B.Sc. Medical Laboratory Sciences

Medical Bacteriology (MLS 2413)

Practical Manual

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Edited and Revised by

Dr. G. I. D. Dushyanthie A. D. Athukorala

Dr. Jananie Kottahachchi

Dr. D. M. B. Thushari Dissanayake

Department of Microbiology

Faculty of Medical Sciences

University of Sri Jayewardenepura

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Complied by

V.G.N.S. Velathanthiri - MLS Unit

A.M.B. Priyadarshani - MLS Unit

M.M. Weerasekara - Dept. of Microbiology

D.M.B.T. Dissanayake - Dept. of Microbiology

Edited by Prof Neluka Fernando

Head/Department of Microbiology

Faculty of Medical Sciences

University of Sri Jayewardenepura

Contents

Contents
Cont…..

Objectives

At the end of Medical Bacteriology unit, students should know,

- Organization and general safety in a clinical microbiology laboratory
- Safety precautions and good laboratory practice in the laboratory
- Staining techniques for identification of medically important micro-organisms and Gram nature of medically important bacteria
- Preparation of different culture media for the growth of medically important bacteria
- Proper techniques of inoculating culture plates, tubes, agar slants and broths
- Correct method of handling and examining cultures
- Incubation requirements for pathogenic bacteria
- Describe the colony characteristics of medically important bacteria
- Describe the necessity of different types of biochemical tests in the identification of medically important bacteria
- Describe the principles and the procedure of the important biochemical tests
- The methods of demonstration of motility of bacteria
    - Hang-drop method
    - Use of semisolid media for the demonstration of motility [Lysine Indole Motility (LIM) medium]
- Bacterial counting methods
    - Standard / viable plate count method (Counting bacteria by dilution and plating)
        - Pour plate method
        - Milse and Misra method
    - Counting chamber
    - Spectrophotometric / Turbidimetric analysis
    - Most Probable Number (MPN) method
    - Membrane filtration

Introduction to Diagnostic Microbiology and Microbiological Techniques

Microbiology involves the study of microscopic organisms. Although microorganisms are beneficial for life, some are pathogenic and cause infectious diseases. The diagnostic microbiology laboratory is engaged in the identification of the infectious agents. These infectious agents are broadly classified as viruses, bacteria, fungi and parasites.

Identification of the infectious agent is the principle function of the diagnostic microbiology laboratory. In addition, the laboratory also provides guidance in therapeutic management. This is particularly true in the case of bacterial infections where the laboratory provides information regarding the most effective antimicrobial agent to be used against a specific organism isolated from the patient.

Safety Procedures

The Microbiology laboratory is a place where clinical specimens and cultures of actively growing microorganisms are handled and examined. This type of activity must be carried out with good aseptic techniques in an organized manner.

Warning

Some of the laboratory experiments included in this manual may be hazardous if you handle material or carry out procedures incorrectly. Safety precautions should be followed when you work with microorganisms, chemicals, glass tubes, hot water baths, sharp instruments etc.

In general, all safety procedures and precautions followed in the Microbiology Laboratory are designed to;

1. Restrict microorganisms present in specimens or culture 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 culture plates and interfering with results of studies.

General Laboratory Directions

1. Always read notices at the entrance of the laboratory before you enter in to the laboratory.

2. Leave bags, books and other items outside (in a locker) which are not necessary for work in the laboratory.

3. Eating, drinking, smoking and applying cosmetics are prohibited inside the laboratory.

4. Before enter into the laboratory, all should wear a fresh, clean, knee length laboratory coat with sleeves up to wrist. Should wear shoes that cover feet (Personal Protective Equipment -PPE). Any cuts or abrasions on body should be covered with adhesive plasters.

5. Learn good personal habits from the beginning. i.e: to tie back long hair away from the shoulder; do not wear bangles, wrist watches, rings to laboratory sessions; keep fingers, objects like pencils handled in the laboratory should be kept away from mouth; do not lick labels with tongue. Use tap water or self-sticking tapes.

6. Read the laboratory protocols before you start your work. Keep the protocols away from the clinical specimens. Use separate marker pens for labeling of specimens.

7. Do not touch your eyes, nose and mouth with contaminated hands.

8. Perform the procedures to minimize splashes and generation of aerosols.

9. Do not forcibly expel material from a pipette and do not pipette by mouth.

10. Wear gloves when handling clinical specimens and cultures. Remove your gloves as soon as you finish the work. When conducting procedures likely to generate aerosols, wear a protective mask and goggles.

11. Wherever possible do the aerosol generating procedures inside a bio-safety cabinet.

12. At the start and the end of each laboratory session, students should clean their assigned bench top area with a disinfectant solution provided. That space should then be kept clean and uncluttered throughout each laboratory period.

13. Discard all cultures and used glassware into the containers provided for contaminated items. Plastic or other disposable items should be discarded separately from glassware.

    - Never place contaminated pipettes or cotton wool plugs on the bench top.
    - Never discard contaminated cultures, glassware, pipettes, tubes or slides in the waste paper baskets or garbage can.
    - Never discard contaminated liquids or liquid cultures in the sink.

14. If any accident occurs inside the laboratory call the officer in-charge immediately.

15. Accidental spillage of bacterial cultures or other contaminated material should be reported immediately to the officer in-charge or the attending tutor.

16. Do not remove specimens, cultures or equipment from the laboratory without permission.

17. Handle the organisms in the respective bio-safety level.

    Hazard group 1 – Organisms that are not known to cause infections in healthy adults.
    Organisms can be handled on open bench in a containment Level 1 laboratory.

    Hazard group 2 - Organisms that may cause human diseases and may be a hazard to laboratory workers but are unlikely to spread in the community. Laboratory exposure rarely causes infections and effective prophylaxis or treatment is usually available. Organisms can be handled inside a class 1 bio-safety cabinet in a containment level 2 laboratory.

Hazard group 3 – Organisms that may cause severe human diseases and present a serious hazard to laboratory workers. They may pose a risk of spread in the community, but there is usually effective prophylaxis or treatment. Organisms can be handled inside class 1, 2 or 3 bio-safety cabinets in a containment level 3 laboratory.

Hazard group 4 – Organisms that cause severe human disease and pose a risk to laboratory workers. They may pose a high risk of spread in the community and there is usually no effective prophylaxis or treatment. Organisms should be handled in a class 3 bio-safety cabinet in a containment level 4 laboratory.

18. Do not sit on the bench top.

19. Wash hands once the work is finished and whenever the hands are contaminated with a clinical specimen or culture of organisms.

20. Do not walk outside the laboratory with overcoat and gloves.

21. Remove gloves before handle any laboratory records.

22. Before leaving the laboratory, remove the over coat and carefully wash and disinfect your hands.

Staining Techniques
Simple Staining: Positive and Negative Stains

Stains are used to recognize bacteria in clinical specimens and in cultures. Simple stains are used to demonstrate the presence of organisms as well as the nature of the cellular contents in exudates. This exercise is useful to practice staining and observing bacteria before doing more complicated stains.
Examples of simple stains include Loeffler's Methylene Blue, Polychrome Methylene Blue and Dilute Carbol Fuchsin. The typical bacteria will be visualized as about 0.5 – 1.0 micrometer (μm) in width to 2-7 μm long and are usually rods, cocci or spiral shaped.

Negative stain is yet another simple stain available in laboratories. This is especially useful to demonstrate the capsules of bacteria. Bacterial capsule is not stained by ordinary staining techniques. When stained with wet India ink method, organisms can be observed under the microscope as clear areas in a black background.
In all types of simple stains you will be able to determine the shape of the bacteria, their arrangement after cell division and characteristics.

Technique of simple stain
Materials

- Heat-fixed bacterial smears (Refer "Staining Techniques in Microbiology" 1st Edition by Dr. Neluka Fernando- Page 2-5).
- Methylene blue, polychrome blue or carbol fuchsin to act as simple stain.
- Staining tray, wash bottles, inoculation loop
- Microscopic glass slides, Bunsen burner, blotting papers, microscopes
- Marking pen, pencils and slide labels

Method

1. Cover the label on the slide with tape.
2. Place the slide on the staining rack and flood the slide with stain for 1minute.
3. Rinse the slide with tap water, tilting the slide slightly to rinse all the stain from the slide. Tap the slide gently to remove excess water.
4. Allow the slide to air dry.
5. Examine the stained smear with the microscope, under oil immersion.

Technique of negative stain
Materials
India ink
Bacterial culture on a solid medium or liquid medium
Wire loop
Microscopic glass slides, Bunsen burner, blotting papers, microscopes
Marking pen, pencils and slide labels

Method

1. Take a new glass slide and place a large loop-full of undiluted India ink on it.
2. Emulsify a very small portion of solid bacterial culture or a small loop-full of liquid culture in the ink.
3. Place a clean cover-slip on the ink drop and press it down through a sheet of blotting paper so that the film becomes very thin and thus pale in colour. The film should be so thin that the bacterial cell with its capsule is gripped between the slide and the cover-slip (Some practice is required in making satisfactory films of the correct thickness).

Exercise

1. Preparation of stains.

   Read a standard protocol for preparation of stains and prepare stain.

   a) Loeffler's methylene blue
   b) Ammonium oxalate – Crystal violet
   c) Carbol fuchsin
   d) Lugol's iodine

2. Prepare a bacterial smear - Follow standard protocols.

3. Stain with simple stain.

4. Observe under microscope (Focus with x 10 objective and observe the details with the oil immersion lens).

5. Prepare a wet India ink smear from the culture isolate of *Streptococcus pneumoniae* and observe under the microscope.

Answer the following questions given to you.

Staphylococcus, Bacillus, Diphtheroids, Enterococcus

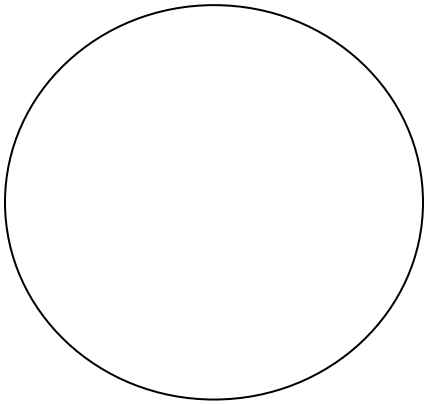
1. Simple stain:

   Shape: ……...

   Arrangement: ……

2. Negative stain

   How did the organism appear?



Read the questions and answers

1. What are the advantages of simple stain?

   (a) Detect the presence of organisms.
   (b) Less time consuming.
   (c) Simple procedure therefore easy to perform.

2. Do you need more or less light when viewing a stained preparation compared to wet mount.

   More light is needed to view stained preparations.

3. What information can you observe in a wet mount that cannot be seen in a stained preparation?

   Motility can be observed in wet mounts but cannot be seen in stained preparations.

4. How does the negative stain differ from simple stain?

   Simple stain uses a single dye to stain the organism which can be seen against the unstained background but in negative stain the organism does not take up the dye and it can be visualize against the darkly stained background as an unstained body.

5. What is the purpose of fixing the smear on a slide before staining?

   Fixing a smear is necessary to prevent it being washed off during the staining procedure. Also it reduces the infectivity of the smeared organisms.

6. List at least two types of bacteria where name reflect their shape and arrangement and state the meaning of each name.

    - Staphylococci –Staphyl in Greek is a bunch of grapes [round bacteria (cocci) arranged in clusters]
    - Streptococci - round bacteria (cocci) arranged in chains.
    - Diplococcic - round bacteria (cocci) arranged in pairs.

7. For what reason do we stain bacteria?

    To visualize;
    - The shape of bacteria.
    - Gram stain characteristics.
    - Special structures of bacterial cell (flagella, spores, volutin granules, glycogen)

Bacteriology
Practical 2 and 3

Differential Stains

Gram Stain

The Gram stain is one of the most useful tools in the microbiology laboratory and is used universally. In the diagnostic laboratory it is not only used to study microorganisms in cultures, but also to smears made directly from clinical specimens. Direct, Gram-stained smears are read promptly to determine the relative numbers and morphology of bacteria and cellular structures in the specimens. The information is valuable to the physician in planning the patient's treatment before culture results are available. It is also valuable to microbiologists, who can plan their culture procedures based on their knowledge of pathogenic bacteria in the specimens.

Purpose

To learn the Gram-stain technique and to understand its value in the study of bacterial morphology.

Materials

24 hours old culture of microorganism on a solid medium
Crystal violet
Lugol's iodine
Iodine acetone
Dilute carbol fuchsin
Staining tray, wash bottles, inoculation loop
Glass slides, Bunsen burner, blotting papers, microscopes
Marking pen, pencils and slide labels

Procedure

1. Make a thin smear of the bacterial culture; air dry and heat fix the smear.
2. Place the slide on the staining rack and flood the smear with ammonium oxalate – crystal violet (primary stain) for 30 seconds.
3. Wash off thoroughly with Lugol's Iodine solution*.
4. Flood the smear with Lugol's Iodine solution (mordent) for 30 seconds.
5. Wash off thoroughly with Iodine-acetone*.
6. Decolourize with few drops of iodine acetone for 30 seconds. (If you use acetone for decolourization do not keep it on the film for more than 2-3 seconds as acetone decolourizes very quickly)
7. Wash the smear thoroughly with water.
8. Counter stain the smear by flooding the smear with dilute carbol fuchsin for 30 seconds. Safranin is used as the counter stain for *Neisseria*.
9. Wash off the smear thoroughly with water and dry the slide and observe under the microscope.

*Water can be used as a substitute for reagents in routine laboratories for washing purposes.

Precautions

- Always use fresh cultures to avoid misleading results.
- Excessive heat should be avoided during heat fixing.
- Smears should be thin and uniform.

Exercise

1. **Prepare the slides from the given culture plates and examine the slides under oil immersion objective. Record the results in the given table.**

Diagram	**Colour (Pink/Purple)**	**Gram stain reaction**	**Name of the organism**

2. **What is the function of the Lugol's iodine solution in the Gram stain? If it was omitted, how would stain results be affected?**

   When we apply Lugol's iodine solution after staining the smear with ammonium oxalate - crystal violet solution, it will form a crystal violet - iodine complex, a larger molecule that precipitates in the cell cytoplasm. The complex formed is larger than crystal violet so it cannot be easily washed out from the intact peptidoglycan layer. Both Gram positive and negative bacteria remain purple after this step.

If Lugol's iodine was removed, there won't be a crystal violet-iodine complex and crystal violet molecules could be easily washed off during decolourization.

3. What is the purpose of the iodine acetone solution in the Gram stain?

When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the thin peptidoglycan layer is left exposed. The crystal violet-iodine complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large crystal violet-iodine complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan.

