etc.

1) Inoculation Needle & Inoculation Loop

•These are the most commonly used tools.

•Inoculation needle/loop is made up of a long platinum wire fixed into a metallic rod.

•A wire loop has a handle with steel screw shaft in which nichrome or platinum wire is to be fitted.

•The straight wire needle is used for transferring culture from solid medium. Even smaller amount of liquid culture can be manipulated by using straight needle.

•The loop and wire are also used for picking small quantities of solid materials from a microbial colony and can be used to inoculate either a liquid or a solid medium. Both the loop and straight wire must be flamed immediately after use to avoid contamination.

2) Bunsen Burner

•Sterilization of tools by using spirit lamp is called incineration.

•Gas enters the burner at the base, and its supply is regulated externally by the gas cock.

•The amount of air can be controlled by rotating a sleeve that fits over the holes in the body of the burner.

•To keep the flame from blowing out special tips are frequently used to fit over the top end of the barrel.

•The proper method of lighting the burner is to close off the air supply, turn on the gas and light. The flame will be large and yellow. Gradually open the air intake until the flame takes a blue colour.

3) Water Bath

•Water bath is an instrument that is used to provide constant temperature to a sample.

•It consists of an insulating box made up of steel fitted with electrode heating coil.

•The temperature is controlled through a thermostat.

•In some of the water baths, plate form rotates, then it is called water bath shaker. It is more useful to microbiologists because it provides a uniform heat to the sample material meant for incubation.

•The main use of water bath is the incubation of samples at a desired and constant temperature.

4) Laminar Air Flow Chamber

•Laminar air flow is an apparatus consists of an air blower in the rear side of the chamber which can produce air flow with uniform velocity along parallel flow lines. There is a special filter system of high efficiency particulate air filter (HEPA) which can remove particles as small as 0.3 mm.

•In front of the blower, there lies a mechanism through which air blown from the blower produces air velocity along parallel flow lines.

•The laminar air flow is based on flow of air current of uniform velocity along parallel flow lines which help in transferring microbial cultures in aseptic conditions. Air is passed through the filters into the enclosure and the filters do not allow any kind of microbe to enter in to the system.

•Inside the chamber one fluorescent tube and another UV tube are fitted. Two switches for these tubes and a separate switch for regulation of the air flow are fitted outside the LAF. Due to uniform velocity and parallel flow of air current, pouring of media, plating, slant preparations, streaking etc. are performed without any kind of contamination.

•Initially, dust particles are removed from the surface of the laminar air flow with the help of smooth cloth containing alcohol. Switch on the UV light for a period of 30 minutes so as to kill the germs, if any present in the area of working space.

•The front cover sheet of the apparatus is opened to keep the desired material inside. The air blower is set at the desired degree so that the air inside the chamber is expelled because the air inside the chamber may be contaminated / bring contaminants.

•Sit properly in front of the chamber and wipe the working table with alcohol to reduce the contaminants. All the work related to pouring, plating, streaking etc. are to be carried out in the flame zone of the burner or spirit lamp.

•In microbiology laboratory, horizontal type of laminar air flow is used to supply the air through filter.

Precautions: Put off the shoes before entering to operate the apparatus. Wash the hands with detergents or soap. One should not talk inside the chamber while performing microbial culture transfer, failing which chances of contamination may be more which may come either through mouth, sneezing or air.

5. Incubator

•An incubator is an instrument that consists of copper/steel chamber around which warm water or air is circulated by electric current or by means of small gas flame.

•The temperature of the incubator is kept constant due to its control by using thermostat.

•The incubator is made up of double walled chamber adjusted to a desired temperature. It is done by using an external knob controlling the thermostat system. The gap between two walls is insulated to check heat condition. A thermometer is inserted from the top for recording the temperature.

•Temperature greatly influences the microbial growth. Therefore, instrument is generally designed that can allow the desired microorganism to grow at a particular temperature.

•It is operated to allow the microbial growth on a suitable medium under proper temperature. In an incubator, the variation in temperature should not be more than one degree.

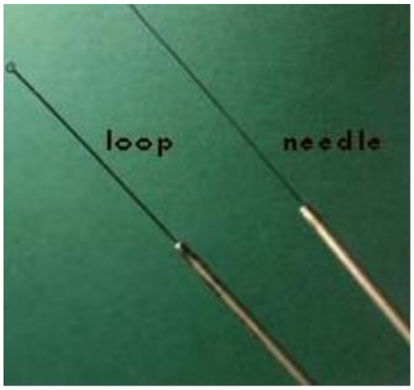
•Small square type incubators are better than large ones. If a lower temperature than the room is

required, the water is circulated around the chamber to pass through an ice chest.
Precautions: the door of the incubator should be opened only when necessary. If the tubes are to be incubated for a long time or at higher temperature, the medium may become too dry due to excessive evaporation. In such cases cotton plug should be pushed inside the neck of the tube. The tube should be covered by a rubber cap so as to cover the plug. If the petriplates are to be incubated for a long time, they
may be placed in moist chamber with a damp sterile cotton wool at the bottom.

6. Colony Counter

- It is a device used for counting small or closely growing colonies on the surface of media.
- For accuracy, the lens fitted or mounted in it helps to see the colonies.
- The lens is movable on the box and can be adjusted to see the colonies.

- The petriplate is kept on a slanting platform meant for it and illuminated with the help of light source
from beneath.
- The numbers of colonies are read out with the support of digital reading meter

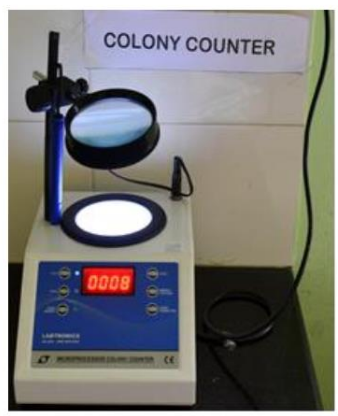


Inoculation Loop & Needle

Bunsen Burner

Water Bath

Laminar Air Flow



Colony Counter

Incubator

Labeling:

You must carefully label your plates and tubes. There will be a lot of plates and test tubes incubating together making clear labeling crucial to avoid chaos. Always label the BOTTOM of the plate so that, even if lids get accidentally switched or broken, we will know what was in each plate. Likewise, do not label tubes on the cap. The label should include your name, the date the plate was struck, and what was put into the plate (microbe name).

The Microscope

A good microscope is an essential tool for any microbiology laboratory. There are many kinds of microscopes but the type most useful in diagnostic work is the compound microscope. By means of a series of lenses and a source of bright light, it magnifies and illuminates minute objects such as bacteria and other microorganisms that would otherwise be invisible to the eye. This type of microscope will be used throughout your laboratory course. As you gain experience using it, you will realize how precise it is and how valuable for studying microorganisms present in clinical specimens and in cultures. Even though you may not use a microscope in your profession, a firsthand knowledge of how to use it is important. Your laboratory experience with the microscope will give you a lasting impression of living forms that are too small to be seen unless they are highly magnified. As you learn about these "invisible" microorganisms, youshould be better able to understand their role in transmission of infection.

Instructions

•Observe that a flat platform, or stage as it is called, extends between the upper lens system and the lower set of devices for providing light. The stage has a hole in the center that permits light from below to pass upward into the lenses above. The object to be viewed is positioned on the stage over this opening so that it is brightly illuminated from below. Note the adjustment knobs at the side of the stage, which are used to move the slide in vertical and horizontal directions on the stage. This type of stage is referred to as a mechanical stage.

•A built-in illuminator at the base is the source of light. Light is directed upward through the abbe condenser. The condenser contains lenses that collect and concentrate the light, directing it upward through any object on the stage. It also has a shutter or iris diaphragm which can be used to adjust the amount of light admitted. A lever is provided on the condenser for operating the diaphragm.

•The condenser can be lowered or raised by an adjustment knob. Lowering the condenser decreases the amount of light that reaches the object. This is usually a disadvantage in microbiological work. It is best to keep the condenser fully raised and to adjust light intensity with the iris diaphragm.

•Above the stage, attached to the arm, a tube holds the magnifying lenses through which the object is viewed. The lower end of the tube is fitted with a rotating nosepiece holding three or four objective lenses. As the nosepiece is rotated any one of the objectives can be brought into position above the stage opening. The upper end of the tube holds the ocular lens, or eyepiece (a monocular scope has one; a binocular scope permits viewing with both eyes through two oculars).

•Depending on the brand of microscope used, either the rotating nosepiece or the stage can be raised or lowered by coarse and fine adjustment knobs. These are located either above or below the stage. On some microscopes they are mounted as two separate knobs; on others they may be placed in tandem with the smaller fine adjustment extending from the larger coarse wheel. Locate the coarse adjustment on your microscope and rotate it gently, noting the upward or downward movement of the nosepiece or stage. The coarse adjustment is used to bring the objective down into position over any object on the stage, while looking at it from the side to avoid striking the object and thus damaging the expensive objective lens. The fine adjustment knob moves the tube to such a slight degree that movement cannot be observed from the side. It is used when one is viewing the object through the lenses to make the small adjustments necessary for a sharp, clear image.

Care and Handling of the Microscope

•Always use both hands to carry the microscope, one holding the arm, other under the base.

•Before each use, examine the microscope carefully and report any unusual condition or damage.

•Keep the oculars, objectives, and condenser lens clean. Use dry lens paper only.

•At the end of each laboratory period in which the microscope is used, remove the slide from the stage, wipe away the oil on the oil-immersion objective, and place the low-power objective in vertical position.

•Replace the dust cover, if available, and return the microscope to its box.

Handling and Examining Cultures

Microscopic examination of microorganisms provides important information about their morphology but does not tell us much about their biological characteristics. To obtain such information, we need to observe microorganisms in culture. If we are to cultivate them successfully in the laboratory, we must provide them with suitable nutrients, such as protein components, carbohydrates, minerals, vitamins, and moisture in the right composition. This mixture is called a culture medium. It may be prepared in liquid form, as a broth, or solidified with agar, a nonnutritive solidifying agent extracted from seaweed. Agar media may be used in tubes as a solid column or as slants, which have a greater surface area. They are also commonly used in petri dishes, or plates.

Solid media are essential for isolating and separating bacteria growing together in a specimen collected from a patient, for example, urine or sputum. When a mixture of bacteria is streaked across the surface of an agar plate, it is diluted out so that single bacterial cells are deposited at certain areas on the plate. These single cells multiply at those sites until a visible aggregate called a colony is formed. Each colony represents the growth of one bacterial species. A single, separated colony can be transferred to another medium, where it will grow as a pure culture. Colonies of several different species are regularly present on the same agar plate when certain patient specimens are inoculated onto them. Working with pure cultures permits the microbiologist to study the properties of individual species without interference from other species.

The appearance of colonial growth on agar media can be very distinctive for individual species. Observation of the noticeable, gross features of colonies, that is, of their colonial morphology, is therefore very important. The colour, density, consistency, surface texture, shape, and size of colonies all should be observed, for these features can provide clues as to the identity of an organism, although final identification cannot be made by morphology alone. In liquid media, some bacteria grow diffusely, producing uniform clouding, whereas others look very granular. Layering of growth at the top, center, or bottom of a broth tube reveals something of the organisms' oxygen requirements. Sometimes colonial aggregates are formed and the bacterial growth appears as small puff balls floating in the broth. Observation of such features can also be helpful in recognizing types of organisms.

Disposal of Laboratory Wastes and Cultures

During laboratory practices, it has been noticed that the untreated waste is generally disposed off by the laboratory staff. It happens due to their unskilled work culture. Any material which contains microorganisms should be treated first and thereafter, with the proper treatment should be thrown properly. The treatment is necessary due to the reasons:

a) If it contains pathogenic microorganisms, the disease may transmit or spread to the healthy persons.

b) It may contaminate soil and causes soil, water and air-pollution. Hence, to check from such hazards, proper treatment is required to kill microorganisms.

The infected material is generally the solid or liquid culture media used for cultivation of microorganisms, or it may also contain cotton plugs, paper, cotton or cotton swabs, gloves, pins, PCR tubes, gel material etc.

Some of the materials such as cotton plugs, paper, napkins, swabs etc. should be autoclaved first and then it is incinerated. But the microbial contaminants containing materials should be treated with some disinfectant and thereafter autoclaved by putting them in suitable containers. The molten material should be discarded.

Sometimes, HCl is also added to hydrolyze the agar, if present in the medium. This is added before their safe disposal. All such laboratory materials should be disposed of after autoclaving.

Experiment 1

Preparation of Culture Media under Aseptic Conditions

Introduction:

Bacterial taxonomy: each species has to be assigned to a genus (binary nomenclature). There are two parts of the name, one defining the genus and the other the species e.g. *Streptococcus pyogenes*. The genus in this case is "*Streptococcus*" and the species is "*pyogenes*"

Note (Naming of microorganisms):

The genus is normally written with an upper case initial letter and the species with a lower case initial letter, e.g. *Staphylococcus aureus* or *Escherichia coli*.

These names are printed in *italics* to designate their status as proper names (in old books: underlined).

Microbial Growth: Refers to an increase in cell number, not in cell size.

Bacteria grow and divide by binary fission, a rapid and relatively simple process.

The requirements for microbial growth are both physical and chemical

1. **Physical requirements:**

    1.1 Temperature: most microorganisms live within restricted ranges of temperature with a range of tolerance (minimum and maximum). The **minimum growth temperature** is the lowest temperature at which a species will grow, **the optimum growth temperature** is the temperature at which it grows best, and **the maximum growth temperature** is the highest temperature at which growth is possible.

    1.2 pH: most bacteria prefer neutral pH (6.5-7.5), Molds and yeast grow in wider pH range, but prefer pH between 5 and 6.

    1.3 Osmotic Pressure: Cells are 80 to 90% water, and normally the salt concentration of microbial cytoplasm is about 1 %

What is the effect of presence of microbes in hypertonic or hypotonic solution….?

    1.4 Other factors: moisture, light and time.

2. **Chemical requirements:**

2.1 Carbon: It is a structural backbone of all organic compounds in microorganism. Sources of carbon: lipids, proteins, carbohydrates and carbon dioxide.

2.2 Nitrogen: Used to form amino acids, DNA, and RNA. Sources of nitrogen are Protein, ammonium and nitrogen gas (N_2)

2.3 Sulfur: Used to form proteins and some vitamins (thiamin and biotin). Sources of sulfur are protein, hydrogen sulfide and sulfates.

2.4 Phosphorus: Used to form DNA, RNA, ATP, and phospholipids. Sources of phosphorous mainly are inorganic phosphate salts and buffers.

2.5 Oxygen: Organisms that use oxygen produce more energy from nutrients than those do not use it. i.e areboic microorganisms produce more energy than anaerobic microorganisms.

Other Elements: Potassium, magnesium, and calcium are often required as enzyme cofactors.

Calcium is required for cell wall synthesis in Gram positive bacteria.

Bacterial culture media

So to grow bacteria, we should provide them with suitable environmental conditions and suitable media. The mixture (in which the nutrients are supplied) is referred to as the growth medium or culture medium**.**

A. A growth medium or culture medium is a liquid or gel designed to support the growth of **microorganisms** or **cells**, or small **plants** like the **moss** ***Physcomitrella patens***. Generally the growth medium contains:

1. Moisture (water)
2. An energy source, for example: glucose, amino acids, nitrite and nitrate
3. Nutritionally suitable sources of carbons, nitrogen, sulfur and oxygen
4. Organic growth factors, for example: amino acids.

Vitamins and nucleosides are other ingredients that may be added to the medium in order to grow the desired microorganisms.

B. Media must be prepared in such a way that it is sterile prior to being inoculated with a bacterial sample, so that when a particular type of bacteria is cultured (cultivated) on that medium; it is the only type of bacteria present.

Classification of culture media:

A. Classification based on consistency

1. Liquid (Broth) medium: these media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. eg. Sugar fermentation tests, MR-VP broth.
2. Solid medium (Agar): is media containing agar (at a concentration of 1.5-2.0%) or some other, agar is mostly inert solidifying agent. Solid medium has physical structure and this allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks).solid medium is useful for isolating bacteria or for determining the characteristics of colonies.
3. Semisolid media: they are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of microaerophilic bacteria or for determination of bacterial motility.

Agar:

The discovery of agar (a polysaccharide derived from red algae) has revolutionized the study of microbiology because of its distinctive properties:

- Agar at a concentration of about 1 – 1.5 %(w/v) will provide a firm gel that cannot be liquefied by the enzymes normally produced during bacterial growth.
- Agar is semi-translucent
- An agarose medium is porous
- Fluid agar solutions set (solidify) at approximately 40°C, but do not reliquefy on heating until the temperature is in excess of 90°C.

Thus agar forms a firm gel at 37°C which is the normal incubation temperature for many pathogenic microorganisms. And when used as a liquid at 45°C is at a sufficiently low temperature to avoid killing microorganisms (this property is important in pour plate counting method).

Forms of solidified agar:

1. Slant (test tube):
    - 1/3 full
    - Solidified in a slant position
2. Stab (test tube):
    - 1/3 – 1/2 full
    - Solidified in an upright position
    - For reduced O_2 environment
    - Or for observation of in-media growth
3. Deeps or pours (Petri-dish)

4. Plates (Petri-dish)

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

1. General purpose media/ Basic media/ Minimum Media

- Contains minimal nutritional requirements for microorganisms
- Supports the growth of only a relatively narrow range of bacteria
- Growth is slow
- Generally used for the primary isolation of microorganisms.

2. Enriched medium (All purpose-medium):

- Contains a wide range of nutrients (sugar, amino acids, fatty acids, vitamins, salts, organic products from animals and plants)
- Supports the growth of a wide range of microorganisms
- Growth is fast

Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media.

3. Selective medium:

- It is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., they select for certain microbes).
- Uses certain dyes, sugars, high salt concentration, or pH to achieve the selectivity.
- Examples: MacConkey agar: Enterobacteriaceae members contains Bile salt that inhibits most gram positive bacteria, EMB (Eosin Methylene Blue) agar

4. Differential medium (indicator medium):

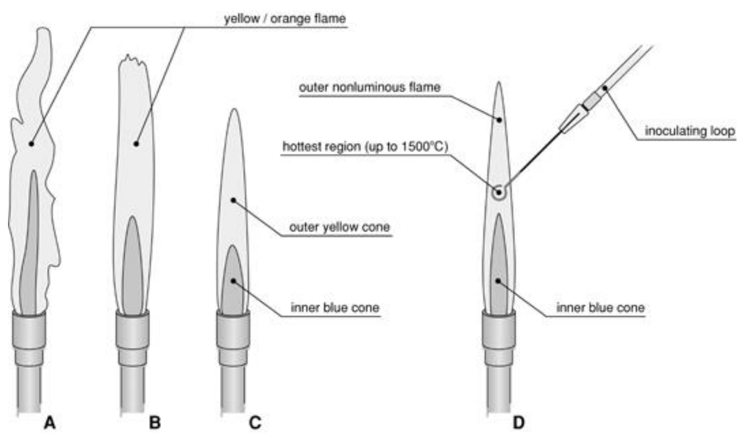
- Allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies, i.e they Support the growth of different microorganisms but in different ways.
- Example: Incorporation of 0.5% mannitol + phenol red (a pH indicator that changes from red to yellow in acidic medium) into a medium that supports the growth of both Staphylococcus aureus and Staphylococcus epidermidis, to differentiate between them.

Since Staphylococcus aureus can only ferment mannitol (unlike Staphylococcus epidermidis) and produce acidic degradation products that would change the pH of the medium to acidic and the color to yellow. In the case of Staphylococcus epidermidis, there is no color change.

Aseptic techniques applied to prepare a sterile culture media

Aseptic technique is a method designed to prevent contamination from microorganisms. It involves applying the strictest rules and utilizing what is known about infection prevention to minimize the risks that you'll experience an infection

1. Aseptic techniques that should be followed to prepare a sterile culture media
2. Doors and windows are kept closed
3. Hand hygiene
4. Decontaminate the surface of your bench at least one time
5. Turn on the Bunsen burner and make sure that the flame is no more than four inches high and that the blue inner cone more than two inches high. (Figure below)
6. Agar plates are held in a manner that minimizes the exposure of the surface to the environment. The Petri dish tops are lifted with the left hand and replaced immediately as the plate is poured
7. When removing lids from tubes, lids are held in the hand and not placed on the countertop during the transfer of materials from one tube to another.
8. Sterilize all needed glassware's in autoclave prior to use.



Autoclave: It is a sterilization procedure performed by means of temperature and pressure. It is used only for the sterilization of heat stable media and equipment. An autoclave is basically a huge steam cocker. Steam enters into a jacket surrounding a chamber. The pressure will go up over 15 pounds per square inch (psi); at this point the timer begins to count down usually for 15 minutes. The high pressure in a closed container allows the temperature to go around 121°C (249 °F). Therefore, the parameters for sterilization with an autoclave are 121°C at ≥15 psi for 15 minutes. Fifteen Minutes is the thermal death time for most microorganisms (except some really hardy spore-formers).

Incubator is a device used to grow and maintain **microbiological cultures** or **cell cultures**

Practical Part:

Preparation and preservation of media

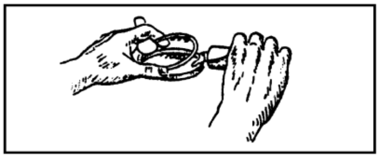
Most culture media are sterilized by autoclaving. Certain media that contain heal liable components like glucose, antibiotics, urea, serum and blood are not autoclaved only they are filtered and may be added separately after the medium is autoclaved.
Prepared media may be held at 4-5 °C in the refrigerator for 1 to 2 weeks. Certain liquid media in screw capped bottles or tubes or cotton plugged can be held at room temperature for weeks.

I. Making Liquid Media

1. Read the label on a bottle of nutrient broth. It specifies the amount of dehydrated powder required to make 1 litter (1000 ml) of medium. Then calculate the amount needed to prepare ____ ml and weight this quantity.
2. Place the required amount of distilled water in Erlenmeyer flask. Add the weighed powder and shake to dissolve.
3. Use a pipette to dispense 5 ml aliquots of the broth media into sterile test tubes.
4. Close it with the cotton plug or cap then place the flask in autoclave (121^{o}C, for 15 min) to sterilize broth media.
5. After that, tubes are ready for culturing.

II. Making Solid media:

1. Read the label on a bottle of nutrient agar. It specifies the amount of dehydrated powder required to make 1 litter (1.000 ml) of medium. Then calculate the amount needed to prepare ___ ml and weight this quantity.
2. Place the required amount of distilled water in Erlenmeyer flask. Add the weighed dehydrated agar while stirring; use a glass rod to prevent lumping, if needed.
3. Set the flask over a Bunsen burner and start heating agar till boiling (solution will become transparent when agar completely liquefies). While heating keep shaking the flask to prevent charring of the precipitated agar.
4. After that remove the flask from the flame or hot plate, close it with the cotton plug or cap.
5. Place in the autoclave (121^{o}c, for 15 min).
6. After sterilizing agar allow it to cool to about 50 °C (the agar should be warm and melted, but not too hot to handle in its flask). Pour agar solution into plate:
    - Pouring procedure should be performed in an aseptic manner to prevent contamination: speech is prohibited and Bunsen burner should be ON
    - Open the cover of Petri-dish with one hand and while still holding the cover over the Petri-dish, pour approximately 20 mL of agar solution into the dish. Cover the dish



- Flame the surface of agar solution to remove any bubbles that formed during pouring and to make the surface smooth. Cover the dish
- When the plates are cool (agar solidified), invert them to prevent condensing moisture from accumulating on the agar surface. If happened the medium could be destroyed.
- To test the sterility of broth and agar plates. Place the inverted agar plates and tubes of sterilized nutrient broth in the incubator at 37 °C. They should be incubated for at least 24 hours to ensure they are sterile (free of contaminating bacteria) before using them.

Report Sheet
Experiment No. 1
Preparation of Culture Media under Aseptic Conditions

Name:	Section:
Group No.	Day and Date:

1. After 24-48 hours of incubation at 37 °C, do your prepared plates and broths appear to be sterile? Explain your results?

2. Evaluate their physical appearance (out of 5).

   A. Plates: any microbial growth/ any air bubbles

   B. Broths: clear/ turbid (degree of turbidity)

3. Compare the composition of agar media and nutrient broth media found on the bottles used in laboratory to the composition we discussed in the previous sections, write down your comments and conclusions.

Experiment 2
Sources of Microbial Contamination and Appropriate Hygienic Practices

Introduction

The term "normal microbial flora" denotes the population of microorganisms that inhabit the skin and mucous membranes of healthy normal persons. For a healthy human, the internal tissues, e.g. blood, brain, muscle, etc., are normally free of microorganisms. However, the surface tissues, i.e., skin and mucous membranes are constantly in contact with environmental organisms and become readily colonized by various microbial species. The mixture of organisms regularly found at any anatomical site is referred to as the normal flora.

Factors Influencing Normal Flora:

1. Local Environment (pH, temperature, redox potential, O_2, H_2O, and nutrient levels…).
2. Diet
3. Age
4. Health condition (immune activity…)
5. Antibiotics,…..etc

Classification of microbial flora

1. The resident flora

Consists of relatively fixed types of microorganisms regularly found in a given area at a given age; if disturbed, it promptly reestablishes itself. It consists of organisms which are regularly present in a particular area and when disturbed it reestablishes itself like Esch.coli is a normal inhabitant of the intestine.
The microbes of the normal resident flora are harmless and may be beneficial in their normal location in the host and in the absence of coincident abnormalities. They may produce disease if introduced into foreign locations in large numbers and if predisposing factors are present.

2. The transient flora

Consists of nonpathogenic or potentially pathogenic microorganisms that inhabit the skin or mucous membranes for hours, days, or weeks; it is derived from the environment, does not produce disease, and does not establish itself permanently on the surface. Members of the transient flora are generally of little significance so long as the normal resident flora remains intact. However, if the resident flora is disturbed, transient microorganisms may colonize, proliferate, and produce disease.

Beneficial Effects of the Normal Flora

Some of the characteristics of a germ-free animals that are thought to be due to lack of exposure to a normal flora are:

1. Vitamin deficiencies, especially vitamin K and vitamin B12
2. Increased susceptibility to infectious disease
3. Poorly developed immune system, especially in the gastrointestinal tract
4. Lack of "natural antibody" or natural immunity to bacterial infection

The overall beneficial effects of microbes are summarized below:

1. Normal flora synthesizes and excretes vitamins in excess of their own needs, which can be absorbed as nutrients by their host. For example, in humans, enteric bacteria secrete Vitamin K and Vitamin B12.
2. Normal flora prevents colonization by pathogens by competing for attachment sites or for essential nutrients.
3. Normal flora may antagonize other bacteria through the production of substances which inhibit or kill non indigenous species.
4. Normal flora stimulates the development of certain tissues, i.e., the caecum and certain lymphatic tissues (peyer's patches) in the GI tract. The caecum of germ-free animals is enlarged, thin-walled, and fluid-filled, compared to that organ in conventional animals. Also, based on the ability to undergo immunological stimulation, the intestinal lymphatic tissues of germ-free animals are poorly-developed compared to conventional animals.
5. Normal flora stimulates the production of natural antibodies. Since the normal flora behaves as antigens in an animal, they induce an immunological response.

Hygienic practices

Hygiene is a set of practices performed for the preservation of health. According to the World Health Organization (WHO), "Hygiene refers to conditions and practices that help to maintain health and prevent the spread of diseases.

A. Surface hygiene.
B. Disinfection of cleansing tools.
C. Hand hygiene (For types of hand hygiene (see table below))

TABLE 1. The U.S. Centers for Disease Control and Prevention's Hand-Hygiene Methods and Indications.^

Method	Agent	Purpose	Duration (minimum)	Indication*
Routine handwash	Water and nonantimicrobial soap (e.g., plain soap†)	Remove soil and transient microorganisms	15 seconds§	Before and after treating each patient (e.g., before glove placement and after glove removal). After barehanded touching of inanimate objects likely to be contaminated by blood or saliva. Before leaving the dental operatory or the dental laboratory. When visibly soiled.¶
Antiseptic handwash	Water and antimicrobial soap (e.g., chlorhexidine, iodine and iodophors, chloroxylenol [PCMX], triclosan)	Remove or destroy transient microorganisms and reduce resident flora	15 seconds§	
Antiseptic hand rub	Alcohol-based hand rub¶	Remove or destroy transient microorganisms and reduce resident flora	Rub hands until the agent is dry¶	
Surgical antisepsis	Water and antimicrobial soap (e.g., chlorhexidine, iodine and iodophors, chloroxylenol [PCMX], triclosan) Water and non-antimicrobial soap (e.g., plain soap†) followed by an alcohol-based surgical hand-scrub product with persistent activity	Remove or destroy transient microorganisms and reduce resident flora (persistent effect)	2–6 minutes Follow manufacturer instructions for surgical hand-scrub product with persistent activity¶**	Before regloving after removing gloves that are torn, cut, or punctured.