4. What is the advantage of the Gram stain over the simple stain?

In simple stain we can find the size, shape and the arrangement of the microorganism. All the cells are stained in the same colour irrespective of the cell wall characteristics. But in Gram stain we can differentiate the microorganism according to their cell wall characteristics (Gram positive and Gram negative) apart from the size, shape and arrangement.

5. **What is the principle of Gram stain reaction?**

Gram positive and Gram-negative bacteria stain differently because of fundamental differences in the structure of their cell walls.
Gram positive cell wall consists of numerous layers peptidoglycan. On the other hand Gram negative cell wall has only 2-3 layers of peptidoglycan and is surrounded by an outer membrane composed mainly of lipopolysacccharides.
When Lugol's iodine solution is applied after staining the smear with ammonium oxalate - crystal violet solution, it will form a crystal violet - iodine complex, a larger molecule that precipitates in the cell cytoplasm. The complex formed is larger than crystal violet so it cannot be easily washed out from the intact peptidoglycan layer.
When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer membrane and the thin peptidoglycan layer is left exposed. The crystal violet-iodine complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large crystal violet-iodine complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan.
When the counter stain is added, the cells without the Gram positive colour will take up the Gram negative colour.

Acid Fast Stain

Members of the bacterial genus *Mycobacterium* contain large amount of lipids (fatty) substances within their cell walls. These fatty waxes resist staining by ordinary methods.

When these organisms are stained with a concentrated solution of a basic dye such as carbol fuchsin, while applying heat, the stain can penetrate the lipid cell wall and reach the cell cytoplasm. Once the cytoplasm is stained it resists decolourization even with harsh agents such as acid alcohol which cannot dissolve and penetrate beneath the mycobacterial lipid wall. Under these conditions of staining the mycobacteria are said to be *acid fast*. Other bacteria of those, cell walls do not contain high concentration of lipid are readily decolorized by acid alcohol after staining with carbol fuchsin and are said to be *nonacid fast*.

The original technique for applying carbol fuchsin with heat is called the Ziehl-Neelsen stain named after two bacteriologists who developed it in the late 1800s.

Purpose

To learn the acid-fast technique and to understand its value when used to stain clinical specimens.

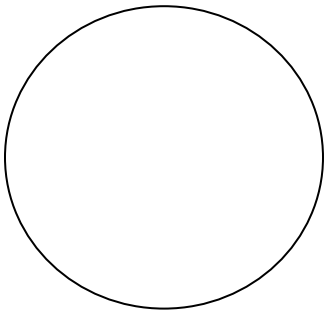
Materials

A young culture slant of *Mycobacterium spp*.

Procedure:

Refer “Staining Techniques in Microbiology” 1st Edition by Dr. Neluka Fernando.

Draw what you see after staining under microscope.



Bacteriology

Special Stains

Special stains are used to detect special structures of micro-organisms.

1. Spore stain
    - Malachite green
    - Modified acid-fast stain

2. Capsular stain
   Muir's method – Refer page 217 of Cowan and Steel's manual for the identification of medical bacteria (3rd edition)

   McFadyean's reaction – The capsular material of *Bacillus anthracis* can be detected by the McFadyean's reaction which involves staining with polychrome methylene blue. The poly-D-glutamyl capsular material is detected by this technique. Positive test creates blue rods in a background of purple/pink-stained capsular material. This appearance is given only by *Bacillus anthracis* and not by other Bacillus species.

3. Stains for volutin (metachromatic) granules
    - Albert – Laybourn stain
    - Neisser's stain

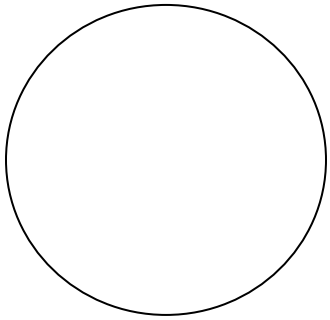
4. Flagella stain
    - Wet mount flagellar stain (By Heimbrook et al 1989)

Exercise

Prepare smears with given bacterial cultures.
Stain with special stains according to standard guidelines.
Observe under microscope and answer the following questions given to you (Refer "Staining Techniques in Microbiology" 1st Edition by Dr. Neluka Fernando).

1) Spore stain; Modified acid-fast.

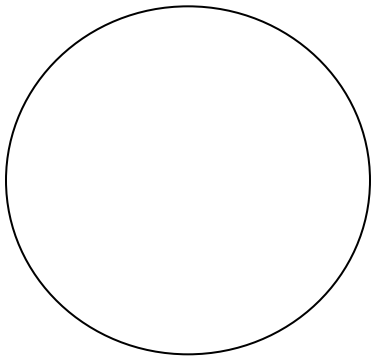
1. Draw a diagram and describe the appearance. Note the position of the spores.



2. Name few spore-forming bacterial species.
   .....
   .....

2) Stains for volutin (metachromatic) granules; Albert – Laybourn stain.
(Volutin granules are well formed in Loeffler Serum medium. You have to use cultures grown on this medium for the staining procedure.)

1. Draw a diagram and describe the appearance.



2. What are the problems encountered in detecting volutin granules?

   The location and the typical appearance of the volutin granules will depend on growth conditions and age of culture.

Bacteriology
Practical - 4

Sterilization and Disinfection

Sterilization of culture media
Sterilization requirements are slightly different from media to media. Follow the instruction given by the manufacturer before the sterilization procedure.

1. A bottle of nutrient agar culture media was prepared and needs to be sterilized.

1.1 What is the most suitable method of sterilization?
Moist heat sterilization – Autoclaving at 121^0C at 15lb for 15 minutes.

1.2 How do you prepare the media for sterilizing?

Pre-sterilizing;

- Close the lid of the bottle containing prepared media; do not tightly fit the lid of the bottle.
- Stick a piece of sterilizing indicator tape on the bottle.
- Keep the media bottle inside the autoclave.
- Autoclave at 121 °C temperature, at 15 lb for 15 min.

1.3 If you want to prepare blood culture media how would you sterilize it? Write the procedure.

- Prepare the basal medium.
- Sterilize the basal medium at 121^0C temperature, at 15 lb for 15 min.
- Mix blood to get a concentration of 5-10% into the sterilized basal medium after cooling it to about 55^0C and pour into plates.

1.4 How will you pour the media into culture plates? Write the steps.

- Fifteen to twenty milliliters of media is poured into the culture plates inside a safety cabinet or near a flame to avoid the environmental contamination.
- Remove and hold lid of the media bottle with little finger, flame the mouth of the bottle and pour the media to the labeled (date/ type of media), sterile petri dishes.
- Flame the mouth of the bottle whenever the lid is replaced.
- Rinse bottle immediately after pouring last plate before the remainder solidifies.
- When plates are solidified, invert them and place in an incubator at 37°C to dry out excess moisture. Store in labeled, sealed plastic bags at 4°C as batches.
- Pre-warm before use.

1.5 How will you sterilize Thiosulphate Citrate Bile salt Sucrose medium (TCBS)?

- Add all the ingredients to the water and bring to the boil to dissolve. Do not autoclave (as it will destroy the sugar).
- Dispense to sterile plates and allow cooling.

1.6 List the processes used by the laboratory to monitor moist sterilization.
Chemical and biological indicators.

1.7 How will you store these plates until use in the laboratory?
Store in labeled, sealed plastic bags at 4°C as batches.

Sterilization of glassware

A set of glass culture bottles that has been used to culture TB specimens needs to be sterilized before re-use.

2.1 What is the method of decontamination?

- Autoclave.

2.2 List the processes that have to be performed before sterilization.

- Autoclave the used bottles with live culture (at 121 °C temperature, at 15 lb for 15 min).
- Discard the autoclaved culture media with organisms into a bin containing clinical waste.
- Wash the bottles with a detergent (Teepol).
- Wash three times with water.
- Place the bottles inside a wire basket while the lids are loosened. Cover the top of the basket with a craft paper; place an indicator tape on the craft paper to monitor the sterility.

2.3 How will you sterilize the plates and store it?

- Dry heat sterilization by hot air oven (160^0C for 2 hours), store inside a closed cabinet.

2.4 How will you sterilize oils and powders?

- In a hot air oven at 160^0C for 2 hrs as thin layers.

Sterilization of metal items

A pair of forceps used to prepare fungal slide cultures and wire loops used in the bacteriology laboratory needs to be sterilized.

3.1. How will you sterilize the above material?

- Forceps - dip in 70% alcohol and hold to the flame until red hot (before performing the procedure). When the procedure is done, wash with detergent, dry, wrap in aluminium foil, dry heat by hot air oven.
- Wire loops – Hold to the flame until red hot, after use do the same and keep.

3.2. What do you do to the used forceps before sterilizing? Write the steps.

- Wash the forceps with disinfectants.
- Dry.
- Wrap individually using aluminium foil.
- Sterilize in the hot air oven (160^0C for 2 hrs).

3.3. Write few other items which you can sterilize using this method.

- Scissors
- Wire loops
- Glass bottles (only the once without a rubber cap)
- Test tubes

3.4. List the processes used by the lab to monitor this form of sterilization.
Biological and chemical indicators.

4. How will one sterilize or disinfect the following instruments / surface?

4.1 Thermometer.

- Wipe with 70% alcohol, store dry.

4.2 A sample of blood that has been spilt on the lab floor.

- Evacuate the area.
- Cover the blood spill with absorbent material and then pour 10,000 ppm (1%) sodium hypochlorite to soak the absorbent material.
- Leave for 20 minutes and wearing protective gloves remove the absorbent material into a clinical waste bag.
- Clean the area with a general purpose disinfectant.

4.3 Swabs that are used in collecting samples

- Autoclave
- Irradiation

4.4 Glass bottle used to collect urine for culture

- Autoclave

4.5 Used needles

- Incineration

4.6 Working tops of biological safety cabinets

- Bench – Disinfect with 70% alcohol before and after use. If used for virological work, disinfect with 0.1% (1000 ppm available chlorine) sodium hypochlorite.
- UV irradiation before and after use.

4.7 Microbiological waste products before discard

- Autoclave or incineration.

4.8 Molecular biology waste: e.g. Ethidium bromide

- Ethidium bromide solutions- charcoal filtration for chemical deactivation.
- Ethidium bromide contaminated gels, gloves, tips – incineration.

Note: AUTOCLAVING

Autoclaving at pressure of 15 pounds per square inch (psi), at temperature of 121^0C for 15 minutes kill vegetative microorganisms, bacterial endospores, viruses as long as they are not protected by biological material (pus, blood, sputum).

Packaging before autoclaving

Canister lids kept loosely to facilitate circulation of air around the material inside.
Bottle/tube lid keep loosely, covered loosely with foil.
Surgical instruments wrapped in cloth.
Do not ever seal the container before autoclaving.

Quality control in autoclaving

1. Pressure sensitive autoclave tape.

2. Commercially available solutions containing bacterial spores.

Bacteriology
Practical – 5 (a)

Media Preparation

To study about the pathogens and commensal organisms of the human body, they need to be cultivated on artificial culture media. Those media generally provide source of carbon, energy and nitrogen, in the form of available carbohydrates and amino acids. In order to obtain a suitable growth, the artificial medium should provide nutrients and a pH approximating to those of tissues and body fluids. Artificial culture media are available in liquid form (broth), in solid form with addition of agar or other solidifying agents or in semi solid form. These are commonly used in tubes or in Petri dishes.

When a mixture of bacteria is streaked (spread) across the surface of an agar plate, it is diluted out so that single bacterial cells are deposited at certain area on the plate. These single cells multiply at those sites until a visible colony is formed. Each colony represents the growth of one bacterial cell. A single colony can be transformed to another medium and it will grow as a pure culture.

Containers for Media and cultures

Following can be used as containers for media and cultures.

- Test tube stoppered with cotton wool plugs or slip-on metal caps
- Screw capped bottles of different capacity and shape
- Petri Dishes
- Flasks stoppered with cotton wool plugs

Read the following questions and answers.

1. Define a culture medium
A culture medium is an artificial environment that provides sources of carbon, energy and nitrogen in the form of available carbohydrates and amino acids for the growth of bacteria.

2. How can you classify culture media?
Bacterial culture media can be classified in different ways; based on consistency, based on nutritional component and based on its functional use.

A) Classification based on consistency
Culture media are liquid, semi-solid or solid and biphasic.

a) Liquid media

Liquid media are sometimes referred as "broths" (e.g. nutrient broth). These are available for use in test-tubes, bottles or flasks. In liquid medium, bacteria grow uniformly producing a turbidity. Certain aerobic bacteria and those containing fimbriae (Vibrio and Bacillus) are known to grow as a thin film called 'surface pellicle' on the surface of undisturbed broth. *Bacillus anthracis* is known to produce a stalactite growth (spikes hanging from the surface) on ghee containing broth. Sometimes the initial turbidity is cleared due to autolysis, which is seen in penumococci. Long chains of Streptococci when grown in liquid media tend to entangle and settle to the bottom forming granular deposits. Liquid media tend to be used when a large number of bacteria have to be grown. These are suitable to grow bacteria when the numbers in the inoculum is suspected to be low. Inoculating a specimen in the liquid medium also helps to dilute any inhibitors of bacterial growth. This is the practical approach in blood cultures. Culturing in liquid medium can be used to obtain viable counts (dilution methods). Colony morphology of bacteria is not visible in liquid media and presence of more than one type of bacteria cannot be differentiated.

b) Solid media

Any liquid medium can be converted to a solid medium by addition of solidifying agents such as agar-agar, egg yolk or serum. While serum and egg yolk are normally liquid, they can be rendered solid by coagulation using heat. Serum containing medium such as Loeffler's serum slope and egg containing media such as Lowenstein Jensen medium and Dorset egg medium are solidified as well as disinfected by a process called inspissation.

Agar-agar (simply called agar) is the most commonly used solidifying agent. It is an unbranched polysaccharide obtained from the cell membranes of some species of red algae. It melts at 95^{o}C and solidifies at 42^{o}C. Agar incorporated in to media can withstand sterilization by autoclaving. Agar doesn't contribute any nutritive property. It is not hydrolyzed by most bacteria and is usually free from growth promoting or growth retarding substances. However, it may be a source of calcium and organic ions. Most commonly, it is used at concentration of 1-3% to make a solid agar medium. New Zealand agar has more gelling capacity than the Japanese agar. Agar is available as fibers (shreds) or as powders.

c) Semi-solid media

Reducing the concentration of agar to 0.2 - 0.5% renders a medium semi-solid. Such media are fairly soft and are useful in demonstrating bacterial motility and separating motile from non-motile strains (U-tube and Cragie's tube). Certain transport media such as Stuart's and Amies media are semi-solid in consistency. Hugh and Leifson's oxidation fermentation test medium as well as mannitol motility medium are also semi-solid.

c) Biphasic media

A culture system may comprises of both liquid and solid medium in the same bottle. This is known as biphasic medium (Castaneda system for blood culture). The inoculum is added to the liquid medium and when subcultures are to be made, the bottle is simply tilted to allow the liquid to flow over the solid medium. This obviates the need for frequent opening of the culture bottle to subculture and minimize the risk of contaminating the medium. This is mainly used when the culture need to be kept for a long period with sub culturing: i.e. – Fungal blood cultures, Brucella blood cultures.

B) Classification based on nutritional component

Media can be classified as simple, complex and synthetic (or defined). While most of the nutritional components are constant across various media, some bacteria need extra nutrients.

Those bacteria that are able to grow with minimal requirements are called non-fastidious and those that require extra nutrients are said to be fastidious.

Simple media such as peptone water, nutrient agar can support most non-fastidious bacteria. Complex media such as blood agar have ingredients in which the exact components are difficult to estimate. Synthetic or defined media are specially prepared media for research purposes where the composition of every component is well known.

C) Classification based on functional use or application

These include simple/basal media, enriched media, enrichment media, selective media, indicator/differential media, transport media and storage media.

a) Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal media.

b) Enriched media

Addition of extra nutrients in the form of blood, serum, egg yolk or sugars to basal media makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. Blood agar, chocolate agar, Loeffler's serum slope, Dorset's egg medium are some of the enriched media. In most instances, enriched media are non selective.

a) Enrichment media

Enrichment media contain a substance that's specially formulated to enhance the growth of the wanted bacteria. Colony number will be more when compared to the number in the original specimen. It controls the growth of unwanted bacteria. Enrichment media are usually broth media. Examples of enrichment media are Selenite F Broth, Tetrathionate Broth and Buffered Listeria Enrichment Broth.

d) Selective media

These are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. It does not enhance the growth of any bacteria. The colony number is same as that is in the specimen. Selective media are generally agar based. Any agar media can be made selective by addition of certain inhibitory agents that do not affect the pathogen. Various approaches to make a medium selective include addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these. Examples of selective media are MacConkey agar, Blood Tellurite agar and Thiosulphate Citrate Bile salt Sucrose agar.

e) Differential media or indicator media

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Examples: MacConkey agar, Cystine Lactose Electrolyte Deficient (CLED) agar, Thiosulphate Citrate Bile salt Sucrose (TCBS) agar, Xylose Lysine Deoxycholate (XLD) agar etc.

Blood agar is also considered differential because it is used to distinguish pathogenic bacteria based on the effect of bacterial enzymes known as haemolysins which lyse red blood cells. But it is a non selective differential medium.

f) Transport media

Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. When the patient stays away from the laboratory and there is a risk that the pathogen in the clinical specimen may not survive or may be overgrown by the commensals during the time the specimen is transported to the laboratory, a transport medium is used. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's and Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors. Cary Blair medium, 1% alkaline peptone water and Venkatraman Ramakrishnan medium are used to transport feces from suspected cholera patients. Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.

g) Anaerobic media

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients. Media for anaerobes may have to be supplemented with nutrients like haemin and vitamin K. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Robertson Cooked Meat Medium (RCMM) that is commonly used to grow *Clostridium* spps contains a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Before use, the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin. Methylene

blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the thioglycollate medium. Under reduced condition, methylene blue is colourless.

h) Storage media

Prepared sterilized media in individual screw capped bottles as broths or nutrient agar are used to store organisms for further studies or future reference. Poured plates of agar media deteriorate quickly when held at room temperature on bench and are often contaminated. They can only be held for short periods not exceeding 7 -10 days. For longer durations of storage, bijou bottles with agar slants or glycerol in small screw capped tubes are used. Screw caps prevent the evaporation of water and keep the agar slants wet.

3. What are the common ingredients of culture media?

Culture media may be prepared in the laboratory from basic ingredients or it may be purchased ready for use. It is important to have a balance of nutrients. Excess of certain nutrients may actually inhibit the growth of certain organisms. Hence a culture medium should have the following properties.

1. Water
2. Gelling agents
3. Essential nutrients- proteins, peptides, amino acids
4. Energy- carbohydrates
5. Essential metals and minerals
6. Buffering agents
7. Indicator substances
8. Selective agents
9. Other components

Water

This is essential for the growth of all micro-organisms. Tap water is often suitable for culture media. It must be free from any chemicals that inhibit bacterial growth. If the local water supply found unsuitable, glass distilled or demineralized water should be used. Small amounts of copper are highly inhibitory to bacterial growth, so that copper distilled water should not be used for preparation of media.

Gelling agents

Agar-agar (simply called agar) is the most commonly used solidifying agent. It is an unbranched polysaccharide obtained from the cell membranes of some species of red algae. It melts at 95^{o}C and solidifies at 42^{o}C. So it remains un-melted at all incubation temperatures. Agar doesn't contribute any nutritive property. It is being decomposed or liquefied by only few varieties of bacteria and is usually free from growth promoting or growth retarding substances. However, it may be a source of calcium and organic ions. Most commonly, it is used at concentration of 1-3% to make a solid agar medium. New

Zealand agar has more gelling capacity than the Japanese agar. Agar is available as fibers (shreds) or as powder.

Peptones
This is a general term for the water-soluble products obtained from the breakdown of animal or plant proteins such as heart muscle, fibrin, soya flour or casein.
Peptones provide nitrogen for growing organisms. Apart from the standard grade bacteriological peptone, some manufacturers supply special grades of peptone recommended for particular purposes (Mycological peptone).

Meat extract
These provide organisms with a further supply of amino acids, and also with essential growth vitamins and minerals including phosphates and sulphates.

Yeast extract
This gives a wide range of amino acids, growth factors, carbohydrates and inorganic salts. This may be substituted from meat extract in culture media.

Carbohydrates
Simple or complex sugars are added to many culture media, to provide bacteria with sources of carbon and energy. Carbohydrates are also added to media to assist in the differentiation of bacteria.
e.g. Lactose is added with an indicator to MacConkey agar and Deoxycholate Citrate agar to differentiate lactose fermenting and lactose non fermenting species of Enterobacteriaceae.

Essential metals and minerals
For cell growth, sulphates are required as sources of sulphur and phosphates are sources of phosphorous. Culture media should also contain traces of magnesium, potassium, iron, calcium and other elements which are required for bacterial enzyme activity. NaCl is also an essential ingredient of most culture media.

3. Describe how you sterilize the petri dishes, glass tubes & other containers, which are used to distribute culture media.

Heating by exposure to hot air is an accepted method for sterilizing loads that can not be reliably penetrated by steam and can tolerate the high temperatures required.
e.g. 160 °C for 2hrs

Method
1. Ensure articles are thoroughly clean and dry.
2. Stainless steel canisters, aluminium foils, wrappings of craft papers and stoppers of cotton wool may be used to retain sterility after processing the items.

3. Plug glass test tubes with cotton wool stoppers and place them vertically in metal baskets. Cover the top of the metal basket with an aluminium foil or a piece of craft paper. Do not put screw capped bottles in a hot air oven unless their caps and liners are made of a material that will resist distortion at the sterilizing temperatures in a hot air oven.
4. Flasks are also plugged with cotton wool stoppers and the top is covered with a piece of craft paper or aluminium foil.
5. Position the individual articles to allow free circulation of hot air between and around the items.

Sterilizing cycle

1. Set the controls to ensure that the sterilization hold time does not start until the sterilizing temperature, detected by thermocouples placed on the load, has been reached.

2. Set the sterilization hold time to160°C for 2 hours, or 170°C for 1 hour or 180°C for 30 minutes.

Cooling

1. Do not attempt to open the chamber door until the chamber and load have been cooled to below 80°C as glassware is liable to crack if cold air is admitted suddenly while it is still very hot.
2. Use protective gloves to remove items from the oven.

Glassware for media such as Koser's citrate, in which there is a single source of element as carbon or nitrogen must be chemically clean. A recommended method of ensuring this is to boil all tubes/bottles in 20% nitric acid for 5 – 10 minutes and then wash and rinse well with glass distilled water. Tubes/bottles are dried in an oven in the inverted position in baskets lined with filter or blotting paper to prevent the mouths of tubes/ bottles touching the metal.

4. Why are culture media sterilized before use? Describe the method you use to sterilize.

Culture media used in the isolation of pathogenic micro organisms or for the study of micro organisms should be free from any micro organisms that could affect the interpretation of the results. So they should be sterilized before use.

The choice of method to be used to sterilize a medium depends on whether the ingredients are decomposed by heat or not. If autoclaving will not damage the medium, it is the best method of sterilization. Follow manufacturer's instructions before you sterilize each culture medium.

Autoclaving

In this method of sterilization, pressure is used to produce high temperature steam. It is based on the principle that, when water is boiled at an increased pressure, the temperature at which it boils and of the steam that it forms, rises. Hot saturated steam rapidly penetrates and gives up its latent heat when it condenses on cooler objects.

Autoclaving is used to sterilize most agar and fluid culture media. It ensures the destruction of vegetative cells as well as bacterial endospores by coagulating and denaturing microbial proteins and enzymes.

Method

1. Add the correct volume of water to the autoclave.
2. Place the wire baskets containing bottles or tubes of culture media, with caps loosened, in the inner chamber of the autoclave. Do not overload.
3. Secure the lid of the autoclave, open the air-cock, and close the draw-off knob.
4. Adjust the safety valve to the required pressure i.e. 10psi to give a temperature of 115°C or 15psi to give 121°C. Larger volumes of media require longer sterilization times. Sometimes lower temperatures such as 115^0C for times ranging 10- 20 minutes are recommended for sterilization of media containing ingredients that are not very stable to heat.
5. Apply heat electrically or use gas or a primus stove.
6. When all the water droplets have been expelled and only steam is emerging, wait for 1 minute, and closes the air-cock. This will cause the pressure to rise and with it, the temperature of the steam.
7. When the required temperature has been reached, and the excess steam begins to be released from the safety valve, reduce the heat and begin the timing.
   Most culture media are sterilized at a holding time of 15 minutes.
8. At the end of the sterilizing time, turn off the heat, and allow the autoclave to cool naturally.
9. Check that the pressure gauge is showing zero. When at zero, open the air-cock and then wait for a few minutes before opening the lid to allow time for the autoclave to become fully vented.
10. After removing the culture media, tighten the caps of bottles.

A bacteriological method of monitoring the performance of an autoclave is to use a strip of filter paper impregnated with spores of *Bacillus stearothermophilus* as an indicator with the sterilizing load and to see whether the organisms grow or not when plated. If the organisms grow, the process of sterilization is unsatisfactory.

Other methods used to sterilize culture media, are steaming at 100°C on several occasions (Tynderlization) and filtration.

Steaming at 100°C on one occasion or several occasions

This is used to sterilize media containing ingredients that would be broken down or inactivated at temperatures above 100°C. This is not a definite way of sterilizing media.
Steaming can be performed in a steam sterilizer, such as an Arnold or Koch steamer. The bottles of media with loosened caps are placed on perforated trays above the boiling water. During boiling all the vegetative cells will be destroyed but not necessarily all the spores. Upon keeping the spores will germinate to form vegetative cells and on subsequent boiling they will also be destroyed. After sterilization, when the medium has cooled, the caps of the bottles are tightened.

Filtration
This provides a means of removing bacteria from fluids. It is used mainly to sterilize additives that are heat sensitive and cannot be autoclaved, or less stable substances that need to be added to a sterilized medium immediately before it is used. Several different types of filters can be used, including those made from sintered glass, asbestos or inert cellulose esters.

5. How do you decontaminate used culture media plates?
All cultures to be discarded must render non infectious by sterilization, even if they are apparently 'negative'. Sterilization is equally important for cultures in reusable containers or in disposable containers such as plastic petri dishes that may be incinerated, as it is of paramount importance that no living infective material leaves the laboratory.

There are 3 practical methods by which cultures may be sterilized.

- Incineration
- Autoclaving
- Chemical disinfection

Autoclaving

Discarded culture plates are usually first collected in stainless steel pails or in autoclavable plastic bags held in pails. They are best autoclaved open, in multipurpose autoclaves with high pre-vacuum or vacuum pulsing to remove all air from the plates, before exposure to pure stream under pressure. Care must be taken to ensure that molten agar does not escape into the chamber drain, which would block the drain when the agar cools.

If a gravity displacement autoclave is used the discard pails must be designed to allow the downward displacement of air from the load. A stainless steel pail with a wire mesh shelf about 4 cm above the bottom and a row of four holes 1 cm in diameter, in the sides just below it is suitable. The holes permit discharge of air and the melted agar and spilt liquids collect in the bottom of the pail. To prevent spillage before autoclaving, the pail must be kept in a shallow solid box or dish while standing in the laboratory and being carried to the autoclave. Closed petri dishes must be placed on their edges in the pail, to assist air discharge.

Cultures are non infectious after autoclaving at 121^0C at 15lb for 15 minutes and can be discarded into a bin containing clinical waste.

Chemical disinfectants are used to disinfect instruments used in the microbiology laboratory. Five percent phenol or 1% hypochlorite is used to disinfect instruments contaminated with organisms (Pasteur pipettes, glass slides, coverslips). When there is a spillage of blood, 10% hypochlorite solution is used.

6. Describe the way you wash the used plates and bottles step by step.

Autoclave the used plates and bottles

↓

Wear protective heavy duty gloves

↓

Remove the agar from the plates manually

↓

Wash Petri dishes and bottles with soap and tap water

↓

Rinse with distilled water

↓

Dry in moderate heat

Petri dishes

Put the Petri dishes into a canister or wrap them in foil paper

↓

Put them into the hot air oven at 160°C for 2 hours or 180°C for 1 hour

Bottles

Loosen the caps of the bottles

↓

Put the bottles in a wire basket

↓

Autoclave the bottles at 121^0C at 15 lb for 15 minutes

↓

Tighten the caps when taken out of the autoclave

7. What is the method you use to store the prepared culture media?

Prepared sterilized media in individual screw capped bottles can be stored at room temperature for weeks, but some deterioration is likely to occur. It is essential to have some form of cold storage in the laboratory for the preservation of blood, serum and culture media.

For a smaller laboratory, one of the domestic refrigerators of 1-2 m^3 capacity is suitable. Larger laboratories require a corresponding larger cabinet, insulated cold room with the refrigerating plant outside. The temperature should be maintained between 4 - 5^oC. It should never be low as freezing since this may be detrimental.