^ Reprinted with permission from the CDC's Guidelines for Infection Control in Dental Health-Care Settings-2003. Morbidity and Mortality Weekly Report. Vol. 52 / No. RR-17, Page 15. Table 2. Hand-hygiene methods and indications. (Numbers in parenthesis below indicate references in the CDC Guidelines.)

* (7,9,11,13,113,120–123,125,126,136–138)

† Pathogenic organisms have been found on or around bar soap during and after use (139). Use of liquid soap with hands-free dispensing controls is preferable.

§ Time reported as effective in removing most transient flora from the skin. For most procedures, a vigorous rubbing together of all surfaces of premoistened lathered hands and fingers for >15 seconds, followed by rinsing under a stream of cool or tepid water is recommended (9,120,123,140,141). Hands should always be dried thoroughly before donning gloves.

¶ Alcohol-based hand rubs should contain 60%–95% ethanol or isopropanol and should not be used in the presence of visible soil or organic material. If using an alcohol-based hand rub, apply adequate amount to palm of one hand and rub hands together, covering all surfaces of the hands and fingers, until hands are dry. Follow manufacturer's recommendations regarding the volume of product to use. If hands feel dry after rubbing them together for 10–15 seconds, an insufficient volume of product likely was applied. The drying effect of alcohol can be reduced or eliminated by adding 1%–3% glycerol or other skin-conditioning agents (123).

** After application of alcohol-based surgical hand-scrub product with persistent activity as recommended, allow hands and forearms to dry thoroughly and immediately don sterile surgeon's gloves (144,145). Follow manufacturer instructions (122,123,137,146).

†† Before beginning surgical hand scrub, remove all arm jewelry and any hand jewelry that may make donning gloves more difficult, cause gloves to tear more readily (142,143), or interfere with glove usage (e.g., ability to wear the correct-sized glove or altered glove integrity).


How to Handrub?
RUB HANDS FOR HAND HYGIENE! WASH HANDS WHEN VISIBLY SOILED
Duration of the entire procedure: 20-30 seconds
1a
1b
Apply a palmful of the product in a cupped hand, covering all surfaces;
2
Rub hands palm to palm;
3
Right palm over left dorsum with interlaced fingers and vice versa;
4
Palm to palm with fingers interlaced;
5
Backs of fingers to opposing palms with fingers interlocked;
6
Rotational rubbing of left thumb clasped in right palm and vice versa;
7
Rotational rubbing, backwards and forwards with clasped fingers of right hand in left palm and vice versa;
8
Once dry, your hands are safe.
World Health Organization
Patient Safety
SAVE LIVES
Clean Your Hands

Practical Part:

Test for environmental contamination

1. Use sterile cotton swab to swab an area from the environment such as a door knob, floor or the bottom of your shoe.
2. Immerse the cotton swab in 5 ml broth solution and mix thoroughly.
3. Use another sterile nutrient broth test tube as a control.

Test for hygienic hand wash

1. Divide the nutrient agar plate into quadrants and label 1 through 4.
2. Quadrant 1 is your negative control. Do not touch it.
3. Touch quadrant 2 with your finger tip.
4. Rinse your hands with tap water only. Dry your hands and touch your finger tips to quadrant 3.
5. Wash your hands with tap water and soup as you're normally would. Dry your hands and touch your finger tips to quadrant 4.

Test for mouth hygiene

1. Divide the nutrient agar plate into 2 halves.
2. Swab the mouth cavity or teeth using a cotton swab. Then streak on the surface of the first half of petri dish.
3. Use a mouth wash for 1 min.
4. Swab the mouth cavity using a new sterile cotton swab. Then streak it on the surface of the second half of petri dish.

Test for water contamination

1. Pour 1 ml of tap water on the surface of nutrient agar plate.
2. Rotate the plate to spread the sample over the whole surface.
3. Cover the plate and leave until the surface dries.
4. Pour 1 ml of sterile water on the surface of another nutrient agar plate.
5. Rotate the plate to spread the sample over the whole surface.
6. Cover the plate and leave until the surface dries.

Test for bench cleaning

1. Divide the nutrient agar plate into 2 halves.
2. Swab the bench surface before cleaning with a disinfectant using a cotton swab. Then streak on the surface of the first half of petri dish.
3. Clean your bench using the disinfectant.
4. Swab the clean bench surface using a new sterile cotton swab. Then streak it on the surface of the second half of petri dish.

Incubate all of your samples at 37 ^{o}C for 24- 48 hrs.

Report Sheet
Experiment No. 2
Sources of Microbial Contamination and Appropriate Hygienic Practices

Name:	Section:
Group No.	Day and Date:

1. After 24 - 48 hrs of incubation at 37^{o}C, do your prepared plates and broths appear to be sterile?

No.	**Sample**	**Sterile / Contaminated**
1	Environmental sample	
2	Un washed hands	
3	Hands washed with tap water	
4	Hands washed with soup and water	
5	Mouth cavity before mouth wash	
6	Mouth cavity after mouth wash	
7	Tap water	
8	Sterile water	
9	Unclean bench	
10	Bench cleaned with a disinfectant	

2. Do you have microbial growth in sterile water plate? Explain?

Experiment No. 3
Culturing Methods and Plating Techniques

CULTURE METHODS

- Culture methods employed depend on the purpose for which they are intended.
- The indications for culture are:
  - To isolate bacteria in pure cultures.
  - To obtain sufficient growth for the preparation of antigens and for other tests.
  - For bacteriophage & bacteriocin susceptibility.
  - To determine sensitivity to antibiotics.
  - To estimate viable counts.
  - Maintain stock cultures.

Culture methods include:

1. **Streak culture**
2. Lawn culture
3. Stroke culture
4. Stab culture
5. **Pour plate method**
6. Liquid culture
7. Anaerobic culture methods
8. **Spread plate method.**

The common plating techniques employed in microbiology are Streak Plate Method, Spread Plate Method and Pour Plate Method.

1) Streak Plate Method

This method is routinely employed for the isolation of bacteria in pure culture. In this method a sterilized inoculating loop or transfer needle is dipped into a suitable diluted suspension of microorganisms which is then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks. The process is known as streaking and the plate so prepared is called a streak plate. The main objective of the streak plate method is to produce well separated colonies of bacteria from concentrated suspensions of cells.

A sterilized inoculating needle with a loop made up of either platinum or nichrome wire is used for streaking. One loopful of specimen is transferred onto the surface of the agar plate in a sterile petridish and streaked across the surface in the form of a zig-zag line. This process is repeated thrice to streak out the bacteria on the agar plate so that some individual bacteria are separated from each other. The first streak

will contain more organisms than the second and the second more than the third and so on. The last streaks should thin so on. The last streaks should thin out the culture sufficiently to give isolate colonies. The successful isolation depends on spatial separation of single cells. Each colony usually represents the growth from a single organism when such a plate is incubated colonies will appear on the surface of the medium. Because of the high concentration of water in agar, some water of condensation forms in petriplate during incubation. Moisture is likely to drip from the cover to the surface of the agar and spread out, resulting in a confluent mass of growth and running individual colony formation. To avoid this, petriplates are routinely incubated bottom side up. Pure colonies can be obtained from well isolated colonies by transferring a small portion of each to separate culture media.

2) Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position. The dispersed cells will develop into isolated colonies. Because the number of colonies will be equal to the number of viable organisms in the sample spread plates can be used to count the microbial population.(fig.exp. no. 6)

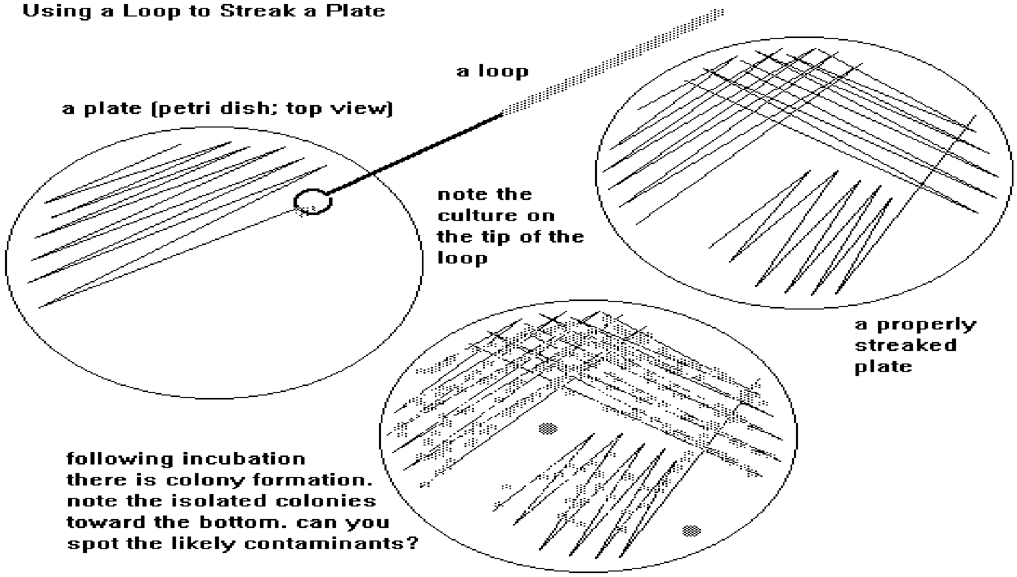
3) Pour Plate Method

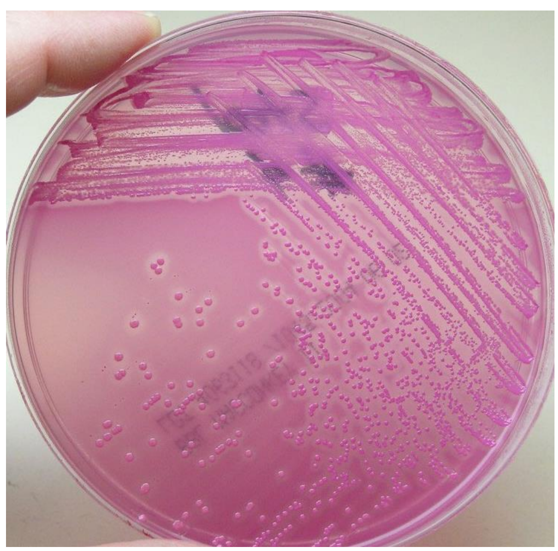
In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petri plate to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify. After incubation, the plates are examined for the presence of individual colonies. The pure colonies may be isolated and transferred into test tube culture media for making pure cultures. This technique is employed to estimate the viable bacterial count in a suspension. .(fig.exp. no. 6)

Practical work : STREAK CULTURE:

- Used for the isolation of bacteria in pure culture from clinical specimens.
- Platinum wire or Nichrome wire is used (inoculum)
- One loopful of the specimen is transferred onto the surface of a well dried plate.
- Spread over a small area at the periphery.

- The inoculum is then distributed thinly over the plate by streaking it with a loop in a series of parallel lines in different segments of the plate.
- On incubation, separated colonies are obtained over the last series of streaks.





Report Sheet
Experiment No. 3
Culturing methods and plating technique

Name:	Section:
Group No.	Day and Date:

1. **Mention the different plating technique**

2. **What is the aim of plate streaking?**

3. **What is the effect of forgetting to flam the inoculum loop after the first quarter plate streak?**

Experiment 4

Bacterial Identification
Simple stain and Gram stain

Simple Staining

Aim
To compare the morphological shapes and arrangements of bacterial cells

Principle
In simple staining, bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. A simple stain that stains the bacteria is the direct stain. The purpose of simple staining technique is to determine cell shape, size arrangement of bacterial cells. Simple
staining is performed by using basic stains which have different exposure time (Crystal Violet 20-60 s, Carbol fuschin 15-30 s and Methylene blue 1-2 miutes). The preparation of the smear is required for many laboratory procedures, including Gram –stain. The purpose making of a smear is to fix the bacteria onto the slide and to prevent the sample from being lost during a staining procedure. A smear can be prepared from solid or broth medium.
You prepare a smear using the **heat fixation process** The heat causes the microorganism to adhere to the glass slide. This is known as fixing the microorganism to the glass slide.

Procedure

- Clean glass slide was taken and was washed and dried.
- Bacterial smears were prepared from the bacterial cultures.
- The slide was kept on the staining tray and 5 drops of stain was added for a designed period.
- The extra stain was poured off and the smear was washed gently under slow running tap water.
- The slide was then blot dried using blotting paper.
- The slide was then examined under 10X, 45X and oil immersions objects respectively.

Observation
On the basis of microscopic observation, bacteria appeared blue, violet and red respectively depending on the stain taken.

Differential staining
Differential staining requires the use of at least 3 chemical reagents that are applied sequentially to a heat fixed smear. Its function is to impart its colour to all cells. In order to establish a colour contrast, the second reagent used is the decolorizing agent. Based on the chemical composition of cellular components the decolorizing agent may or may not remove the primary stain from the entire cell or from any cell structure.
The final reagent is the counter stain. Following discoloration, if the primary stain is not washed out, the counter stain cannot be absorbed and neither the cell nor its components will retain the colour of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting colour of the counter stain. In this way, cell type or their structure can be distinguished from each other. On the basis of the stain that is retained the most important differential

stain used in
bacteriology is the Gram stain.

Gram-Staining

Aim

To differentiate two principal groups of bacteria.

Bacteria are divided into two groups designated Gram-positive and Gram-negative according to their reaction to a staining procedure developed in 1884 by Christian Gram.

Gram staining is a common technique used to differentiate two large groups of bacteria based on their different cell wall constituents. The Gram stain procedure distinguishes between Gram positive and Gram negative groups by coloring these cells red or violet. Gram positive bacteria stain violet due to the presence of a thick layer of peptidoglycan in their cell walls, which retains the crystal violet these cells are stained with. Alternatively, Gram negative bacteria stain red, which is attributed to a thinner peptidoglycan wall, which does not retain the crystal violet during the decolorzing process.