Plates of agar media can be kept for several days not exceeding 7- 10 days at room temperature or in a refrigerator. However when kept at room temperature, changes in hydration with concentration of ingredients may occur, the pH may alter and bacterial or fungal contamination may occur.

When stored in the refrigerator, care should be taken to prevent evaporation of water from the medium. This can be achieved by packing the plates in plastic re-sealable bags. When storing in containers, it is advisable to store them in a screw capped container than a cotton plugged container to minimize evaporation.

Strong light is detrimental to most media and storage in a dark place is preferable especially for those containing dyes.

Egg media should be kept stored for long periods (about 2 weeks) before use to provide the opportunity to the contaminants that grow only at room temperature to develop visible colonies.

Bacteriology
Practical – 5 (b)

Culture Procedures and Colony Morphology

Culture procedures are performed to isolate pathogens from sterile sites or non sterile sites. Even from a sterile site you may get a mixture of organisms. It is important to get a pure growth of organisms when we go for the identification and antibiotic sensitivity of them. The effort in this chapter is to give you the information of obtaining isolated colonies and pure cultures of organisms during cultivation of them on artificial media.

You should wash your hands thoroughly with soap and water after handling bacterial cultures and as and when your hands get contaminated with bacterial cultures or chemicals.

Instruments used to seed culture media

- Wire loops – Original type of inoculating wire was platinum. But nichrome wires are widely used now. They are re-usable. The wire is sterilized by holding it almost vertically in a Bunsen flame until the entire wire become red-hot and is used to pick colonies once it is cooled. Plastic disposable wire loops of different diameters are available for use now. Wire loops are used to pick and spread colonies on agar media, to transfer and spread clinical specimens on agar surfaces and to transfer colonies into broth media.
- Straight wires – Used for stab cultures or picking off single colonies (Sterilize using a Bunsen flame before use).
- Sterilized wooden sticks – Used for spread inoculums on agar surface.
- Pipettes – Used to inoculate agar media and broth media.
- Bunsen flame – Used to sterilize wire loops, straight wires and tips of the pipettes and mouths of the containers used in the procedure.
- Agar media/broth media – Pre-sterilized and use to inoculate specimens and colonies of organisms.

The bench used for inoculation should be free from dust and wiped with a disinfectant at least before the start, when contaminated with clinical material or cultures of organisms and at the end of each day's work.

Air currents should be minimized by closing doors and windows and restricting movements of people in the room during procedures.

During inoculation, culture medium should be uncovered only for few seconds.

Bunsen burner and the inoculating instruments should be placed on the side of your dominant hand on the bench and the cultures and media to the back and on the opposite side of the bench.

Plate cultures and unseeded plates should be placed with the lid on the bench and the bottom containing the medium upper most. Caps or plugs of tubes and bottles should be loosened for easy removal.

Media to be seeded should be labeled indicating the date, inoculums/specimen number with a glass marking pen, pencil or self-adhesive label. Labeling should be done on the bottom of the Petri dishes, on bottles and on tubes rather than on lids or caps which can be mistakenly placed on other cultures. Labels should be checked for accuracy while media are seeded.

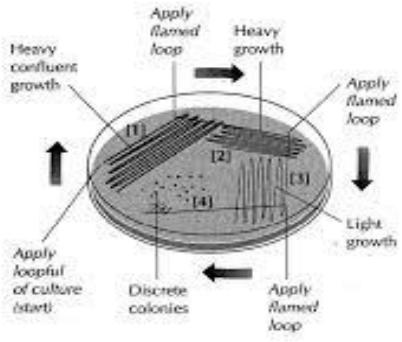
Seeding agar plates

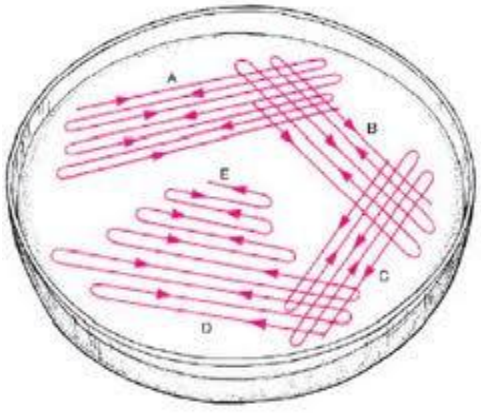
This can be done manually or using machines when automated procedures are used.

In the manual method, it is better to use a loop to seed a plate as straight wires tend to cut agar surface. Lift the bottom of the Petri dish containing medium from its lid with your non dominant hand and hold it round with your thumb and middle finger. Using your dominant hand take a source of microorganisms and spread the material using a sterilized wire loop over a small area of the agar medium to give a "well-inoculum" or "heavy inoculum". Re-sterilize the wire loop and draw about 3-4 parallel lines from the well inoculum outwards on to the fresh agar surface. This process is repeated to complete 4 sets of parallel lines remembering to re-sterilize the wire loop intermittently to obtain single isolated colonies.

Another quicker method of obtaining isolated colonies is to use a 4 mm wire loop after spreading the heavy inoculum. Sterilize the loop and use one side of the loop to spread the heavy inoculum on to fresh agar and then the other side as a fresh sterile surface to do the next spread.

The isolated colonies will allow you to observe specific characteristics such as colony size, shape and color. If special media are you used, you can also determine other characteristics such as haemolysis (if streaked on blood agar) or ability to ferment lactose (if MacConkey agar is used).



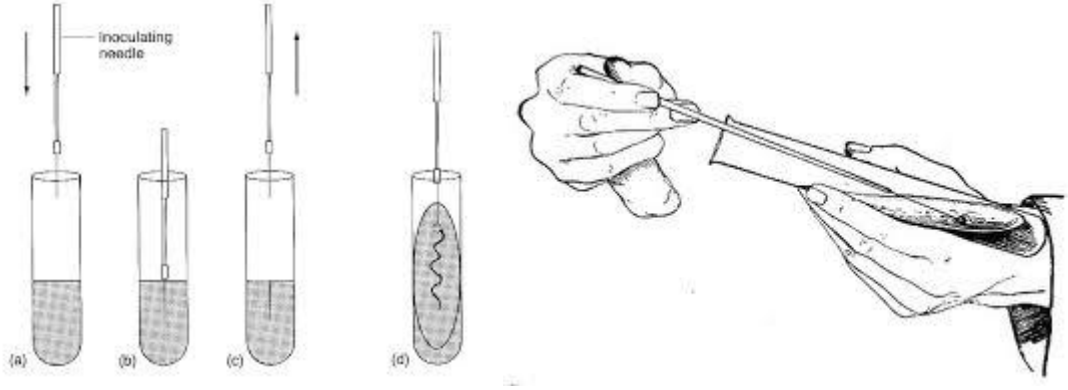


Seeding a tube fitted with a plug or cap or a bottle with a screw cap

It is unwise to attempt to manage more than one tube/bottle and its cap at a time. When the tubes are fitted with cotton wool plugs the mouths of the tubes should be flamed before and after the procedure as the rims of the tubes are not covered by plugs and probably would get contaminated during replacing plugs as organisms could pushed down from the rim into the tube.

When you need to seed the agar surface of the tube/bottle, pick up the tube/bottle with your non-dominant hand and remove the plug or the cap with the crooked 4^{th} and 5^{th} fingers of your dominant hand while holding the charged wire loop with thumb, index and middle fingers of your dominant hand.

Stab cultures in solid media are inoculated by plunging the straight wire into the center of the medium and withdrawing it in the same line to avoid splitting the medium. Slants of solid media are seeded by lightly smearing the surface of the agar with a loop or a straight wire in a zigzag manner taking care not to cut the surface of the agar. Liquid cultures are usually inoculated from a culture on a solid medium by inclining the tube/bottle at an angle of about 45^0 and depositing the inoculum on its wall above the surface of the liquid at its lower end, so that when the tube/bottle is returned to the vertical position, the inoculums is below the surface of the liquid.



After inoculation, withdraw the loop, wire or the pipette, re-sterilize them or place in a discard jar. Flame the mouth of the tube and replace the cap or the plug. Incubate the plates, tubes and bottles as needed, and check 18-24 hours later for growth.

Seeding several tubes and bottles of media from a single colony

For the identification of Enterobacteriaceae we have to use a series of liquid and solid media with different ingredients. It is best to use a loop to pick a single isolated colony and suspend it in saline. Using a sterile Pasteur pipette put a drop into each of the tube. Straight wire is used to stab media such as Kligler Iron (KIA) agar, Lysine Indole Motility (LIM) medium, Citrate and Urea butts.

Incubation conditions

Generally incubation of cultures at 35^0C to 37^0C is the practice to isolate bacteria pathogenic to human. Slightly different temperatures may be required to culture some bacteria and show different characteristics of them. Eg. 43^0C for Campylobacter, 30^0C for Leptospira, 22^0C for the demonstration of motility in Listeria and 44^0C to differentiate Enterococci.

Most of the pathogenic bacteria grow aerobically, but species such as *Streptococcus pneumonia* and *Neisseria gonorrhoeae* needs 5-10% CO_2 for their better growth. Furthermore species such as *Brucella abortus* need extra CO2 for their growth. To obtain CO_2, dedicated CO_2 jars or CO_2 generating kits are used. Certain species like *Campylobacter jejuni* need microaerophilic conditions with 5-7% of O_2 in the atmosphere. This could be achieved by using special gas packs which are commercially available. Anaerobic bacteria need anaerobic atmosphere for their growth. Anaerobic jars or anaerobic cabinets are used to achieve this.

For the air and CO_2 to enter into the tubes, caps should be loosened during incubation.

1. Colony morphology

Colony morphology will vary with the medium on which the organism is grown. But it will give an idea the organism in question.

Differentiating colonies:

i. Colony morphology gives important clues as to the identity of their constituent microorganisms.
ii. Important classes of colony characteristics include;
    1. Size
    2. Shape
    3. Surface
    4. Margin
    5. Elevation
    6. Texture
    7. Translucency (Clear, translucent or opaque)
    8. Colour
    9. Pigmentation
    10. Changes brought about in the medium (Haemolysis)

a) Colony size

- Colony size is dependent not just on the type of organism but also on the growth medium and the number of colonies present on a plate (colonies tend to be smaller when greater than as certain amount are present) and on culture medium characteristics.

- **Usually stabilizes after few days**
  i. Colony size usually stabilizes after a day or two of incubation.
  ii. Exceptions include,
    1. Slow growing microorganisms
    2. During growth under conditions that promote slow growth

b) Shape of the colony

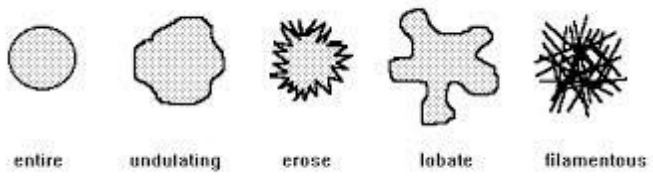
In most of the instances colonies are circular or oval. Colonies of *Corynebacterium diphtheriae* biotype gravis may appear as radially striated colonies (Daisy head).

c) Surface

Can be smooth or irregular. Colonies of *Streptococcus pneumonia* are depressed centrally in. After 48 hours of anaerobic incubation, *Actinomyces israelii* will appear as molar tooth colonies.

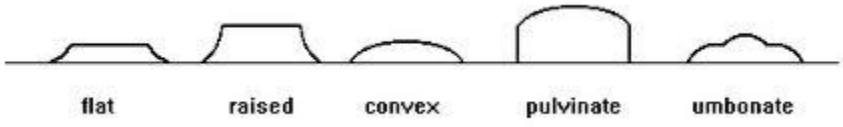
d) Margin/Edge

- Colonies can vary in their margins.
- See illustration below.



e) Colony elevation

Colonies can vary in their elevations according to types of microorganisms and growth conditions.



f) Colony texture

- **Surface appearance**
  Colonies can vary in their texture. Possible textures include

    1. Shiny to dull
    2. Smooth to wrinkle
    3. Rough
    4. Granular
    5. Mucoid

- A shiny, smooth, and/or mucoid appearance tends to be associated with the presence of capsular material.

g) Colony light transmission

a. The *light transmission* through colonies can range from
    i. Complete (transparent)
    ii. Through intermediate (translucent)
    iii. Through completely lacking (opaque)

2. Colony pigmentation

Majority of the colonies are none pigmented where as some give characteristic pigmentation. For example, *Serratia marcescens* gives a non diffusible red pigment; *Pseudomonas* species give a range of diffusible pigments such as brown, yellow-green, red and blue-green.

Bacteriology
Practical – 6

Biochemical Testing

Oxidase test

Principle

This test indicates the presence of enzyme cytochrome oxidase. When the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.

Required items

Freshly prepared tetramethyl-p-phenylenediamine dihydrochloride (oxidase) reagent

To make 10 ml

Tetramethyl-p-phenylenediamine dihydrochloride	0.1 g
Distilled water	10 ml

Dissolve the chemical in the water. The reagent is not stable. It is therefore best prepared immediately before use.

Procedure

Place a piece of filter paper in a clean Petri dish and add 2-3 drops of freshly prepared oxidase reagent.

Using a piece of sterile stick or glass rod (not an oxidized wire loop), remove a colony of the test organism and smear it on the filter paper.

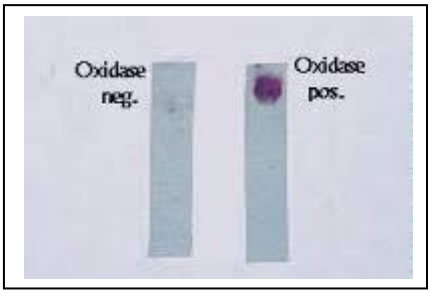
Look for the development of a blue-purple colour within a few seconds.

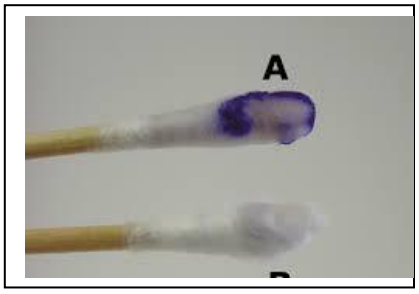
Same can be done using a sterile cotton bud impregnated with the oxidase solution.

Results

Blue-purple colour - Oxidase positive
(Within 10 seconds)

No blue-purple colour - Oxidase negative
(Within 10 seconds)





Controls

Positive control - *Pseudomonas aeruginosa*

Negative control - *Escherichia coli*

Carbohydrate fermentation test

Principle

The identification of some bacteria is based on that what nutrients the bacteria can utilize and the end products produced in the process. These characteristics are controlled by the enzymes which the bacteria produce. The pattern of sugars fermented may be unique to a particular species or strain as the type of enzyme(s) produced by bacteria is genetically controlled. Fermentation products are usually acid (lactic acid, acetic acid etc.), neutral (ethyl alcohol etc.), or gases (carbon dioxide, hydrogen, etc.).