Gram-Positive Cell Wall:

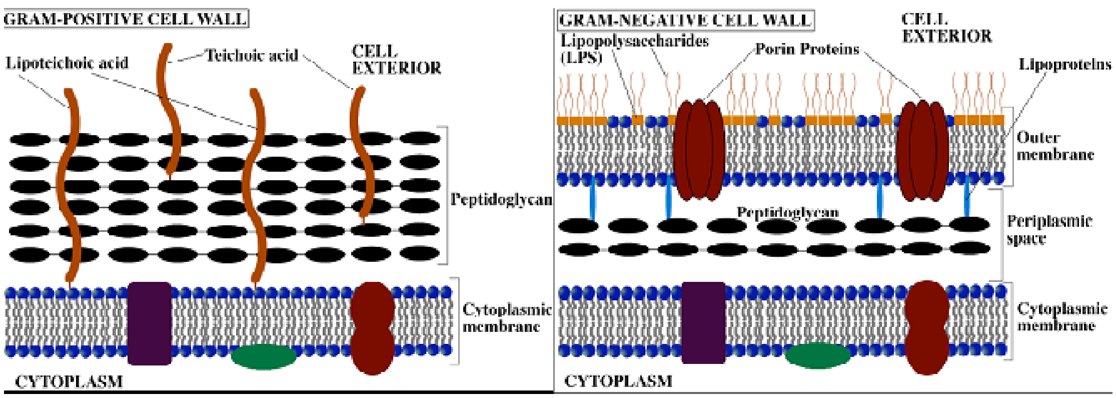
The cell walls of Gram-positive bacteria are quite thick (20-80 nm) and consist of between 60% and 80% peptidoglycan, which is extensively cross-linked in three dimensions to form a thick polymeric mesh.
Gram-positive walls frequently contain acidic polysaccharides called teichoic acids. Because they are negatively charged, teichoic acids are partially responsible for the negative charge of the cell surface as a whole.
Cell wall proteins, if present, are generally found on the outer surface of the peptidoglycan.

Gram-Negative Cell Wall:

The wall, or more correctly, envelop of Gram-negative cells is far more complicated structure. Although they contain less peptidoglycan (10 – 20%) of wall, a second membrane structure is found outside the peptidoglycan layer. This outer membrane is asymmetrical, composed of proteins, lipoproteins, phospholipids and a component unique to Gram-negative bacteria, lipopolysaccharide (LPS).

Table 1 Gram-positive and Gram-negative cell wall composition

Feature	Gram-positive cells	Gram-negative cells
Peptidoglycan	60 – 80%	10 – 20%
Teichoic acid	Present	Absent
Lipoteichoic acid	Present	Absent
Lipoprotein	Absent	Present
Lipopolysaccharide	Absent	Present
Protein	c. 15%	c. 60%
Lipid	c. 2%	c. 20%

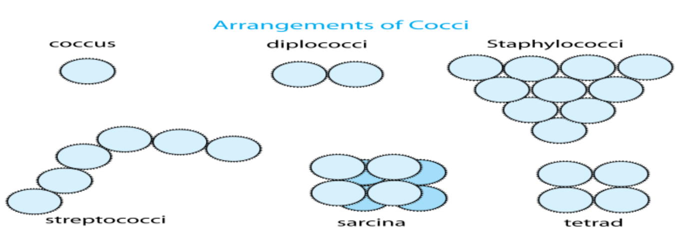


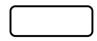
Cell wall structure of Gram+ and Gram-

Bacterial morphology and arrangement: there are three basic shapes of bacteria

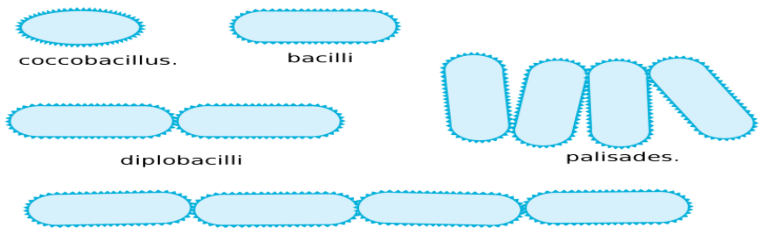
1. **Coccus (pleural: Cocci):** Spherical bacteria ◯ that may occur in:

- ✓ Single (coccus)
- ✓ pairs (diplococci)
- ✓ Groups of four (tetracocci)
- ✓ Grape like clusters (staphylococci),
- ✓ Chains (streptococci)
- ✓ Cubical arrangements of eight or more (sarcinae).



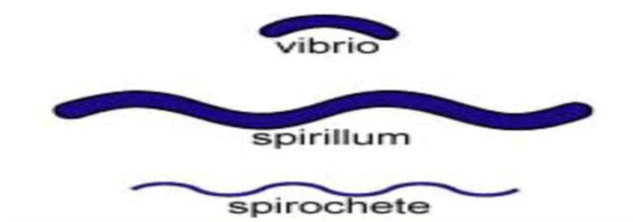
2. **Bacillus (pleural: Bacilli):** Rod- shaped bacteria  that:

- ✓ Generally occur singly (bacillus)
- ✓ Also found in pairs (diplobacilli)
- ✓ Or in chains (streptobacilli).



3. **Spiral shape:** come in one of three forms

- ✓ Vibro: curved or comma- shaped
- ✓ Spirillum: thick, rigid spiral
- ✓ Spirochete species: a thin, flexible spiral



Some Special Features of Common Microorganisms in Laboratory

➢ ***Escherichia coli***
Section : Facultative, Gram negative rods
Family : Enterobacteriaceace
Genus : *Escherichia*
Species : *coli*
•Common coliform bacterium used in laboratory practices
•The bacterium is rod shaped generally 1.3 x 2.5 µm in size
•Gram negative, facultatively anaerobe, motile having peritrichous flagella
•It causes diarrhea due to the presence of enterotoxins.
•It is catalase positive and oxidase negative

Colony Characteristics
a) On Nutrient Agar

Small, regular, circular, translucent colonies
b) On Mac conkey Agar
Small, regular, circular, lactose fermenting colonies

- ***Staphylococcus aureus***
  Section : Facultative, gram positive cocci
  Family : Staphylococcaceae
  Genus : *Staphylococcus*
  Species : *aureus*
  They appear round (cocci) and form in grape-like clusters.
  - Non-Motile
  - It is a common cause of skin infections, respiratory disease and food poisoning.
  - It is catalase and coagulase positive

- *Colony Characteristics*
  a) On Nutrient Agar
  Small, regular, circular, entire, smooth, convex, opaque, golden yellow colonies
  b) On Mac conkey Agar
  Small, regular, circular, entire, smooth, convex, opaque, lactose fermenting colonies

Practical Part:

To a bacterial smear the following chemicals are applied to make a Gram-stain:

4. **Gram's Crystal Violet Stain (primary stain)**

   The smear is flooded with Crystal Violet for **60 seconds**. Crystal violet is a purple chemical that sticks to the peptidoglycan layer of the bacterial cell wall. After 60 seconds, crystal violet is rinsed off using **distilled water**.

5. **Iodine (mordant)**

   Flood the smear with iodine for 60 seconds the wash immediately with distilled water. This causes crystal violet to stick to peptidoglycan like mortar causes bricks to stick together.

6. **95% Ethanol (decolorizing)**

   95% ethanol is added drop by drop for 10 seconds then washed immediately with water. The Acetone/Alcohol washes crystal violet out of the Gram-negative cell wall. The Gram-positive cell wall retains crystal violet as long as the acetone/alcohol wash lasts not more than a few seconds. The acetone/alcohol wash is the **differential step** in the Gram-stain process. After this step Gram-positive cells look purple while Gram-negative cells look clear.

7. **Safranin Stain (secondary or counter stain)**

The smear is flooded with Safranin for 60 seconds then wash immediately with water. Safranin is a stuck to cytoplasmic component of the cell. All cells become stained with safranin. After this step gram-positive cells end up looking purple while gram negative cells, which were cleared in the previous step, look red.

8. **Drying the slide**

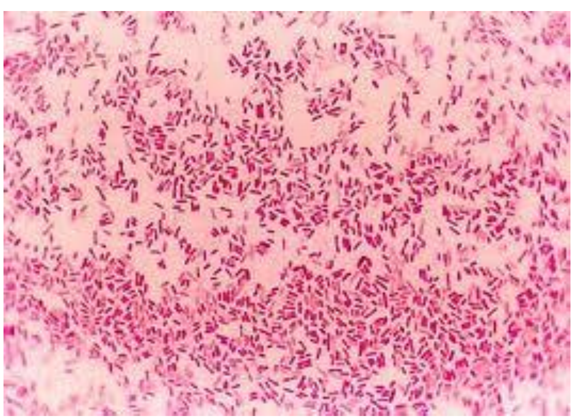
   Dry the smear by blotting a filter paper. Then add one drop of oil on the smear to observe the result under the light microscope using oil-immersion objective lens (100x).

Interpretation of results:

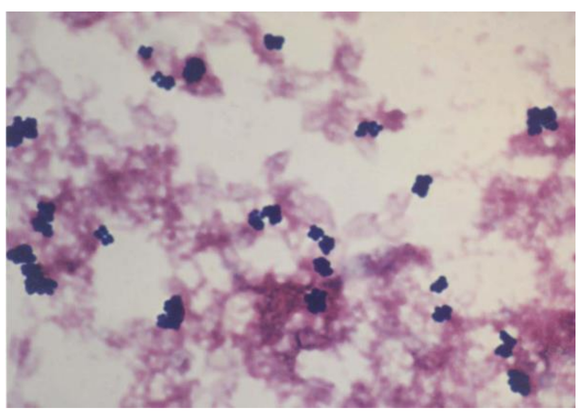
1. **Color:** either gram positive or gram negative
2. **Shape and arrangement**
3. Older cells might have been dead or with damaged cell wall; giving false results. Gram-positive cells would have mixed results (purple and red) because not all cells might be damaged.
4. Excessive decolorization (> 10 seconds) would affect Gram-positive cells.
5. No proper decolorization (<10 seconds) would affect Gram-negative cells.

GRAM STAIN

Procedure	Reagent	Cell Color	
		Gram Positive	Gram Negative
Fixed cells on slide		COLORLESS	COLORLESS
Primary stain	Crystal Violet	PURPLE	PURPLE
Mordant	Iodine	PURPLE	PURPLE
Decolorizer	Alcohol	PURPLE	COLORLESS
Counterstain	Safranin	PURPLE	RED



Gram Stain of *Escherichia coli* (pink) bacilli.



Gram Stain: *Staph. Epidermidis* (note **clusters of Gram positive cocci)**

Report Sheet

Experiment No. 4
Gram-Staining

Name:	Section:
Group No.	Day and Date:

1. **After you examine the slides under microscope, describe the following:**

Bacteria type	Color	Shape	Arrangement
Staphylococcus aureus			
Escherichia coli			

2. **Draw the shape of each type of bacteria as you observed under microscope:**

Staphylococcus aureus	*Escherichia coli*

3. **Do the microorganisms have the appropriate expected appearance, or there was deviation in appearance, if yes, describe the problem you had, the probable causes and suitable corrective actions you can make.**

Experiment 5

Sterilization Methods and Principles

Introduction

Sterilization: can be defined as any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses and prions) from a surface, equipment, foods, medications, or biological culture medium. In practice sterility is achieved by exposure of the object to be sterilized to chemical or physical agent for a specified time.

Decontamination: is the process of cleansing an object or substance to remove contaminants such as micro-organisms or hazardous materials, including chemicals, radioactive substances, and infectious diseases.

Survivor curves: They are plots of the logarithm of the fraction of survivors (microorganisms which retain viability following a sterilization process) against the exposure time or dose.

Methods of Sterilization are:

1. Physical Method

    1. Thermal (Heat) Sterilization methods

Heat Sterilization: is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents. In this processes both dry and moist heat are used for sterilization. The process is more effective in hydrated state. This method of sterilization can be applied only to the thermostable products.

1.1 Dry Heat Sterilization

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 glassware and metal surgical instruments. It is also used for sterilizing non-aqueous thermostable 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.

1.2 Moist Heat Sterilization:

Moist heat sterilization involves the use of steam in the range of 121-134 °C. Steam under pressure is used to generate high temperature needed for sterilization. Autoclaves use pressurized steam to destroy microorganisms, and are the most dependable systems available for the decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents. This method of sterilization works well for many metal and glass items but is not acceptable for rubber, plastics, and equipment that would be damaged by high temperatures (Figure 1).

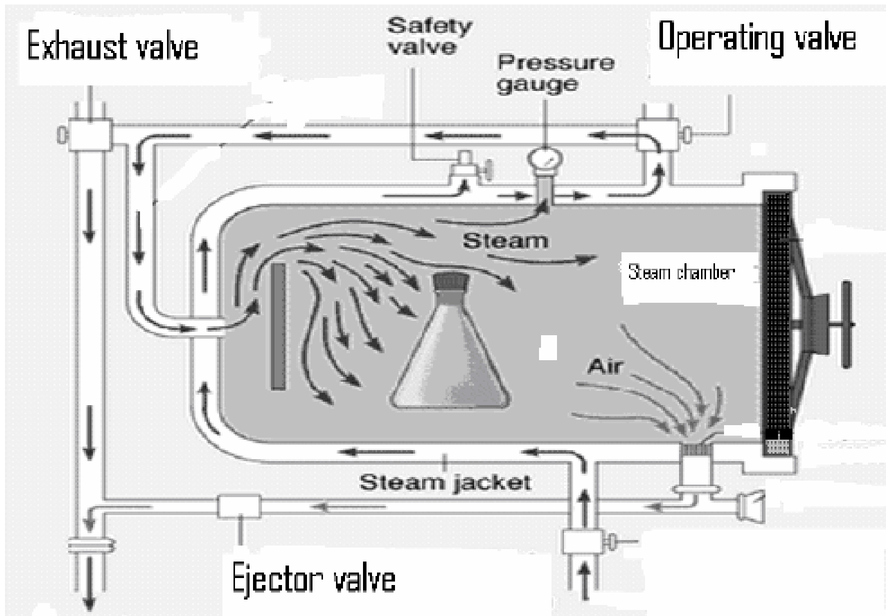


Fig. 1: An Autoclave

2. Radiation Sterilization method

Radiation Sterilization many types of radiation are used for sterilization like electromagnetic radiation (e.g. gamma rays and UV light), particulate radiation (e.g. accelerated electrons).The major target for these radiations is microbial DNA. Radiation sterilization with high energy gamma rays or accelerated electrons has proven to be a useful method for the industrial sterilization of heat sensitive products. But some undesirable changes occur in irradiated products, an example is aqueous solution where radiolysis of water occurs. Radiation sterilization is generally applied to articles in the dry state; including surgical instruments, sutures, prostheses, unit dose ointments, plastic syringes and dry pharmaceutical products. UV light, with its much lower energy, and poor penetrability finds uses in the sterilization of air, for surface sterilization of aseptic work areas, for treatment of manufacturing grade water, but is not suitable for sterilization of pharmaceutical dosage forms.

3. Filtration Sterilization method

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.