Procedure

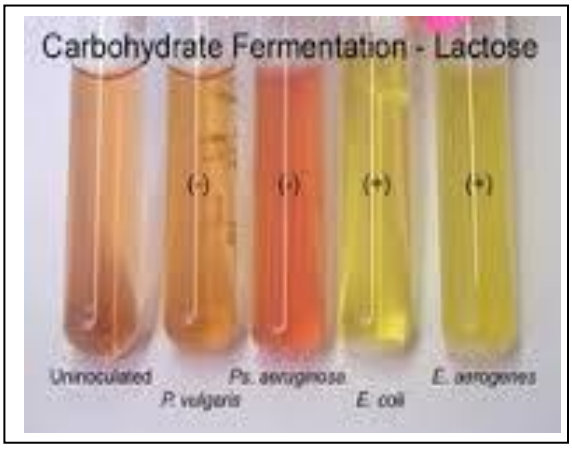
Prepare a carbohydrate fermentation broth at pH 7.4. This broth contains 3 essential ingredients: 0.5%-1.0% of the carbohydrate to be tested (e.g. lactose or glucose), nutrient broth, and the pH indicator phenol red or Andrade's indicator. The nutrient broth supports the growth of most organisms whether they are able to ferment the sugar or not.

The test organism is inoculated into a broth containing the test sugar and incubated. A bright reddish pink colour (in the presence of Andrade's indicator) indicates the production of acid products from fermentation of the sugar to drop the pH to 6.9 or less. A yellow colour (in the presence of phenol red indicator) indicates the production of acid products from fermentation of the sugar.

Results

Yellow colour growth - Fermentation of acid - Positive results
Light red colour growth - No fermentation of acid - Negative results
} With Phenol red indicator

Bright reddish pink colour growth - Fermentation of acid - Positive results
Light red colour growth - No fermentation of acid - Negative results
} With Andrade's indicator



Indole test

Principle

Certain bacteria are able to breakdown the amino acids tryptophan into indole. The test organism is cultured in a tryptophan containing medium and indole production is detected by Kovac's reagent which contains (p)-dimethylaminobenzaldehyde. This reacts with the indole to produce a red colour compound.

Procedure

Inoculate peptone water (or any other tryptophan containing media) with the test organism.

Incubate at 35-37°C overnight.

Add 0.5 ml of Kovac's reagent down the inner wall of the tube.

Shake well and examine about 1 minute later. A red colour in the reagent layer indicates the production of indole.

Results

Bright red colour ring - Indole positive

No colour change - Indole negative

Controls

Positive control - *Escherichia coli*

Negative control - *Enterobacter cloacae*

Methyl Red (MR) test

Principle

The test detects the ability of some organisms to produce sufficient acidity from the glucose fermentation, after prolonged incubation, overcoming the pH buffering system of the medium.

Procedure

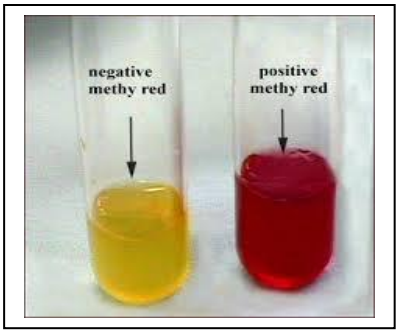
Inoculate a colony of the test organism in 2 ml of sterile glucose phosphate broth.

Incubate at 35-37°C overnight.

Add 5 drops of Methyl Red solution.

Mix and read immediately.

Appearance of a red colour at the surface indicate a positive reaction.



Results

Bright red colour - Methyl Red positive

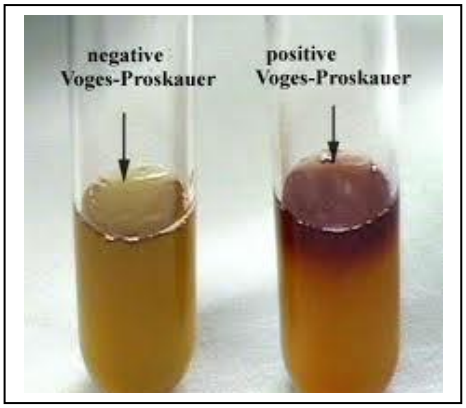
Orange / Yellow colour - Methyl Red negative

Controls
Positive control - *Escherichia coli*
Negative control - *Enterobacter cloacae*

Voges-Proskauer (VP) test
Principle
Some organisms produce acetoin (acetylmethylcarbinol), a neutral-reacting end product, as the main end product of the glucose fermentation. Acetoin is oxidized into diacetyl in the presence of atmospheric oxygen and alkaline condition which forms a pink compound with the creatine.

Procedure
Inoculate the test organism in to 2 ml of sterile glucose phosphate peptone water.
Incubate at 35-37°C for 48 hours.
Add 0.6 ml of α-naphthol solution followed by 0.2 ml of 40% KOH aqueous solution.
Shake well for maximum aeration.
Remove the bottle cap and leave for 1 hour at room temperature.
A pink – red colour indicates a positive reaction.

Results
Pink-red colour -VP positive
No pink-red colour -VP negative



Controls
Positive control - *Klebsiella pneumoniae*
Negative control - *Escherichia coli*

Citrate test
Principle
The test detects the ability of an organism to utilize citrate as the sole carbon and energy source and ammonia as sole source of the nitrogen.

Procedure
Make a light suspension of the organism in sterile water or saline.
Inoculate Koser's or Simmons' citrate medium using a sterile straight wire.
Incubate at 35-37°C for up to 7 days, checking daily for growth.
In Koser's medium look for the turbidity; in Simmons' medium look for a colour change.

Results

Simmon citrate

*Blue colour and growth on the streak line - Citrate positive

*No colour change and no growth - Citrate negative

Koser's Citrate

*Medium becomes turbid – Citrate positive

*No turbidity - Citrate negative

Controls

Positive control - *Klebsiella pneumonia* sub species *aerogenes*

Negative control - *Escherichia coli*

Urease test

Principle

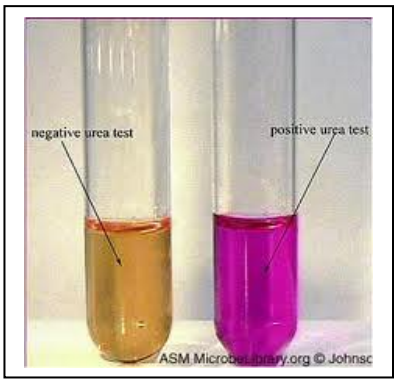
The test detects the ability of an organism to produce enzyme urease. The test organism is cultured in a medium which contains urea and the indicator phenol red. If the organism produces urease, the enzyme will breakdown the urea to ammonia and carbon-dioxide. The medium becomes alkaline when ammonia is released which is indicated by the red-pink colour.

Procedure

Inoculate a tube of urea medium (broth and agar both available) with a smooth colony of the test organism by using a sterile wire.

Stopper the tube and incubate at 35-37°C overnight.

Examine for urease production by red-pink colour of the medium.



Results

Red-pink medium - Urease positive

No red-pink colour - Urease negative

Controls
Positive control - *Proteus vulgaris*
Negative control - *Escherichia coli*

Phenylalaninedeaminase test / Phenylpyruvic acid (PPA) test
Principle
The test is based on the ability of organism to breakdown phenylalanine by oxidative deamination to produce phenylpyruvic acid. Phenylpyruvic acid is detected by adding $FeCl_3$ which produces a green colour on the surface of the culture.

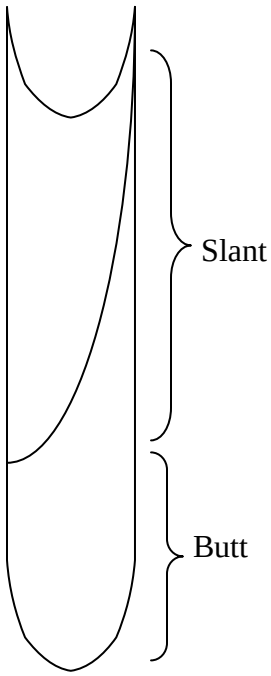
Procedure
Inoculate the slope of phenylalanine agar with the test organism.
Incubate at 35-37°C overnight.
Add 0.2 ml of freshly prepared 10% aqueous solution of $FeCl_3$ to the culture.
Allow the reagent to run down the slope.
Watch immediately.
A positive reaction is indicated by a dark green colour which fades quickly.

Results
Green colour (within 5 minutes) - Positive test
No green colour - Negative test

Controls
Positive control - *Proteus vulgaris*
Negative control – *Klebsiella pneumoniae* sub species *pneumoniae*

Kligler Iron Agar (KIA)
Principle
KIA is used to look for the ability of an organism to ferment lactose and glucose and to produce hydrogen sulphide. It is further used to check the production of gas while fermentation of glucose and lactose. There is an indicator (phenol red) which will change the colour of medium when acid is produced.
Ratio of lactose to glucose is 10:1 in the medium.
An alkalinity is produced in the slant due to the oxidative deamination of the amino acids. The organism that has the ability to ferment glucose changes the colour of the butt to yellow. But the acidity produced by glucose is not sufficient to overcome the alkalinity

produced by oxidative deamination of the amino acids in the slant and change the colour of the slant.
The organism that has the ability to ferment lactose changes the colour of the butt as well as the slant.



Un-inoculated medium is light orange.

Different patterns in KIA

1. Un-changed slant and butt: No fermentation of glucose or lactose.
   This is seen with most strains of *Acinetobacter*.

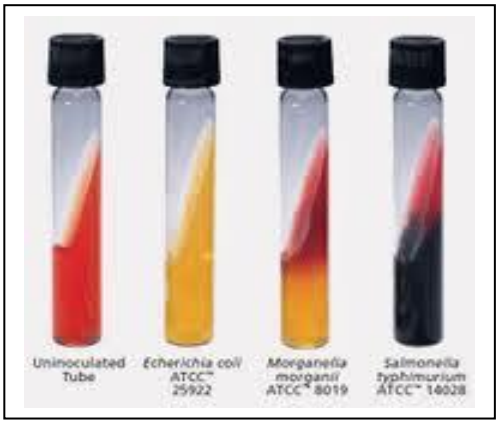
2. Red slant with unchanged butt: Oxidative deamination of amino acids and no fermentation of glucose or lactose.
   This is seen with most strains of *Pseudomonas*.

3. Red slant and yellow butt: Oxidative deamination of amino acids, fermentation of glucose and no fermentation of lactose.
   This is seen with most strains of non lactose fermenting Enterobacteriaceae.

4. Yellow slant and yellow butt: Fermentation of lactose and glucose.
   This occurs with *Escherichia coli* and other lactose fermenting enterobacteriaceae.

5. Cracks and bubbles in the medium: Gas production from glucose fermentation.

6. Blackening along the stab line or throughout the medium: Production of hydrogen sulfide.
   Salmonella Typhi produces small amount of blackening whereas other *Salmonella* species and few other species of enterobacteriaceae causes extensive blackening.



Oxidation-Fermentation (OF) test

Principle

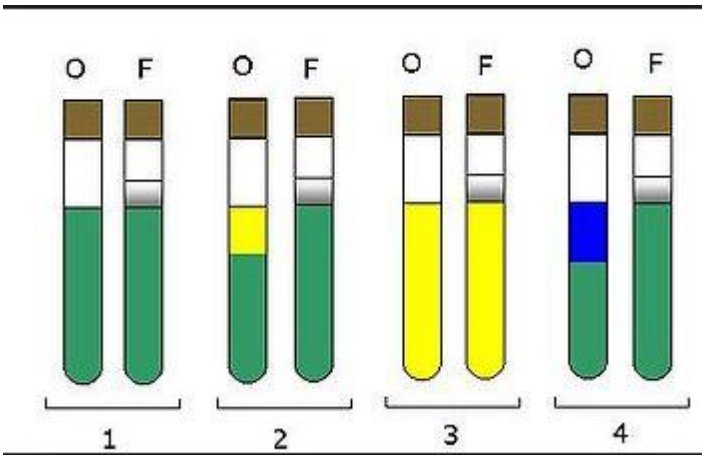
The test is used to differentiate those organisms that oxidize carbohydrate (aerobic utilization) from those organisms that ferment carbohydrate (anaerobic utilization).
Fermentative organisms utilize the carbohydrate in both the open and sealed tubes as shown by a change in colour of the medium from green to yellow. Oxidative organisms are able to utilize the carbohydrate only in the open tube.
Note: Tubes contain tryptone or peptone agar medium containing carbohydrate (glucose or other carbohydrate) and the indicator is bromothymol blue.

Procedure

Inoculate the test organism to the bottom of two tubes of sterile O-F medium (always use a heavy inoculum).
Cover the inoculated medium of one tube with a 10 mm deep layer of sterile paraffin oil or molten wax.
Incubate the tubes at 35-37°C for 14 days.
Examine daily for carbohydrate utilization.