2. Chemical Method
    1. Gaseous method

Gaseous Sterilization the chemically reactive gases such as formaldehyde (CH_2O) and ethylene oxide $(CH_2)_2O$ possess biocidal activity. The mechanism of antimicrobial action of the two gases is assumed to be through alkylations of sulphydryl, amino, hydroxyl and carboxyl groups on proteins and amino groups of nucleic acids. Both of these gases being alkylating agents are potentially mutagenic and carcinogenic

2. Liquid method

In a low-temperature liquid chemical sterile processing system, several steps must be followed for effective sterilization:

1. Pre-cleaning of the devices is necessary because many devices have small connected lumens.
2. Leak testing is done to ensure there are no leaks that could allow fluid to enter/leak the ampoules/vials and cause damage.
3. The appropriate tray/container must then be selected, and if the device has lumens, the appropriate connector attached.
4. The sterilant concentrate is provided in a sealed single- use cup and requires no pre-mixing or dilution.

The disadvantages of this method of sterilization are that the devices must be immersible, must fit in the appropriate tray, and must be able to withstand the 55°C temperature the process uses.

Hydrogen Peroxide Sterilization: in this method disperses a hydrogen peroxide solution in a vacuum chamber, creating a plasma cloud. This agent sterilizes by oxidizing key cellular components, which inactivates the microorganisms. The plasma cloud exists only while the energy source is turned on. When the energy source is turned off, water vapor and oxygen are formed, resulting in no toxic residues and harmful emissions. The temperature of this sterilization method is maintained in the 40-50°C range, which makes it particularly well-suited for use with heat-sensitive and moisture-sensitive medical devices.

<u>Inoculating loop:</u>

Sterilization: the inoculating loop or needle is sterilized by heating it in the flame.

Hold the instrument like a pencil and at an angle so that the wire pointing downward.

Pass the entire wire portion through the flame starting at the base, where it is attached to the holder, and continue all the way through the loop or end of the needle. Heat the wire until glowing to red then allow it to cool before picking up any microorganisms. The instrument is now considered to be sterile. Do not touch the wire to any surface, or it will become contaminated and it will be necessary to repeat the sterilization procedure.

Inoculation: Hold the culture tube in one hand and gently resuspend the organisms. Do not shake the tube up and down as the culture may spill. In your other hand, hold the sterilized inoculating loop, remove the cap of the culture tube with the little finger of your loop hand. Never lay the cap down or it may become contaminated. Very briefly flame the lip of the culture tube.

Keep the tube at an angle, insert the loop and remove a loopful of material. Then flame the lip of the culture tube again and replace the cap.

Practical Part

- **Test for red heat sterilization**
    1. Divide the nutrient agar plate into four quadrants and label 1 through 4.
    2. Quadrant 1 is your negative control. Do not touch it.
    3. Gently resuspend the *S. aureus* bacterial suspension provided to you.
    4. Sterilize the wire loop by Bunsen burner. Then allow it to cool before picking up any microorganisms.
    5. Use the sterilized wire loop to inoculate a sample.
    6. Streak it on the surface of the second quadrant of petri dish.
    7. Sterilize the wire loop by Bunsen burner. Then allow it to cool before picking up any microorganisms.
    8. Use the sterilized wire loop to inoculate a sample.
    9. Immerse the loop in ethanol 70% in a 50 ml beaker.
    10. Wait to dry, and then Streak it on the surface of the third quadrant of petri dish.
    11. Sterilize the wire loop by Bunsen burner. Then allow it to cool before picking up any microorganisms.
    12. Use the sterilized wire loop to inoculate a sample.
    13. Sterilize the wire loop by Bunsen burner. Then allow it to cool before picking up any microorganisms.
    14. Streak it on the surface of the fourth quadrant of petri dish.

- **Test for moist heat sterilization at temperature above 100^{o}C (Autoclaving)**

    1. Pour 1 ml of decontaminated (autoclaved) bacterial suspention provided to you, on the surface of nutrient agar plate.
    2. Rotate the plate to spread the sample over the whole surface.
    3. Cover the plate and leave it until the surfaces dries.

- **Test for moist heat sterilization at atmospheric pressure (temperature 100^{o}C)**

    1. Place the two bacterial suspension test tubes provided to you in a water bath at 100 ^{o}C.
    2. After 15 min, take one test tube, wait to cool and pipette 1 ml, and then pour it on the surface of nutrient agar plate.
    3. Rotate the plate to spread the sample over the whole surface.
    4. Cover the plate and leave it until the surfaces dries.
    5. After 30 min, take the second test tube, wait to cool and pipette 1 ml, and then pour it on the surface of nutrient agar plate.
    6. Rotate the plate to spread the sample over the whole surface.
    7. Cover the plate and leave it until the surfaces dries.

Incubate all of your samples at 37 ^{o}C for 24-48 hrs

Report Sheet
Experiment No. 5
Sterilization Methods and Principles

Name:	Section:
Group No.	Day and Date:

1. After 24-48 hrs of Incubation at 37 °C, do your prepared plates appear to be sterile?

Test	Sample	Sterile/ contaminated
Red heat sterilization	Second Quadrant	
	Third Quadrant	
	Fourth Quadrant	
Moist heat sterilization at temperature above 100^{o}C (Autoclaving)	Autoclaved bacterial sample	
Moist heat sterilization at atmospheric pressure (temperature 100^{o}C)	Bacterial suspension after 15 min boiling in water bath	
	Bacterial suspension after 30 min boiling in water bath	

2. Wire loop sterilization testing plate:

A. Rank the quadrants from that with the most growth to the least growth.

B. Was ethanol 70% sufficient for sterilization of the wire loop, explain?

C. Was red heat sufficient for sterilization of the wire loop, explain?

3. **Moist heat sterilization at atmospheric pressure (temperature 100^{o}C) testing plates:**
   Compare the two plates after 15 and 30 min and write down your comments and conclusion.

4. **Based on your results, compare between the efficacy of autoclaving, red heat and moist heat sterilization at atmospheric pressure (temperature 100^{o}C):**

   A. Sterilization or decontamination efficacy.

   B. The time required to achieve sterilization.

Experiment 6
Bacterial Count Methods and Isolation Techniques

Many studies require the quantitative determination of bacterial populations. Therefore number of methods has been developed to count the bacterial culture:

1. **Viable or plate count method**
2. **Spectrophotometric (turbidimetric) analysis**
3. **Microscopic count method**
4. **Automated method (coulter counter)**

1. Viable or plate count method

This method represents the number of colony forming units (cfu) per g (or per ml) of the sample rather than cells per g (or per ml). For accurate determination of the total number of viable cells, it is critical that each colony comes from only one cell. A viable cell count is usually done by diluting the original sample, plating aliquots of the dilutions onto an appropriate culture medium, and then incubating the plates under proper conditions so that colonies are formed. After incubation, the colonies are counted and, from acknowledge of the dilution used, the original number of viable cells can be calculated.

This method of counting is also called the Minimal Count, because:

- It counts only the cells that can multiply.
- Certain microorganisms clump (aggregate) and each clump gives a rise to a single colony, regardless how many bacterial cells are present in that colony.

A. Direct Count Method

Serial dilution is the basic technique. The original sample (unknown concentration) will be diluted several times (by 10 folds each time). A small volume from each diluted sample will be incorporated on a solid culture

1. **The spread plate technique**

The number of bacteria in a solution can be readily quantified by using the spread plate technique. In this technique, the sample is appropriately diluted and a small aliquot is transferred to an agar plate. The bacteria are then distributed evenly over the surface by a special streaking technique. After colonies are grown, they are counted and the number of bacteria in the original sample is calculated. The end point of our analysis is the number of colony forming units per mL (CFU/mL) since we are counting the number of colonies rather than the actual number of bacteria.

2. **The pour plate technique**

Pour plate technique also sometimes called the loop dilution method, involves the successive transfer (serial dilution) of bacteria from the original culture to a series of tubes of liquified agar. Basically, a loopful of your original culture is transferred to a tube of liquifeid agar and mixed. As a result of this transfer, the concentration of bacteria in the first tube is lower than the concentration in the original culture; in effect you have diluted the original culture. A loopful of material from the first tube of liquified agar is then transferred to the second tube, effecting an additional dilution of the bacterial culture. The process is repeated for a third tube of liquified agar. Following inoculation of the tubes of liquified agar, the contents of each tube is poured into a separate Petri plate. After incubation, one of the plates should have an appropriate number of colonies to allow separation and isolation.

The pour plate technique is technically easier than the streak plate (surface dilution) technique; although, it is not certainly foolproof. In addition to all of the concerns regarding aseptic technique, a major concern in preparing pour plates is the temperature of the agar.

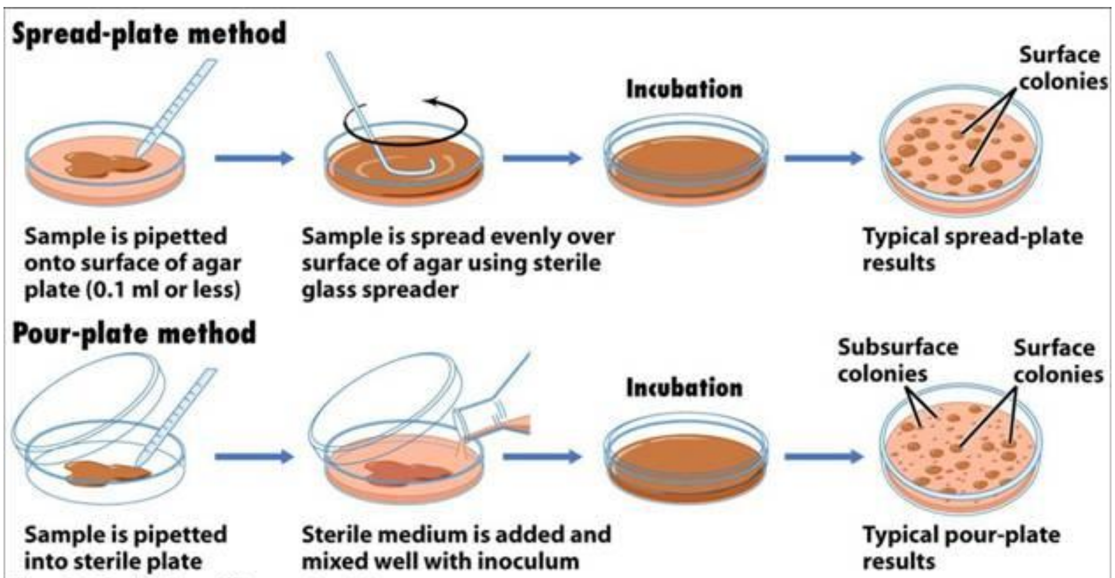


Figure 6-10 Brock Biology of Microorganisms 11/e
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B. **Indirect Count Method (Membrane Filtration Method)**

- Equipment: A filter paper (made from methyl cellulose) with a pore size of 0.22 -0.45 μm in diameter + Buckner funnel + Erlenmeyer flask.
- Methodology: A sample of bacteria (a bacterium is 0.5μm in diameter) is filtered under an aseptic technique (with aid of vacuum to speed up the process), and then the filter paper (bacteria are hindered by) is placed on a solid agar medium, and incubated at 37 °C for 18 -24 hours.
- The result: Colonies to be counted and the concentration to be calculated (CFU/mL).

Practical Part (Direct Viable Count Method):

A. Serial Dilution Technique:

1. Withdraw 1 mL from the original bacterial suspension (unknown concentration) and add it to 9 mL NB or NS.
2. Mix by vortex then withdraw 1 mL from this first diluted test tube and add it to 2nd test tube (contains 9 mL NB).
3. Continue diluting the suspension serially to prepare 9 dilutions of the original sample (from 10^1 to 10^9 dilution factor).

B. Surface Inoculation Technique:

1. Withdraw 0.1 mL from the last diluted test tube (has a dilution factor of 10^9) and pipetted it into a ready-made agar culture medium.
2. Bacteria are spread over the culture medium via a glass rod (L-shaped).
3. Repeat the above 2 steps for (10^8, 10^7, 10^6) dilutions, respectively.
4. Incubate the cultured plated (inverted) at 37 °C for 18 – 24 hours.

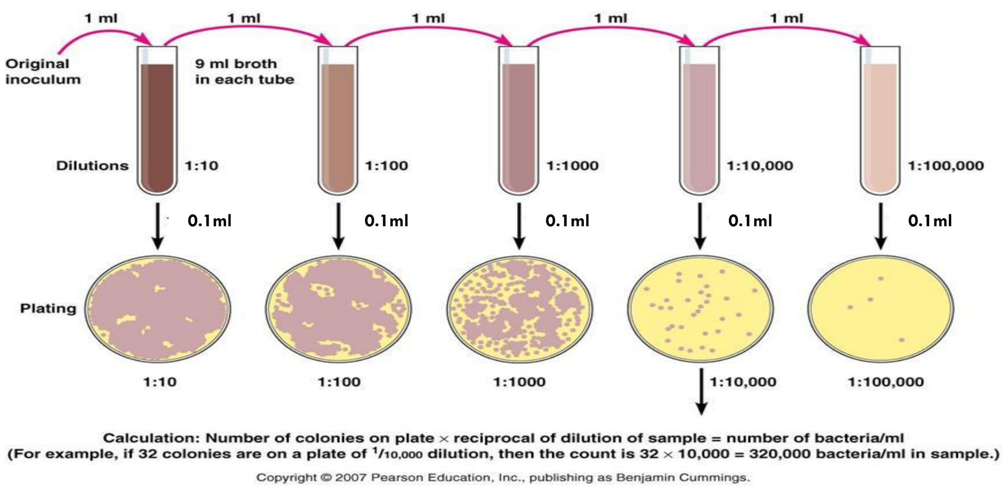


Figure 6.15

- **Interpretation of Results:**

- Count bacterial colonies ranging from 30 to 300 per plate.
- Counts more than 300 colonies; are reported as TNTC (Too Numerous to Count).
- Counts less than 30 colonies; are not considered and reported as SI (Statistically Invalid).
- If 2 or more readings from the resultant table are to be considered (are within 30 – 300 range); Select the reading that is closest to 300.

- The Bacterial Count in CFU unit (of the original sample) = # of colonies * D.F
- The Bacterial Concentration in CFU/mL unit (of the original sample) = (# of colonies * D.F)/ inoculated volume on agar surface

Note: Dilution (mathematically) is the reciprocal of Dilution Factor (DF). For example 10-6 is the dilution related to 106 DF.