Results

Open tube	Sealed tube	Interpretation
Yellow	Green	Oxidative organism
Yellow	Yellow	Fermentative organism
Green/Blue	Green	No utilization of carbohydrates



Controls

Oxidative control - *Pseudomonas aeruginosa*

Fermentative control - *Escherichia coli*

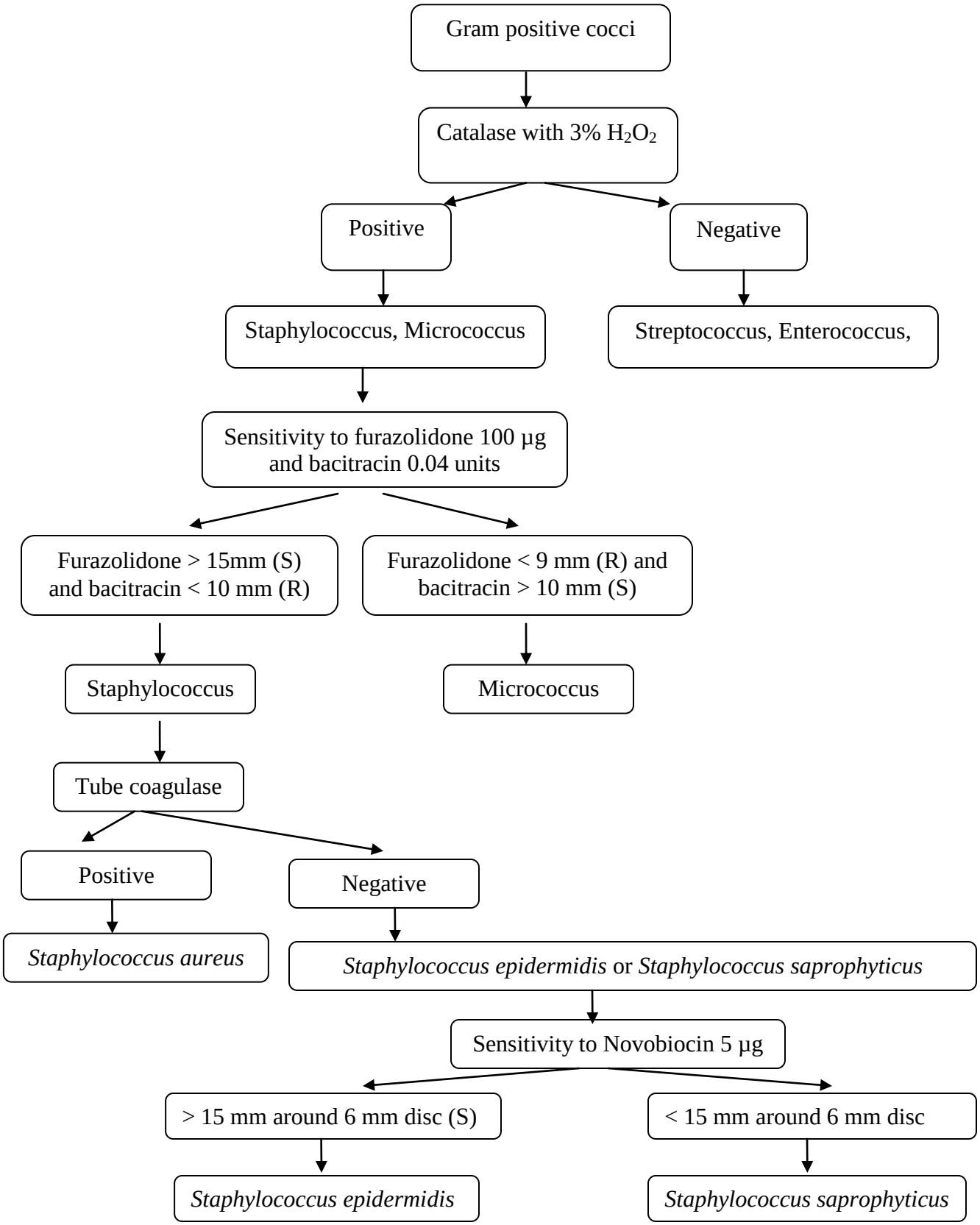
Bacteriology
Practical – 7 and 8

Identification of Gram positive cocci

Question 1
Observe the colony characteristics of the organisms on different culture media and note them down. Note down the Gram stain characteristics of each of them.

Species	Gram stain characteristics	Colony morphology on blood agar	Colony morphology on chocolate agar	Colony morphology on MacConkey agar
Staphylococcus aureus				
Staphylococcus epidermidis				
Staphylococcus saprophyticus				
Streptococcus pyogenes				
Streptococcus agalactiae				
Streptococcus pneumoniae				
Viridans group Streptococci				
Enterococcus species				

Key to identify Gram positive cocci



Catalase test

Principle

Catalase test will help to differentiate those bacteria that produce the enzyme catalase such as staphylococci from non-catalase producing bacteria such as streptococci. This enzyme catalyses the release of oxygen from H_2O_2.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

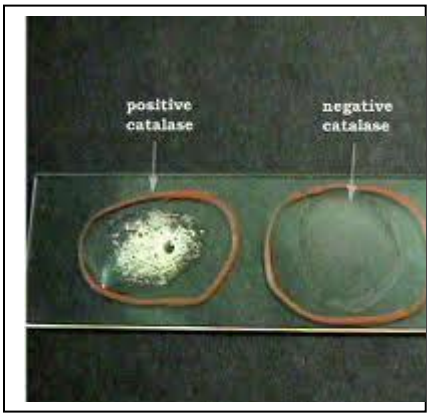
Negative and positive control should be included with the test sample.

False positive reaction can occur when using organisms from blood agar plate or when an iron wire loop is used.

Procedure

There are three methods.

1. Slide method
2. Tube method
3. Plate method



Slide method

Using a sterile wooden stick, plastic wire loop or glass rod take a little amount of colony and place on a clean glass slide.

Put a drop of 3% H_2O_2 on to the colony.

Look for immediate bubbling.

Tube method

Collect 1 ml of 3% H_2O_2 in to a test tube.

Immerse the test colony in to the 3% H_2O_2 in to a test tube by using a sterile glass rod, plastic wire loop or a wooden stick.

Look for bubbling.

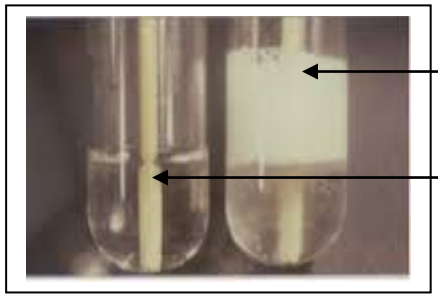


Plate method

Flood or pour 3% H_2O_2 on to the test colony on the culture plate.

Look for bubbling.

Do positive and negative controls with the test.

Blood agar and other blood containing media are not suitable for

the test.

Results
Active bubbling - Positive test
No release of bubbles - Negative test

Controls
Positive control - *Staphylococcus aureus*
Negative control - *Streptococcus pyogenes*

Coagulase test

Principle
Coagulase is an enzyme mainly found in *Staphylococcus aureus*. Therefore this test will help to distinguish *Staphylococcus aureus* from other commonly isolated *Staphylococci*. Two forms of coagulase exist. One is bound to the cell and other one is excreted from the cell as an enzyme. Bound coagulase is known as "clumping factor" which acts directly on the fibrinogen in plasma and convert it to fibrin to form a coagulum. When the coagulase is released as an enzyme, also called "free coagulase", reacts with the coagulase reacting factor (CRF) in plasma to form thrombin that then acts on fibrinogen in the plasma to form a fibrin clot.

$$\text{Fibrinogen} \xrightarrow{\text{Coagulase}} \text{Fibrin}$$

EDTA blood is used. Citrated blood may give false positive result.

Procedure
Slide method
Place two separate drops of physiological saline on a clean slide.
Emulsify a colony and make two thick suspensions.
If there is no auto agglutination add a drop of plasma to one of the suspensions and mix gently.
Look for clumping within 10 seconds in the plasma added suspension.
If auto agglutination is present this slide test cannot be read.

Tube test

Method 1

Prepare 1:10 diluted plasma in physiological saline.
Add 0.5 ml of diluted plasma in a tube.
Add 0.1 ml of an 18-24 hour broth culture of the organisms.
Mix gently and incubate at 35-37°C.
Examine for formation of a coagulum after 1 hour; if no coagulum, examine at 2 and 6 hours.
Negative tubes should be left at room temperature over night and re-examined.
Use negative and positive controls.

Method 2

Mix 0.5 ml undiluted plasma with an equal volume of an 18-24 hour broth culture. Incubate at 37^{o}C for 4 hours.
Examine after 1 and 4 hour for coagulum.
Negative tubes should be left at room temperature overnight and then re-examine.

Results

Formation of coagulum - Positive test
No coagulum - Negative test

Controls

Positive control - *Staphylococcus aureus*
Negative control - *Staphylococcus epidermidis*

Deoxyribonuclease (DNase) test

Principle

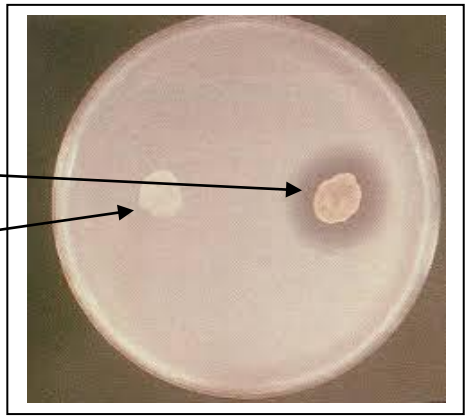
Deoxyribonuclease hydrolyses DNA. The test organism is cultured on a DNA containing medium. After overnight incubation, the colonies are tested for DNase production by flooding the plate with a 1N HCl solution. The acid precipitate unhydrolyzed DNA. DNase producing colonies are surrounded by clear areas indicating DNA hydrolysis.

Procedure

Spot or streak inoculate the test organism on to a medium containing DNA.
Incubate the plate at 35-37^{0}C overnight.
Cover the surface of the plate with 1 N solution of HCl and tip off the excess acid.
Use negative and positive controls.

Results

Look for clearing around the colonies within 5 minutes.



Controls

Positive control - *Staphylococcus aureus*

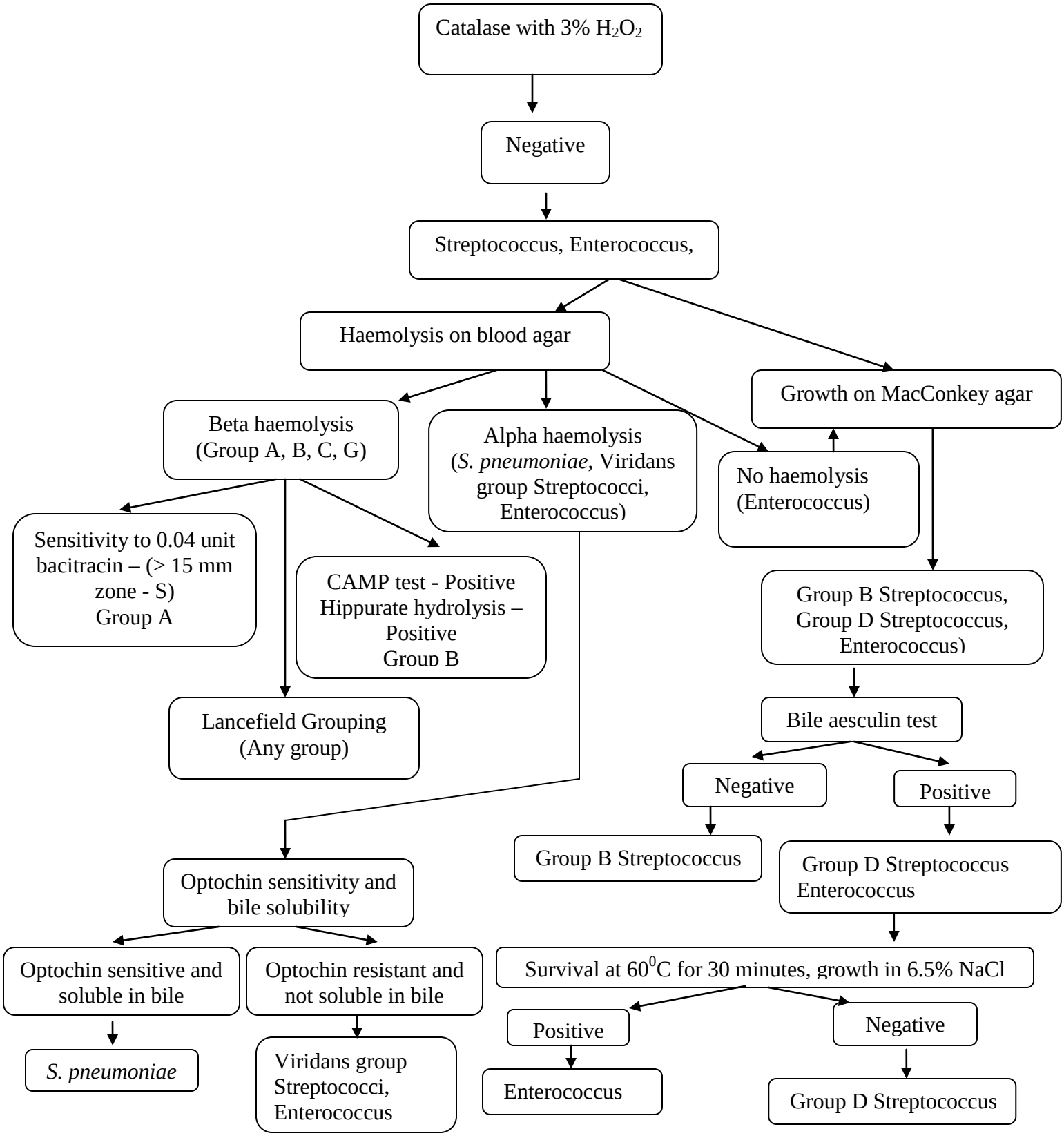
Negative control - *Staphylococcus epidermidis*

Question 2

Note down the appropriate test results for each of the organism in the list.

Organism	**Catalase with 3% H_2O_2**	**Slide coagulase test**	**Tube coagulase test**	**Sensitivity to 100 µg Furazolidone**	**Sensitivity to 0.04 U Bacitracin**	**Sensitivity to 5 µg Novobiocin**	**DNase test**
Staphylococcus aureus							
Staphylococcus epidermidis							
Staphylococcus saprophyticus							
Micrococcus species							

Identification of Streptococci

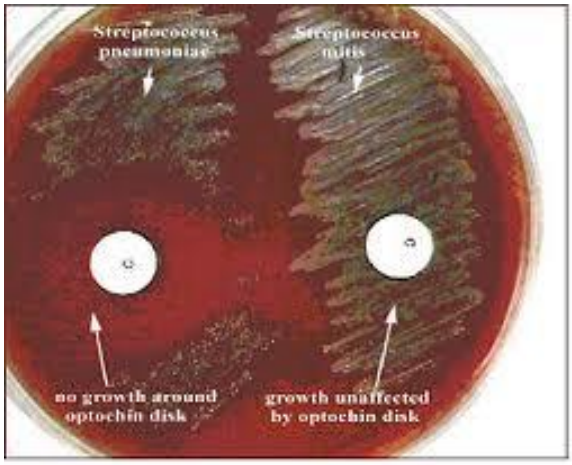


Optochin sensitivity test [Ethylhydrocuprein hydrochloride (EHCH) sensitivity test]

Place a disc impregnated with EHC 0.05 mg optochin on a surface of a blood agar plate inoculated with test organism.

Incubate at 35°C for overnight in 5-10% CO_2 and examine for inhibition zone.

Do a positive and a negative control along with the test.



Interpretation

Zone of inhibition of 14 mm or more around a 6 mm disc or 16 mm or more around a 10 mm disc, presumptively identifies the test organism as *Streptococcus pneumoniae*.

Zones smaller than these should be identified using bile solubility test. If the organism is soluble in bile, it is identified as *Streptococcus pneumoniae*.