Report Sheet
Experiment No. 6
Bacterial Count Methods and Isolation Techniques

Name:	Section:
Group No.	Day and Date:

- **After 24-48 hours of incubation at 37ºC, Record the following?**

Dilution of test tube	Dilution factor	No. of colonies	Comment

Calculate the bacterial **count (CFU)** and **concentration (CFU/ml)** of the original sample:

Self-Reading

2. Turbidmetric Measurement:

A rapid and quite useful method of estimating cell numbers is by turbidity measurements. A cell suspension looks cloudy (turbid) to the eye because cells scatter light passing through the suspension. The more cells that are present, the more light is scattered, and hence the more turbid the suspension. Turbidity can be measured with a spectrophotometer device that pass light through a cell suspension and detect the amount of unscattered light. Spectrophotometers measure only unscattered light. The measurements must be correlated initially with cell number

However the method is very rapid and simple to perform and provides reliable results when used with care, the tedious and laborious work make it replaced by **McFarland turbidity standards** that are a set of tubes with increasing concentration of barium sulfate suspension. The cloudiness or turbidity created by barium sulfate's white precipitates is used as a point of comparison of bacterial suspensions to known bacteria's turbidity.

McFarland turbidity standards preparation:

React different volumes of 1% solution (w/v) anhydrous barium chloride ($BaCl_2$) and 1% solution (v/v) sulfuric acid (H_2SO_4). Use the ratio in the following table to obtain desired McFarland scale:

McFarland standard no.	mLs of (1% $BaCl_2$)	mLs of (1% H_2SO_4)	Approx. cell density ($1X10^8$ cells/ ml)
0.5	0.05	9.95	$1.5 * 10^8$
1	0.1	9.9	$3 * 10^8$
2	0.2	9.8	$6 * 10^8$
3	0.3	9.7	$9 * 10^8$
4	0.4	9.6	$12 * 10^8$

It is essential that the standards be thoroughly shaken to ensure complete suspension of the barium sulfate each time they are used. Visual comparison of turbidity is easily and accurately made by viewing the test suspensions and standard against a white background with a black line running horizontally at midpoint.

3. Microscopic Count Method:

- Equipment: Microscope & Hemocytometer (a special slide used to count blood cells)
- Methodology: A loopful (0.001 mL using a wire loop, made from nichrome) of a bacterial suspension is placed on an area of 1 cm^2 of the slide, and then bacteria are counted under microscope.
- The result: This method counts living and dead cells.

4. Automated Method (Coulter Counter):

- Equipment: A capillary tube, (down it) there is a light source + a photodetector, which sends a signal to a digital counter.
- Methodology: 100 mL of a bacterial suspension added into the capillary tube, any interruption of the light source would be detected and so that a signal is sent to the digital counter to count 1, and so on.
- The result: This method counts everything (living cells, dead cell, debris of dead cells, and even dust).

Experiment 7
Testing of Disinfectants

Disinfectionis the process of elimination of most pathogenic microorganisms (excluding bacterial spores) on inanimate objects. Disinfection can be achieved by physical or chemical methods. Chemicals used in disinfection are called disinfectants. Different disinfectants have different target ranges, not all disinfectants can kill all microorganisms. Some methods of disinfection such as filtration do not kill bacteria, they separate them out. Sterilization is an absolute condition while disinfection is not

Disinfectants are those chemicals that destroy pathogenic bacteria from inanimate surfaces. Some chemical have very narrow spectrum of activity and some have very wide. Those chemicals that can sterilize are called chemisterilants. Those chemicals that can be safely applied over skin and mucus membranes are called antiseptics.

An ideal antiseptic or disinfectant should have following properties:

- Should have wide spectrum of activity
- Should be able to destroy microbes within practical period of time
- Should be active in the presence of organic matter
- Should make effective contact and be wettable
- Should be active in any pH
- Should be stable and have long shelf life
- Should have high penetrating power
- Should be non-toxic, non-allergenic, non-irritant or non-corrosive
- Should not have bad odor
- Should not leave non-volatile residue or stain
- Efficacy should not be lost on reasonable dilution
- Should not be expensive and must be available easily

Such an ideal disinfectant is not yet available. The level of disinfection achieved depends on contact time, temperature, type and concentration of the active ingredient, the presence of organic matter, the type and quantum of microbial load.
The chemical disinfectants at working concentrations rapidly lose their strength on standing.

Classification of disinfectants:

1. Based on consistency

a. Liquid (E.g., Alcohols, Phenols)
b. Gaseous (Formaldehyde vapor, Ethylene oxide)

2. **Based on spectrum of activity**

   a. High level
   b. Intermediate level
   c. Low level

Spectrum of activity

	Vegetative cells	Mycobacteria	Spores	Fungi	Viruses	Examples
High level	+	+	+	+	+	Ethylene Oxide, Gluteraldehyde, Formaldehyde
Intermediate level	+	+	-	+	+	Phenolics, halogens
Low level	+	-	-	+	+/-	Alcohols, quaternary ammonium compounds

3. **Based on mechanism of action**
   a. Action on membrane (E.g., Alcohol, detergent)
   b. Denaturation of cellular proteins (E.g., Alcohol, Phenol)
   c. Oxidation of essential sulphydryl groups of enzymes (E.g., H_2O_2, Halogens)
   d. Alkylation of amino-, carboxyl- and hydroxyl group (E.g., Ethylene Oxide, Formaldehyde)
   e. Damage to nucleic acids (Ethylene Oxide, Formaldehyde)

Factors Influencing the Action of Disinfectants

1. Type of disinfectant
2. Concentration of disinfectant
3. Contact time: the longer the contact time, the greater the kill
4. Temperature
5. Type of organisms
6. Concentration of organisms
7. Nature of suspending liquid

Suspension tests:

In these tests, a sample of the bacterial culture is suspended into the disinfectant solution and after exposure it is verified by subculture whether this inoculum is killed or not. Suspension tests are preferred to carrier tests as the bacteria are uniformly exposed to the disinfectant.
There are different kinds of suspension tests: test for the determination of the phenol coefficient (Rideal and Walker),the qualitative suspension testsand the quantitative suspension tests.

✓ **Determination of phenol coefficient:**

Phenol coefficient (PC or PE) of a disinfectant is calculated by dividing the dilution of test disinfectant by the dilution of phenol that disinfects under predetermined conditions.

Rideal Walker method: Phenolic disinfectant is diluted from 1:400 to 1:800 and the phenol is diluted from 1:95 to 1:115. Theirbactericidal activity is determined against *Salmonella typhi* suspension. Subcultures areperformed from both the test and phenol at intervals of 2.5, 5, 7.5 and 10 minutes. The plates areincubated for 48-72 hours at 37°C. Then PC is calculated

PC or PE = *(highest DFof disinfectant required to eliminate bacteria within 7.5 minutes but not in 5 minutes)* /*(highestDFof phenol required to eliminate bacteria within 7.5 minutes but not in 5 minutes*)

Disinfectant	Dilution	Growth of test organism in subculture after exposure for:			
		2.5 mins	5 mins	7.5 mins	10 mins
Test disinfectant	1:400	NG	NG	NG	NG
	1:500	G	NG	NG	NG
	1:600	G	G	NG	NG
	1:700	G	G	G	NG
	1:800	G	G	G	G
Phenol	1:95	G	NG	NG	NG
	1:100	G	G	NG	NG
	1:105	G	G	G	NG
	1:110	G	G	G	NG
	1:115	G	G	G	G

Example:

To calculate phenol coefficient of the tested disinfectant in the above table. After 7.5 minutes, the test organism was killed by the test disinfectant at a dilution of 1:600. In the same period the test organism was killed by phenol at a dilution of 1:100.

$$\text{Phenol coefficient} = \frac{600}{100} = 6$$

This result indicates that the test disinfectant can be diluted six times as much as phenol and still possess equivalent killing power for the test organism.

Test interpretation:

1. If a phenol coefficient no greater than 1 indicates that this agent is equal to or less effective than phenol.
2. If a phenol coefficient greater than 1 indicates that this agent is more effective than phenol.

Disadvantages of the Rideal-Walker test are:

1. No organic matter is included
2. The microorganism *Salmonella typhi* may not be appropriate
3. The time allowed for disinfection is short also the presence of time skips
4. It should be used to evaluate phenolic type disinfectants only.

✓ **The qualitative suspension tests**

This was done in a qualitative way. A loopful of bacterial suspension was brought into contact with the disinfectant and again a loopful of this mixture was cultured for surviving organisms. Results were expressed as ‘growth (+)’ or ‘no growth (-)’

Practical Part A (Qualitative Suspension Test)

This test is followed only by the DGHM guidelines.

Procedure (under aseptic conditions):

1. 0.1 mL of a bacterial suspension of 1*108 cells/mL is exposed to 10 mL of disinfectant with various dilutions (95%, 70% or 35% Ethanol).
2. After certain cumulative exposure times (2, 4, 8, 15 and 30 minutes), take a 0.001 mL sample (using inoculating loop: flamed and cooled) from the reaction mixture and add it to 5 mL NB. This step is both neutralization and subculture.
3. Incubate test tubes overnight at 37°C.

Interpretation of Results:

After incubation; each test tube (each subculture) should be examined whether it is turbid (+) or clear (-).

✓ **The Quantitative Suspension Test:**

In quantitative methods, the number of surviving organisms is counted and compared to the original inoculum size. By subtracting the logarithm of the former from the logarithm of the latter, the decimal log reduction or microbicidal effect (ME) or Germicidal Effect (GE) is obtained. An ME of 1 equals to a killing of 90% of the initial number of bacteria, an ME of 2 means 99% killed. A generally accepted requirement is an ME that equals or is greater than 5: at least 99.999% of the germs are killed. Even though these tests are generally well standardized, their approach is less practical.

The Microbicidal Effect (ME):

The Decimal Reduction Rate or the Microbicidal Effect (ME) can be calculated by using the following formula:

$ME = \text{Log } NC - \text{Log } ND$

NC: Number of colony forming units (CFU) developed in the control series, in which the disinfectant is replaced by sterile normal saline

ND: Number of colony forming units (CFU) developed in the disinfectant series, after exposing the bacterial cells to the disinfectant

The 5, 5, 5 Test:

The common name for the standard suspension test is the 5, 5, 5 test.

- 1st (5): Five test microorganisms are originally tested
  1. *Pseudomonas aeruginosa*
  2. *Staphylococcus aureus*
  3. *Escherichia coli*
  4. *Bacillus cereus*
  5. *Saccharomyces cerevisiae*

- 2nd (5): The exposure time is 5 minutes.
- 3rd (5): Determination of the bacterial activity (after the 5 minutes exposure to disinfectant) will be by decimal reduction rate (5 logarithms). The criterion for activity is a germicidal effect of 5 logarithms.

Practical Part B (Quantitative Suspension Test):

Procedure: (under aseptic conditions)

1. Expose 0.1 mL of bacterial suspension to 10 mL of disinfectant (70% ethanol). Add another 0.1 mL to the control (sterile NS or sterile DS)
2. After 5 minutes, neutralize 1 mL of reaction mixture using 9 mL NB.
3. Serially dilute the neutralized sample 4 times (by 10x dilution factors).
4. Subculture the neutralized sample and the followed 4 dilutions on agar plates for counting, using surface inoculation technique.

Interpretation of Results:

Counts are selected to be from 25 – 250 colonies. For results having more than one accepted count; the number closer to 250 (in control series), and the number closer to 25 (in disinfectant series) are used in the ME equation.

- Note: Do not forget to multiply the selected number of colonies by the corresponding dilution factor.

Example:

Calculate the ME for 70% ethanol which was used during a quantitative suspension test, knowing that the subculture step was performed using surface inoculation technique:

Dilution Factor	# of CFUs	
	Control Series	Disinfectant Series
10^1	TNTC	TNTC
10^2	TNTC	95
10^3	310	33
10^4	240	28
10^5	120	0

ME = Log NC – Log ND
ME = Log ($240 * 10^4$) – Log ($33 * 10^3$)
ME = 1.86

Report Sheet
Experiment No. 7
Testing of Disinfectants

Name:	Section:
Group No.	Day and Date:

Part A (Qualitative Suspension Test)

- After 24 hrs of incubation at 37ºC, record the following?

Concentration of ethanol disinfectant	Growth in subculture after specified time (G/NG)				
	2 min	4 min	8 min	15 min	30 min
95 %					
70 %					
35 %					

- Discuss the results you obtained and write your comments about them.

Part B (Quantitative Suspension Test)

Dilution Factor	# of CFUs	
	Control Series	Disinfectant Series
10^1		
10^2		
10^3		
10^4		
10^5		

ME Calculation:

Self-Reading

Different Protocols of Suspension Test:

Countries differ in their ways to evaluate the efficacy of disinfectants according to the type of disinfectant and its use (clinical, veterinary, or household). There are four well-known protocols in the world:

1. In the United States of America: the American Association of Official Analytical Chemists (**AOAC**)
2. In Germany: the German Society of Hygiene and Microbiology (**DGHM**; German Abbreviation)
3. In France: the French Association of Normalization (**AFNOR**; French abbreviation)
4. In the United Kingdom: the British Standards Institution (**BSI**)

The main differences between those tests reside in the following:

- Type of microorganisms to be tested: each country has its own point of view for the microorganisms that represent the vegetative bacteria that should be eliminated by the disinfectant.
- The initial bacterial inoculum (concentration and volume) to be tested.
- The neutralization method
- Determination of the end point: Qualitative or Quantitative

Experiment 8
Methods of Antimicrobial Susceptibility (Sensitivity) Testing
Disc Diffusion Method

Introduction

Resistance to antimicrobial agents (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs. Although defining the precise public health risk and estimating the increase in costs is not a simple undertaking, there is little doubt that emergent antibiotic resistance is a serious global problem. The results of *in-vitro* antibiotic susceptibility testing, guide clinicians in the appropriate selection of initial empiric regimens and drugs used for individual patients in specific situations (although sensitivity tests measure antimicrobial activity against bacteria under laboratory conditions it cannot be assumed that the results of *in vitro* tests will be necessarily the same *in vivo*). The selection of an antibiotic panel for susceptibility testing is based on the commonly observed susceptibility patterns, and is revised periodically.

Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system. They include:

1) Diffusion method (used to classify the microorganisms qualitatively according to their susceptibility to antimicrobial agents)

- **a.** Stokes method
- **b.** Kirby-Bauer method

2) Dilution method (<u>used to determine the minimum inhibitory concentration (MIC)</u>)

- a. Broth dilution
- b. Agar Dilution

3) Diffusion&Dilution method (<u>used to determine the minimum inhibitory concentration (MIC)</u>)

- a. E-Test method

Kirby-Bauer Disc Diffusion method

Kirby–Bauer (K-B) antimicrobial sensitivity test allows for the rapid determination of the efficacy of a drug (antibiotic) against certain type of bacteria by measuring the diameter of the zone of inhibition that resulted from diffusion of the drug from its disc into the surrounding medium.

K-B Test Standards:

A. Microorganisms are isolated from infected tissues. For example:
   - Septicemia: a blood sample is obtained
   - Upper respiratory tract infections: a throat swap or a sputum sample

   The obtained bacterial suspension should be **standardized** by comparing it with McFarland standard # 0.5 or # 1 (according to the type of bacteria). This would be equivalent to a bacterial concentration of $1.5 * 10^8$ cells/mL or $3 * 10^8$ cells/mL.

B. The used medium should be **standardized**. Mueller-Hinton Agar is used. This medium with a *p*H of 7.2 to 7.4 contains high percent of proteins, which will facilitate diffusion from the antibiotic disc and improve bacterial growth.

C. The antibiotic disc is made from filter paper. The concentration of certain antibiotic (antibiotic amount per disc area) should be **standardized** to ensure consistency in results. Knowing that antibiotic concentration is a factor affecting the diffusion rate from disc into the surrounding medium.

D. The incubation temperature should be **standardized** to be 37 °C. Since temperature is also a factor affecting rate of diffusion (proportional relationship).

E. The incubation time should be **standardized** to be from 18 to 24 hours (overnight incubation).

Those are the standards for the most commonly tested bacteria (*Enterobacteriaceae*, *Enterococci*, *Pseudomonas aeruginosa*).

Preparing MUELLER HINTON Agar

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method.

Bauer, Kirby, Sherris and Tuck[4] recommended Mueller Hinton Agar for performing antibiotic susceptibility tests using a single disk of high concentration. This un supplemented medium has been selected for several reasons. This medium is low in sulfonamide, trimethoprim and tetracycline inhibitors, and provides satisfactory growth of most non-fastidious pathogens .

Principles of the procedure:

Beef Extract and **Acid Hydrolysate of Casein** provide nitrogen, vitamins, carbon, and amino acids in Mueller Hinton Agar. **Starch** is added to absorb any toxic metabolites produced. **Agar** is the solidifying agent.

Formula/Liter

BeefExtract..2g
Acid Hydrolysate of Casein..17.5 g
Starch..1.5 g

Agar..17 g

Final pH 7.3 ± 0.1 at25°C

Directions

1. Suspend 38 g of the medium in one liter of purified water.
2. Heat with frequent agitation and boil for one minute to completely dissolve the medium.
3. Autoclave at 121°C for 15 minutes. Cool to room temperature.
4. OPTIONAL: Supplement as appropriate. Pour cooled Mueller Hinton Agar into sterile petri dishes on a level, horizontal surface to give uniform depth. Allow to cool to room temperature.
5. Check prepared Mueller Hinton Agar to ensure the final pH is 7.3 ± 0.1 at 25° C.

Prepared Appearance: Prepared medium is hazy and light to medium yellow.

Procedure (using aseptic techniques)

1. Prepare an E. coli bacterial suspension equivalent to 0.5 McFarland turbidity standard in a sterile broth media.

2. Immerse a sterile cotton swab in the bacterial suspension, rotate and remove the excess fluid by pressing and rotating the cotton against the inside of the tube above the fluid level.

3. Inoculate the surface of Mueller-Hinton agar palates (3 plates) by streaking the swab over the entire agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60^o each time to ensure an even distribution of inoculum. As a final step, the rim of the agar is swabbed.

4. Leave the plate to dry (for about 5 min, but not more than 15 min) to allow any excess surface moisture to be absorbed before applying the drug impregnated discs.

Note: Extremes in inoculum density must be avoided. Never use undiluted overnight broth cultures or other unstandardized inoculums for streaking plates.

5. Using sterilized forceps (by ethanol followed by heating), gently divide each plate into four quadrants and place 4 discs on each plate, the discs must be distributed evenly and not too close to the plate edge.

Notes:

I. The minimum distance between disc center to neighboring disc center is 24 mm, and also no closer than 10-15 mm from the edge of Petri-dish). A maximum of 6 discs may be placed on a 9-cm Petri-dish, and 12 discs on a 15-cm dish. Reduce the number of applied discs per plate if overlapping zones of inhibition are encountered.

II. A disc should not be relocated once it has come into contact with the agar surface because some of drug diffuses instantaneously. Instead, place a new disc in another location on the agar.

6. Gently press each AB disc down by forceps, to ensure that discs adhere to the surface of agar. Do not press the discs into agar.

7. Incubate the inverted plates at 37oC, within 15 min after applying the discs.

Zone of inhibition measurement and interpretation

1. After 16 to 18 hours of incubation, each plate is examined. If the plate was satisfactorily streaked, and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be a confluent lawn of growth. If individual colonies are apparent, the inoculum was too light and the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, including the diameter of the disc. Zones are measured to the nearest whole millimeter, using sliding calipers or a ruler, which is held on the back of the inverted Petri plate. The Petri plate is held a few inches above a black, nonreflecting background and illuminated with reflected light.

2. The zone margin should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be subcultured, re-identified, and retested.

3. The sizes of the zones of inhibition are interpreted by referring to: **Zone size interpretive table for the Kirby Bauer test.**

Antimicrobial Agent	**Disc Content**	**Zone of Inhibition (Diameter in mm)**		
		Resistant	**Intermediate**	**Sensitive**
Amikacin	30 mcg	14 or less	15 - 17	18 or more
Amoxicillin	25 mcg	22 or less	23 - 30	31 or more
Ampicillin for cocci	20 mcg	22 or less	23 - 30	31 or more
Ampicillin for bacilli	20 mcg	12 or less	13 - 14	15 or more
Bacitracin	10 IU	8 or less	9 - 12	13 or more
Cephalexin	30 mcg	14 or less	15 - 17	18 or more
Chloramphenicol	30 mcg	12 or less	13 - 17	18 or more
Ciprofloxacin	5 mcg	15 or less	16 - 20	21 or more
Clindamycin	2 mcg	14 or less	15 - 16	17 or more
Cloxacillin	5 mcg	9 or less	10 - 13	14 or more
Co- Trimoxazole	25 mcg	11 or less	12 - 16	17 or more
Doxycycline	30 mcg	14 or less	15 - 18	19 or more
Erythromycin	15 mcg	13 or less	14 - 17	18 or more
Gentamicin	10 mcg	12 or less	13 - 14	15 or more
Kanamycin	30 mcg	13 or less	14 - 17	18 or more
Lincomycin	2 mcg	9 or less	10 - 14	15 or more
Nalidixic acid	30 mcg	13 or less	14 - 18	19 or more
Penicillin G for cocci	10 IU	20 or less	21 - 28	29 or more
Piperacillin	100 mcg	14 or less	15 - 17	18 or more
Streptomycin	10 IU	11 or less	12 - 14	15 or more
Tetracycline	30 mcg	14 or less	15 - 18	19 or more
Tobramycin	10 mcg	12 or less	13 - 14	15 or more

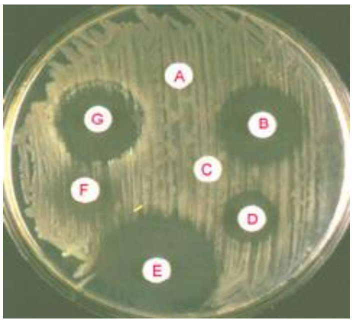
Classification of microorganisms according to susceptibility test results

1. **Susceptible (S):** an organism is called susceptible to a drug when the infection caused by it is likely to respond to treatment with this drug, at the recommended dosage.

2. **Intermediate Susceptibility (I):** covers two situations

    1. The organism cannot be classified as either susceptible or resistant, but is interpreted as being or intermediate (I) Susceptibility to a given drug.
    2. The clinical interpretation of this category is that the organisms tested may be inhibited by the antimicrobial agent provided that either:
        a. Higher doses of drug are given to the patient, or
        b. The infection is at a body site where the drug is concentrated.

3. **Resistant (R):** this term implies that the organism is expected not to respond to a given drug, irrespective of the dosage and of the location of the infection.



Factors that affect diameter of inhibition zone:

1. The susceptibility of the microorganism to the antibiotic used.
2. The diffusion rate of the drug through the agar medium.
3. The concentration of bacteria spread onto agar plate.

Troubleshooting during measurement of inhibition zone diameter:

1. If distinct colonies are observed, the colonies should be sub- cultured to check purity and test repeated if necessary
2. If fuzzy zone edges we should ignore them as much as possible (examine the plate against a dark background, do not hold the plate up to light or use a magnifying glass and estimate where the zone edge is)

INTERPRETATION:

1. Place the metric ruler across the zone of inhibition, at the widest diameter, and measure from one edge of the zone to the other edge. HOLDING THE PLATE UP TO THE LIGHT MIGHT HELP.
2. Use millimeter measurements. The disc diameter will actually be part of that number.
3. If there is NO zone at all, report it as 0--- even though the disc itself is around 7 mm.
4. Zone diameter is reported in millimeters, looked up on the **chart,** and result reported as sensitive, resistant, or intermediate.

5. Record the results for your table, as well as other tables, in the table.

Report Sheet

Experiment No. 8
Methods of Antimicrobial Susceptibility (sensitivity) Testing
Disc diffusion method

Name:	Section:
Group No.	Day and Date:

- After 24 hrs of incubation at 37^{o}C, record the following?

Antimicrobial Agent Name	**Disc Content**	**Zone's diameter (mm)**	**Classification (S,R,I)**

- Record the presence of individual colonies within zones of inhibition, the occurrence of fuzzy zones or irregular zones (deviation from round shape). Mention the antibiotic(s) that had these phenomena.

- According to your results, to which antibiotic E. coli is most susceptible. Explain your answer?

Experiment 9
Methods of Antimicrobial Susceptibility (Sensitivity) Test
(Broth Dilution Methods)

Introduction

In vitro susceptibility tests are performed on microorganisms suspected of causing disease, particularly if the organism is thought to belong to a species that may exhibit resistance to frequently used antimicrobial agents. The tests are also important in resistance surveillance, epidemiological studies of susceptibility and in comparisons of new and existing agents.

Minimum Inhibitory Concentration (MIC)

Diffusion tests widely used to determine the susceptibility of organisms isolated from clinical specimens have their limitations

In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing dilutions of the antimicrobial agent. The lowest concentration of an antimicrobial agent (in mg/L) that, under defined *in vitro* conditions, prevents the appearance of visible growth of a microorganism within a defined period of time is known as the MIC.

MIC and MBC determination

Each bacterium has a level of antibiotic which will inhibit growth but not kill the organism. This is called the minimum inhibitory concentration (MIC). Related to this, a higher antibiotic concentration will kill the organism. This is called the minimum bactericidal concentration (MBC).

Bacteriocides are expected to have equal or very close MIC and MBC values, but bacteriostatics have a difference in these values mainly MBC concentrations are higher than MIC concentrations.

MIC is defined as "the lowest concentration of antimicrobial agent required to inhibit or stop visually the organism from growing after 18-24 hrs incubation". It is usually expressed in (mg/L or µg/ml).

MBC is the lowest concentration of a given antimicrobial that will kill 99.9 % of the organism after 18-24 hrs of incubation. It is the lowest concentration of antimicrobial agent that will prevent the growth of an organism after subculture on to antibiotic- free media.

Now that we know the MIC and MBC for certain organism and antibiotic, we can put the patient on oral antibiotics and see what antibiotic levels can be achieved in the patient's bloodstream. The peak plasma level should be several times higher (e.g. 8 times higher) than the MBC, depending on the type of infection. If such levels cannot be obtained by oral antibiotics, then I.V antibiotics must be maintained for the duration of therapy.

For highly resistant microorganism with continuously changing profile of resistance, there are no constant parameters (MIC & MBC) identified, and thus their MIC & MBC should be checked all the time.

Examples:

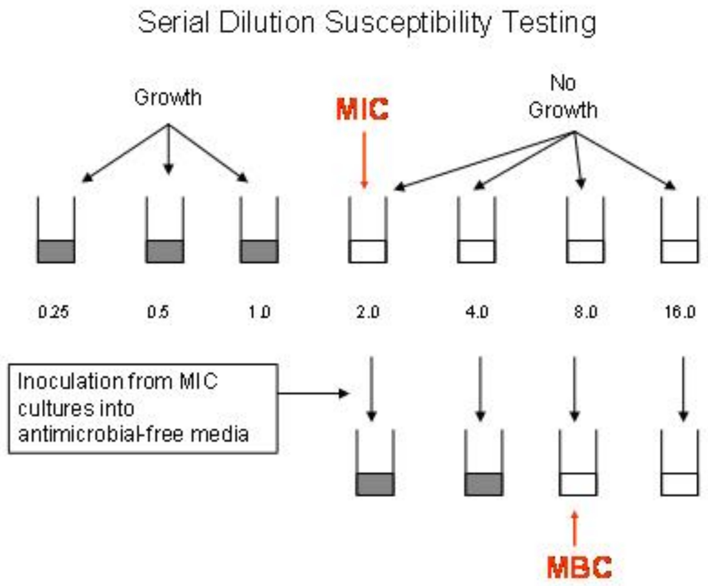
Bactericidals	**Bacteriostatics**
Gentamicin	Chloroamphenicol
Bacitracin	Clindamycin
Amoxicillin	Ethambutol
Isoniazid	Erythromycin
Metronidazole	Nitrofurantoin
Ciprofloxacin	Sulfamethoxazole
Rifampin	Tetracycline
Vancomycin	Trimethoprim

To determine MBC : all visually negative tubes (no visible growth after 18-24 hrs incubation) are sub-cultured into agar plates or antimicrobial free broth media and incubated for another 18-24 hrs at 37^{o}C. The lowest dilution which shows no viable growth on subculture is the MBC. Sub-culturing on an agar plate using the surface spread plate method will dilute the antibiotic and if there were still surviving bacteria, growth on the plate will determine it.

These sub- cultures into agar may show the following results after incubation:

- Similar number of colonies indicating bacteriostasis only.
- A reduced number of colonies indicating a partial or slow bacteriocidal activity.
- No growth if the whole inoculum has been killed bacteriocidal activity.

Figures below are an example on serial dilution susceptibility testing for MIC and MBC determination.



Procedure:

- Using a set of test tubes containing 1 mL NB, prepare a series of dilutions for the two antimicrobials by serially diluting1 mL of the AB stock solution through the set of test tubes. Notice that a single test tube would not contain an antimicrobial dilution (this test tube is called the control).
- Add 1 mL of the bacterial inoculum (bacterial suspension of 1*106 cells/mL) to each test tube, including the control.
- Incubate the test tubes at 37°C for 18-24 hours.
- Determine MIC for each anti- microbial agent

Interpretation of Results:

- After incubation: Compare each AB dilution test tube with the control test tube; to decide whether it is turbid (T) or clear (C).
- The AB concentration of the first clear test tube is considered as the MIC.
- To determine the MBC, the first 2 to 4 clear test tubes are subcultured on agar plates (using the surface inoculation technique). This will dilute the antibiotic to levels below the MIC, and if there were still surviving bacteria; growth on the plate would be seen as colonies.