No zone of inhibition – Viridans group Streptococci.

Controls

Positive control - *Streptococcus pneumoniae.*

Negative control – *Enterococcus faecalis*

Bile solubility test

Principle

The test helps to differentiate *S. pneumoniae* which is soluble in bile and bile salts from viridians streptococci which are insoluble.

Procedure

There are 2 methods.

1. Plate method
2. Tube method

Plate method

Touch a suspected colony on the primary culture plate with a loop charged with 10% sodium deoxycholate solution.

Incubate the plate at 35^0C for 15 minutes.

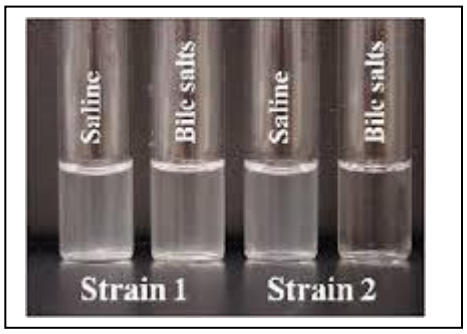
Colonies of *Streptococcus pneumoniae* disappears leaving an area of α-haemolysis on the blood agar plate.

Tube method

Prepare a milky suspension (1 McFarland) of the suspected organism from an overnight culture in 1 ml of saline.
Divide the suspension in to 2 tubes – 0.5 ml in each.
Add 0.5 ml of 2% sodium deoxycholate solution to one tube (test) and 0.5 ml of sterile saline to the other (control).
Vortex and incubate both tubes at 35^0C up to 2 hours (inspect after 10 – 30 minutes initially).
Put known positive and negative controls with the test.

Results

Clearing of turbidity -Positive test
No clearing of turbidity -Negative test



Controls

Positive control - *Streptococcus pneumoniae*
Negative control - *Enterococcus faecalis*

Bacitracin sensitivity test

Inoculate the test organism on to a blood agar plate.
Apply 0.04 units bacitracin disc.
Incubate at 35°C overnight.
Look for inhibition zone.
Group A Streptococci give a large (> 15 mm) zone of inhibition around the disc.

Note: 5% - 10% of group B, C, G streptococci can also give zones of inhibition with 0.04 units bacitracin disc.

CAMP test (Christie, Atkins & Munch-Petersen test)

Principle

The CAMP factor produced by Group B streptococci lyses sheep red blood cells pretreated with β-toxin of *Staphylococcus aureus*.

Procedure

Use layered 10 % sheep blood agar plates.

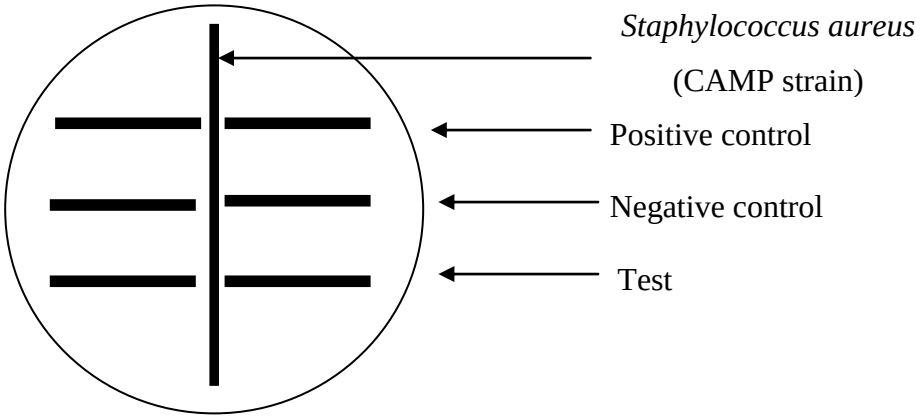
Streak *Staphylococcus aureus* (NCTC 7428) in a line across diameter of the plate.

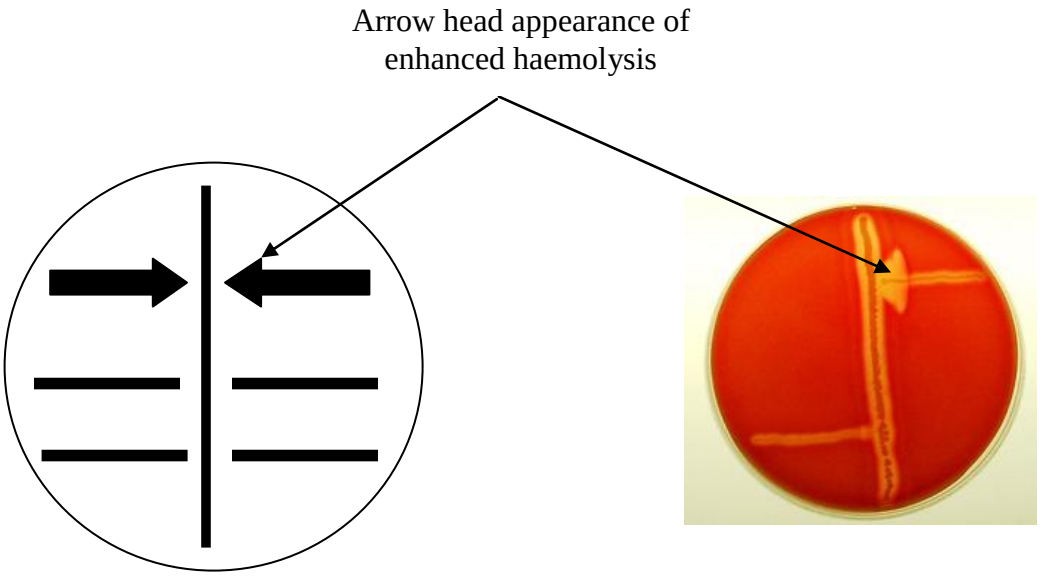
At right angles to the *Staphylococcus aureus* inoculum streak the test cultures without touching the staphylococcal inoculum.

Similarly, streak the positive and the negative controls.

Incubate the plates at 35^0C aerobically or in the presence of 5% CO_2.

Incubate for 24 hours and observe the results.





Controls

Positive control – *Streptococcus agalactiae*

Negative control – *Enterococcus faecalis*

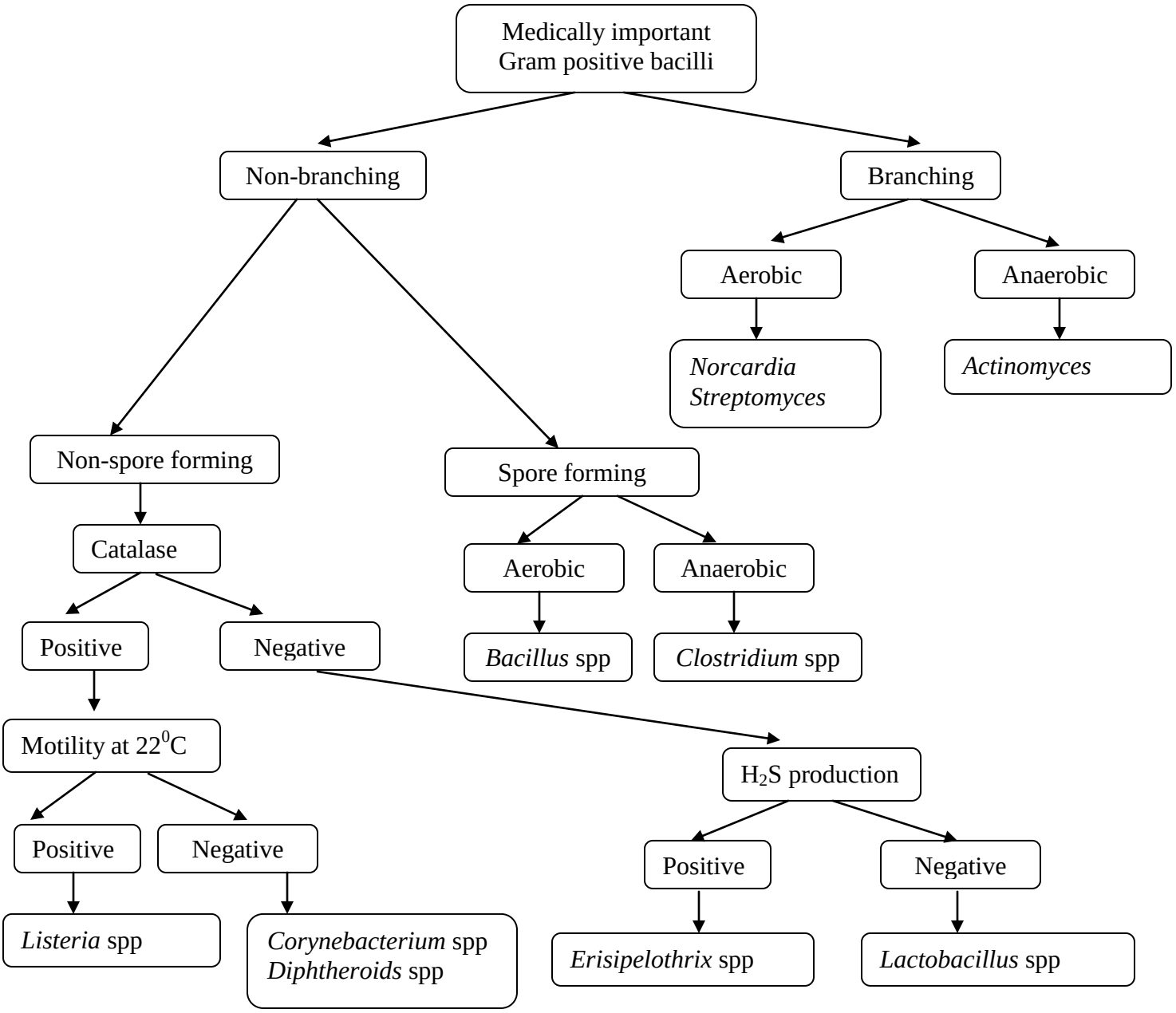
Question 3

Note down the appropriate test results for each of the organism in the list.

Organism	**Catalase with 3% H_2O_2**	**Bacitracin 0.04 U sensitivity (S/R/Not done)**	**Optochin sensitivity (5µg disc) S/R/Not done**	**Bile solubility**	**CAMP test**	**Growth in 6.5% NaCl**	**Bile aesculin test**	**Heat resistance test (60^0C, 30 minutes)**	**Growth on 40% bile agar**	**Growth at 45^0C**
Streptococcus pyogenes										
Streptococcus agalactiae										
Streptococcus pneumoniae										
Viridans group Streptococci										
Enterococcus species										
Group D *Streptococcus*										

Bacteriology
Practical – 9

Identification of Gram positive bacilli



Question 1

Observe the colony characteristics of the organisms on different culture media and note them down. Note down the Gram stain characteristics and other biochemical reactions of each of them.

Species	*Corynebacterium* spp	*Diphtheroids* spp	*Bacillus* spp	*Listeria* spp	*Lactobacillus* spp
Gram stain characteristics					
Methylene blue stain			-	-	-
Colony morphology on blood agar					
Colony morphology on blood tellurite agar		-	-	-	-
Colony morphology on chocolate agar					
Colony morphology on MacConkey agar					

Test for the detection of toxigenicity of *Corynebacterium diphtheriae* (Elek plate test)

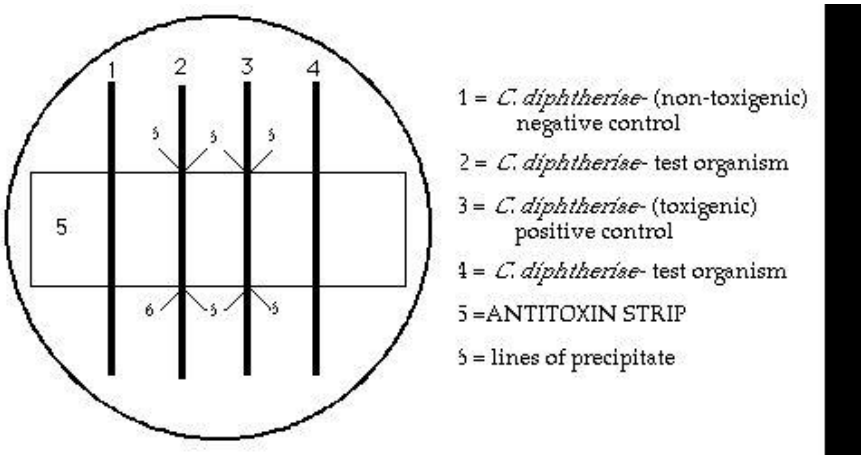
Procedure

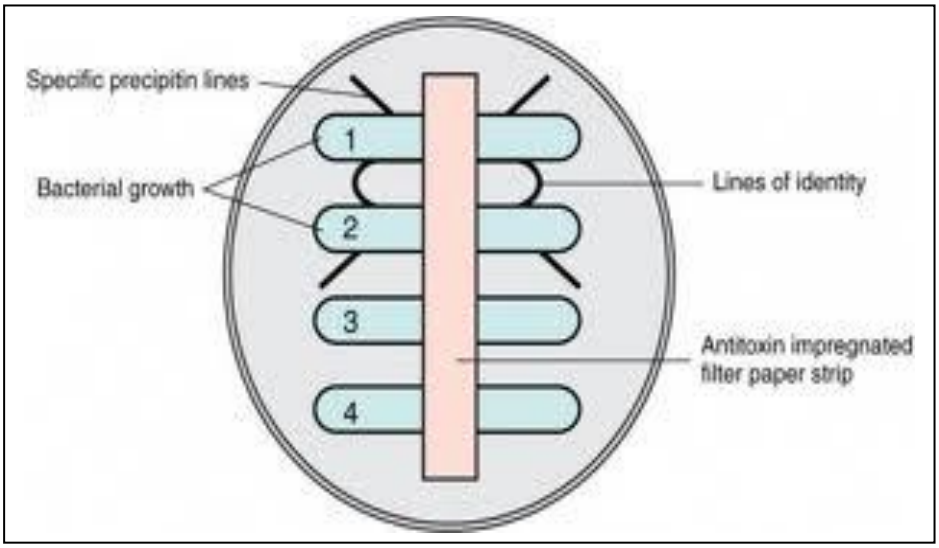
This is an immunodiffusion test (double diffusion precipitation test) first described by Elek. Unknown isolates and known positive and negative controls are streaked on media with low iron content to optimize the toxin production.