Example:

An antimicrobial susceptibility testing – the tube (broth) dilution method was performed as following:

- 1mL broth solution was first added to 8 test tubes.
- 1mL of Gentamicin was added from a stock solution 512 µg/mL to the 8 test tubes by following a serial dilution.
- 1mL of bacterial inoculum is added after that to each test tube.

1. Calculate the concentration of each test tube before the addition of bacteria.

Original Conc.	1st test tube	2nd	3rd	4th	5th	6th	7th	8th
512	256	128	64	32	16	8	4	2

2. Calculate the concentration of each test tube after the addition of bacteria.

Original Conc.	1st test tube	2nd	3rd	4th	5th	6th	7th	8th
512	128	64	32	16	8	4	2	1

3. These test tubes were incubated over night at 37°C, after result inspection, the turbidity started at the 5th diluted test tube. Determine the MIC.

Original Conc.	1st test tube	2nd	3rd	4th	5th	6th	7th	8th
Not Incubated	C	C	C	C	T	T	T	T

Note: C: Clear, T: Turbid

MIC = 16 µg/mL (4th test tube)

4. The first four test tubes were sub-cultured on an agar plate using surface inoculation technique. The 3rd and 4th test tubes showed bacterial growth while the 2nd and 1st test tubes showed no growth at all. Determine the MBC.

Original Conc.	1st test tube	2nd	3rd	4th
Not Sub-cultured	Zero colonies	Zero colonies	6 colonies	10 colonies

MBC = 64 µg/mL (2nd test tube)

Report Sheet
Experiment No. 9
Methods of Antimicrobial Susceptibility (sensitivity) Testing
(Broth dilution methods)

Name:	**Section:**
Group No.	**Day and Date:**

- **After 24 hrs of incubation at 37°C, record the following?**

Chloramphenicol:

Test tube no.	Conc. (μg/ml)	Visual appearance after incubation (growth/ no growth)
1		
2		
3		
4		
5		
6		
7		
8		

Gentamicin:

Test tube No.	Conc. (μg/ml)	Visual appearance after incubation (growth/ no growth)
1		
2		
3		
4		
5		
6		
7		
8		

- **What is MIC for the Chloramphenicol and Gentamicin**

- **Write your expectations for the MBC values for both antibiotics, do you expect to have values similar to MIC or different values, explain your answer.**

Experiment 10

Methods of Antimicrobial Susceptibility (Sensitivity) Test
(The Agar Dilution Method)

Because quantitative MIC information is usually preferred to classify bacteria as resistant, intermediate and susceptible, dilution method, agar diffusion method and the E- test are favored over disk diffusion method

Agar dilution method: antimicrobial agent should be added to molten muller hinton agar at a tempreture of 45- 50 ^{o}C. The plates are inoculated with a bacterial inoculums prepared in sterile normal saline. The inoculum should be standardized to have a concentration of 1-2 x 10^8 CFUlml and diluted to inoculate the plates.

The endpoint should be taken as the plate on which no growth occurs. Appearance of five or less colonies can be ignored.

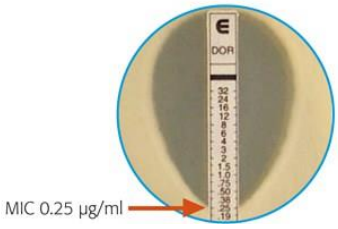
The Epsilometer test (E- test)

The E- test is a quantitative method used to determine MIC which applies both the dilution of and diffusion of antibiotic into the medium. E-test is a 'ready-to-use' reagent strip with a predefined gradient of antibiotic for the determination of precise MIC values of a wide range of antimicrobial agents against different organism groups, when E-test is applied to the surface of an agar plate inoculated with the test strain, there is an instantaneous release of the antimicrobial gradient from the plastic carrier to the agar to form a stable and continuous gradient beneath and in the immediate vicinity of the strip.

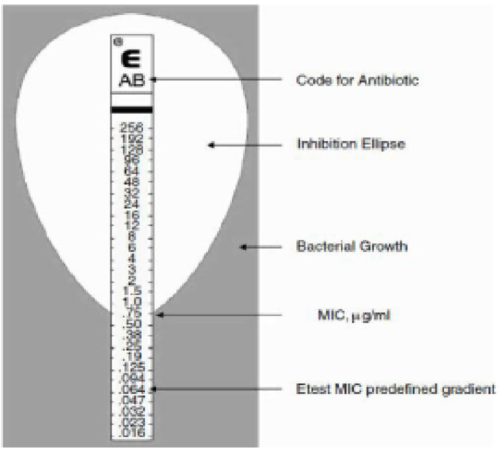
E-test incubation and reading times have been determined based on the intrinsic growth characteristics of the organism, and the specific incubation conditions. Therefore, for reliable and reproducible results, the stability of the gradient must be maintained for many hours. The predefined E-test gradient remains stable for at least 18 to 24 hours; that is, a period that covers the critical times of many species of fastidious and non-fastidious organisms.

When the E-test strip is placed on an agar surface, the antibiotic gradient on the strip is simply transferred to the agar matrix creating an imprint of the gradient on the strip in the agar. The bacterial growth becomes visible after incubation and a symmetrical inhibition ellipse centered along the strip is seen. The MIC value is read from the scale in terms of µg/mL where the ellipse edge intersects the strip.

After the required **incubation period**, and only when an even lawn of growth is distinctly visible, the MIC value can be read where the edge of the inhibition ellipse intersects the side of the strip. The plate should not be read if the culture appears mixed or if the lawn of growth is too light or too heavy. E-test MIC endpoints are usually clear-cut although different growth/inhibition patterns may be seen



The E-test consists of a thin reagent strip that carries a continuous concentration gradient of stabilized and dried drug. The E-test then uses the principle of **agar diffusion** to perform quantitative testing. In order to determine an MIC with the E test, the surface of an agar plate is swab inoculated with an adjusted bacterial suspension in the same manner as a disk diffusion test. One or more E test strips for the antimicrobial agents to be tested are then placed on the inoculatated agar surface.



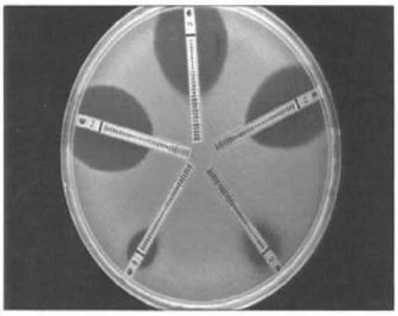
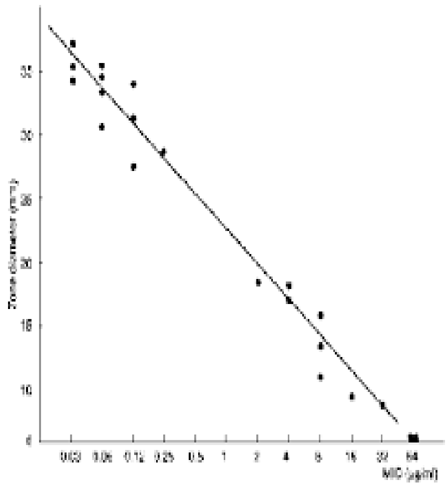


Figure 1-Photograph of a 15 centimeter long Mueller-Hinton plate with five E tests strips (ciprofloxacin, ceftazidime, piperacillin, tircacillin/clavulanic acid and trimethroprim/ sulfamethoxazole. The microrganism being tested was Xanthoma maltophilia.

Determination of MIC by agar diffusion (Cup- plate) method

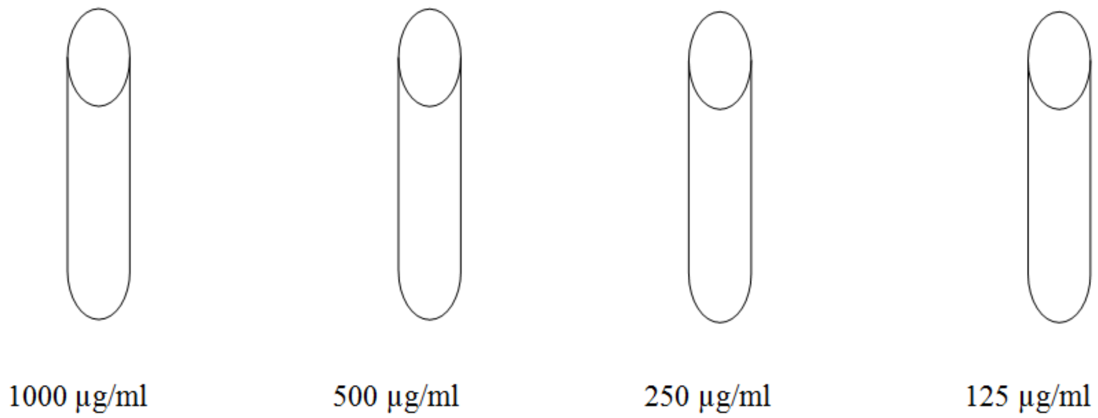
It was found that there is an approximately linear relation between **log MIC** and the inhibition zone diameter in the diffusion test as shown below.



The agar diffusion method is a quantitative method for antimicrobial susceptibility testing that applies both the dilution of and diffusion of antibiotic, it correlates between antimicrobial agent concentration and inhibition zone distance.

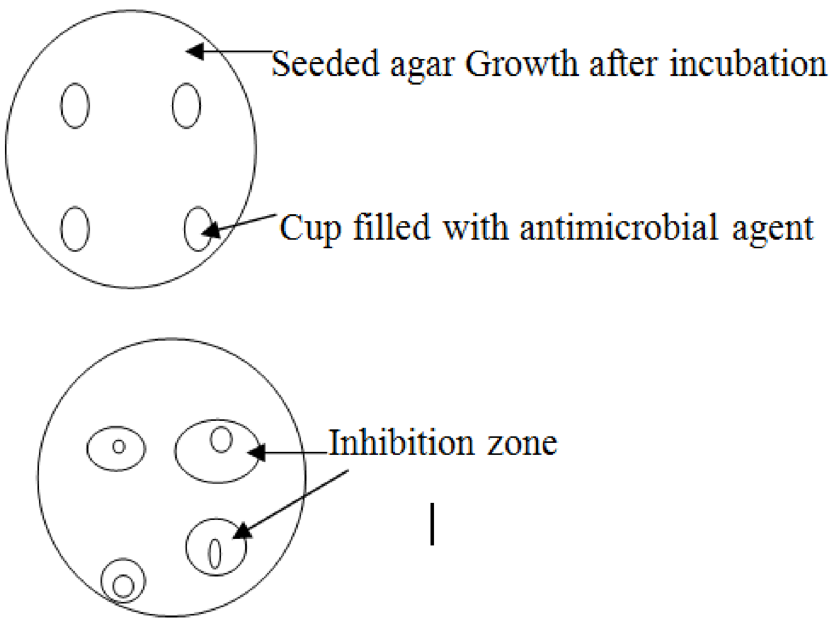
Principle: By means of a cork borer a cup is made in a seeded agar plate (prepared by pour plate method).

The agar disk is removed and the cup is filled with an antimicrobial agent which diffuses from the cup to certain extent and inhibits the growth of the organism, creating an inhibition zone.

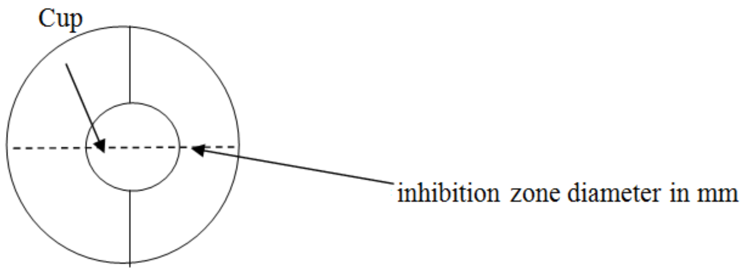


Result interpretation and calculations

1. After incubation the inhibition zone diameter is measure using ruler (the higher the concentration of the antimicrobial agent, the larger the inhibition zone diameter)

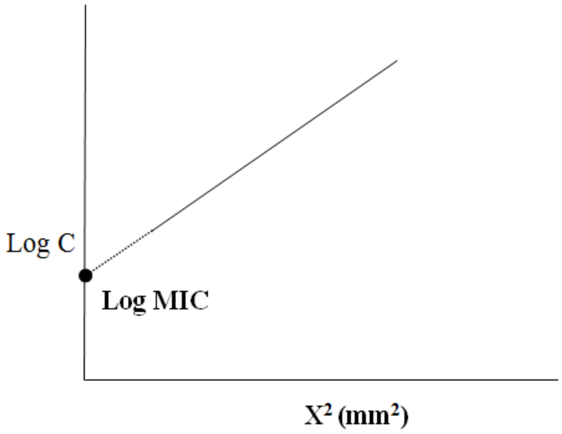


2. The following parameters are calculated as shown below



x= inhibition distance in mm = (inhibition zone diameter – cup diameter) /2

3. Plot log C (C: is the concentration of antimicrobial agent) against X^2 and extrapolate the line.
4. The intercept is log MIC.
5. MIC (µ/ml or mg/L) = antilog the intercept. (If use semi- log paper, then no need to calculate log concentration).



<u>Comparison between different types of antimicrobial susceptibility testing methods</u>

Disk diffusion method is relatively inexpensive and easy to perform and does not require any special experiment. The disadvantages that disk diffusion measures the inhibition zone size which is then converted to categories of susceptible/ intermediate/ resistant, this method is unable to obtain MIC values. It is qualitative only

Agar dilution and broth dilution methods are able to overcome some of the limitations of the disk diffusion method. They are capable of drawing quantitative conclusions by determining the MIC values for antimicrobials.

The advantage of broth dilution is the generation of a quantitative result (MIC) and the ability to test MBC values. The principal disadvantages of the dilution method is the tedious manual work in preparing the antibiotic solutions for each test, and the possibility of errors in preparation of the antibiotic solutions and the lack of automation of the test, it is a manual test.

The E- test has been reported to be a simple and accurate alternative method for determining the antimicrobial susceptibilities of various microorganisms. The disadvantage is the E- test strip is an expensive approach if more than a few drugs are tested. This method is best suited to situations in which an MIC for only one or two drug(s) is needed. Generally, E- test results have correlated well with MICs generated by broth or agar dilution methods.

The agar diffusion method has the advantage that any contaminant can be easily seen by the naked eye. It is not expensive and less tedious than dilution methods. Additionally, it can be used in assay tests to determine the potency of unknown samples. The disadvantages are: it is not suitable for non-diffusible substances and it is affected by the diffusion rate of drug.