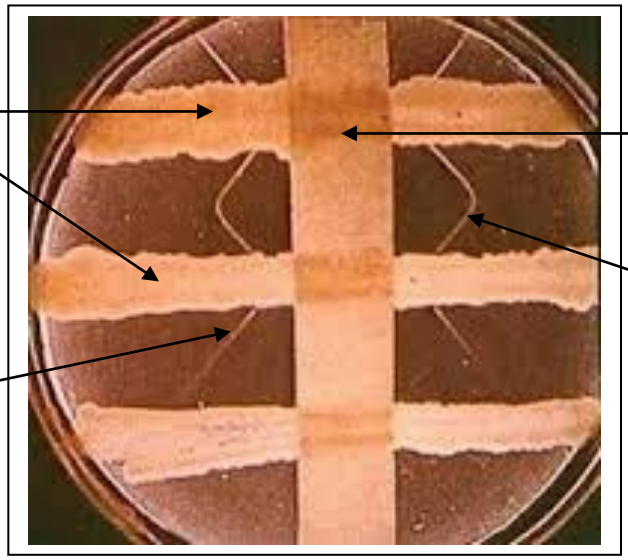
The test is performed on a plate of clear serum nutrient agar. A filter paper strip impregnated with diphtheria anti-toxin (100 units/ml) is placed at the center of the serum agar plate using a sterile forceps before the agar has completely set. The plate should be used within 2 hours of preparation. Streak the test strains as well as the positive and negative control strains at right angles to the strip. Take care not to touch the strip of antitoxin with the cultures.

Toxin diffusing from a streak culture of suspected *C. diphtheriae* is demonstrated by the formation of a white line of precipitate at an angle of 45^0 to the line of inoculums, where it meets with diphtheria antitoxin diffusing from the strip of filter paper embedded in the agar. This line may fuse with the line of precipitate from a positive control forming a line of identity.

Success of the test is dependent on the quality of the medium and the antitoxin, which must be checked in preliminary tests.




Specific precipitin lines
Bacterial growth
1
2
3
4
Lines of identity
Antitoxin impregnated
filter paper strip


Bacterial growth
Antitoxin impregnated
paper strip
Lines of identity
Specific precipitin lines

Bacteriology
Practical – 10

Identification of Gram negative cocci

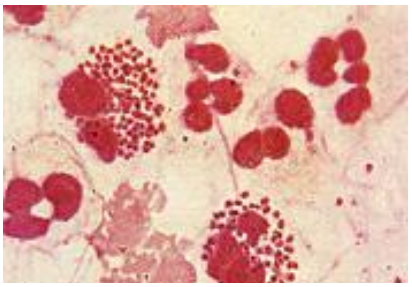
Question 1

Observe the colony characteristics of the organisms on different culture media and note them down. Note down the Gram stain characteristics and other biochemical reactions of each of them.

Species	*Neisseria meningitidis*	*Neisseria gonorrhoeae*	*Moraxella caterrhalis*
Gram stain characteristics			
Colony morphology on blood agar			
Colony morphology on chocolate agar			
Colony morphology on Thayer Martin medium	-		-
Colony morphology on MacConkey agar			
Catalase test			
Oxidase test			
Betalactamase test			

Gram stain

Describe the Gram nature and morphology.



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How would you differentiate species of Neisseria and Moraxella biochemically in the diagnostic laboratory?

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Carbohydrate fermentation test

Principle

The identification of some bacteria is based on that what nutrients the bacteria can utilize and the end products produced in the process. These characteristics are controlled by the enzymes which the bacteria have. The pattern of sugars fermented may be unique to a particular species or strain as the type of enzyme(s) produced by bacteria is genetically controlled. Fermentation products are usually acid (lactic acid, acetic acid etc.), neutral (ethyl alcohol etc.), or gases (carbon dioxide, hydrogen, etc.).

Procedure

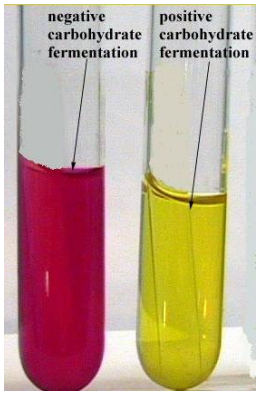
Prepare a carbohydrate fermentation broth at pH 7.4. This broth contains 3 essential ingredients: 0.5%-1.0% of the carbohydrate to be tested (e.g. lactose or glucose), nutrient broth with 10% sterile serum, and the pH indicator phenol red. The nutrient broth, which is a light red color, supports the growth of most organisms whether they are able to ferment the sugar or not.

The test organism is inoculated into a broth containing the test sugar and incubated. A bright yellow color indicates the production of enough acid products from fermentation of the sugar to drop the pH to 6.9 or less.

Results

Yellow colour - Fermentation of acid - Positive results

Light red colour - No fermentation of acid - Negative results



Bacteriology
Practical – 11 and 13

Identification of Gram negative short bacilli and Gram negative curved bacilli

Question 1

(1) Observe the culture characteristics of *Haemophilus influenzae* on chocolate agar plate and note them down.

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(2) Observe the culture characteristics of *Haemophilus influenzae* on blood agar plate with *S. aureus* stabs and note them down.

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(3) Perform the Gram stain from a colony and note down the appearance?

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(4) Describe what are X and V factors.

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(5) a) Perform the satellitism test and write down the steps of the procedure.

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b) What is the principle behind the satellitism?

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(6) Perform the X, V and XV factor requirement test to find out the species of the given isolate. Note down the steps of the procedure.

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Question 2

(1) Observe the culture characteristics of *Acinetobacter* species on blood agar, chocolate agar and MacConkey agar and note them down.

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(2) Perform the Gram stain from a colony and note down the appearance?

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(3) Perform the biochemical tests to identify the isolate as far as possible and note down the results of the tests performed.

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Question 3

(1) Gram stained slide of *Helicobacter pylori* is given to you. How would you describe the slide?

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(2) What are the media used to grow *Helicobacter pylori*?

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(3) Write a special growth requirement for *Helicobacter pylori,* observe the anaerobic jar.

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(4) Observe the CLO test and write the procedure. What is the principle behind this rapid test?

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Question 3

A Gram stained photo of a *Campylobacter jejuni* isolated from faecal specimen is given to you.

(1) How would you describe this slide?

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(2) Write a name of a selective culture media which is used to culture *C. jejuni.*

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(3) Write a special growth requirement for this organism.

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Bacteriology
Practical – 12

Bacterial Motility

Bacterial motility is a very useful character when speciation of bacteria is done. There are several ways that we can demonstrate the motility of bacteria.

- Detection of motility on semi-solid agar
- Detection of motility by microscopy
    - Hanging drop preparation
    - Unstained wet film

Detection of motility on semi-solid agar

In semi-solid media, motile bacteria swarm and give a diffuse spreading growth which is easily recognized by the naked eye. This method detects bacterial motility more easily than the microscopic method.

The correct concentration of agar to demonstrate motility depends on the brand of agar used for the preparation of the medium. If it is Japanese agar, the concentration should be 0.4% and if it is New Zealand agar the concentration should be 0.2%. Final medium should be quite clear and transparent for you to observe motility after inoculating the medium.

Using a sterile straight wire, inoculate the medium with a single stab down the center of the tube to about half the depth of the medium. Incubate at required temperature and observe. Non-motile bacteria generally give growths that are confined to the stab-line with a surrounding clear medium where as motile bacteria typically give diffuse hazy growths that spread throughout the medium making it slightly opaque.

Hanging drop and wet mount preparation

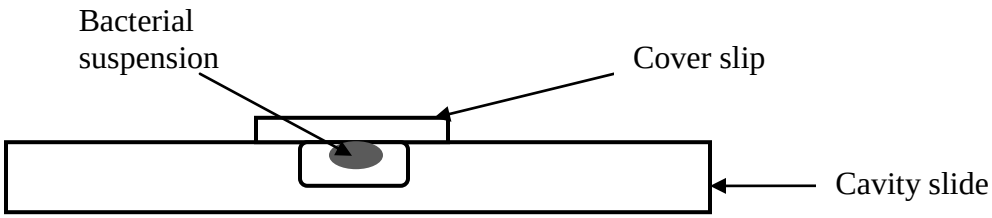
The simplest method for examine living microorganisms is to suspend then in a fluid (water, saline or broth) and prepare either a “hanging drop” or simple “wet mount”. The slide for a hanging drop is with a concave well in the center; the cover glass holds a drop of the suspension. When the cover glass is inverted over the well of the slide, the drop hangs from the glass in the hollow concavity of slide.

Microscopic study of wet preparation can provide useful information. Primarily the method is to determine whether an organism is motile or not but it also permits a view of natural pattern of all grouping and of individual cell shape.

Hanging drop preparation can be observed for a fairly long term, because the drop does not dry up quickly. Wet mounted preparations are primarily used to detect microbial motility rapidly.

Note:

It is important to distinguish the motility from “Brownian movement” a form of movement caused by molecules in the liquid striking a solid object. Brownian movement is the back and forth movement of bacterial cells due to the movements of water molecules. If the bacterial cell is truly motile you will observe its directional movement through point A to B.



In the unstained wet film method, a drop of liquid containing organisms are placed on a clean glass slide and a cover-slip is applied over the drop without creating air bubbles to give a thin smear. Then the motility of organisms is observed under high power dry objective (X40). Drawing a line on the slide before the procedure with a glass marking pen or a pencil will make it easier to focus the unstained drop of liquid.

Exercise

Record the following results.

	Proteus vulgaris	***Staphylococcus epidermidis***	***Klebsiella pneumoniae***	***Escherichia coli***
Shape/ Grouping				
Motile				
Non motile				

Read the questions and answers

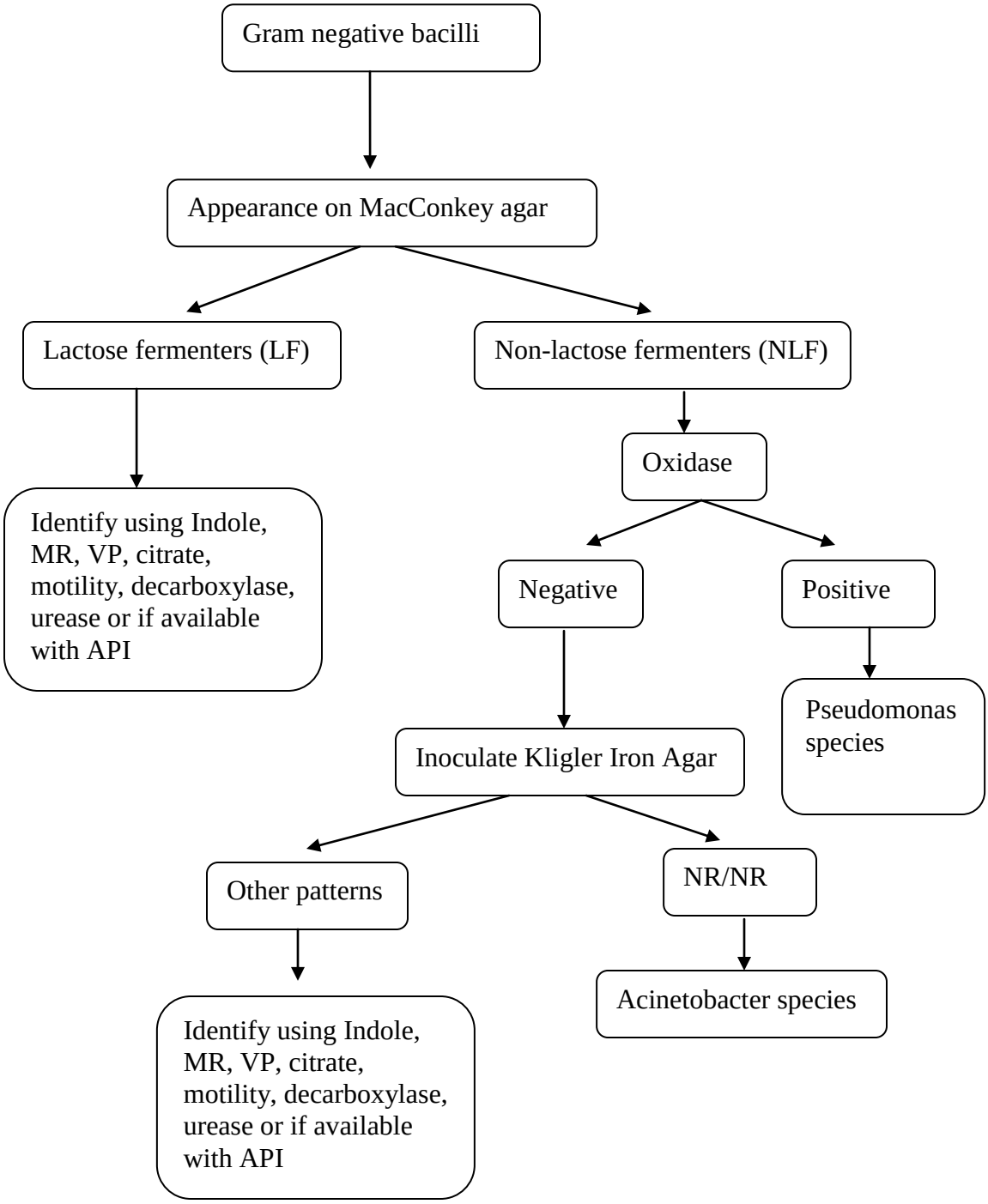
1. How does true motility differ from Brownian movement?
   If the bacterial cell is truly motile you will observe its directional movement through point A to B. In Brownian movement, the movement caused by molecules in the liquid striking a solid object and the bacterial cell will move back and forth.

2. What morphological structure is responsible for bacterial motility?
   Flagella.

3. Why is a wet preparation discarded in disinfectant solution?
   As it contains infectious micro organisms.

Bacteriology
Practical – 14 and 15

Identification of Enterobacteriaceae



Question 1

Observe the colony characteristics of the organisms on different culture media and note them down.

Species	Colony morphology on blood agar	Colony morphology on MacConkey agar	Colony morphology on chocolate agar
Escherichia coli			
Klebsiella species			
Proteus species			
Citrobacter species			
Enterobacter spp			
Serratia spp			

Question 2

Note down the Gram stain characteristics and other biochemical reactions of each of them.

Test	***Escherichia coli***	***Klebsiella species***	***Proteus species***	***Citrobacter species***	***Enterobacter* spp**	***Serratia* spp**
Catalase test						
Oxidase test						
Motility test						
KIA pattern						
Indole						
MR						
VP						
Citrate						
PPA						
Urease						
ONPG						

Bacteriology
Practical – 16 and 17

Identification of Salmonella and Shigella

Question 1
Observe the colony characteristics of the organisms on different culture media and note them down.

Species	***Salmonella* species**	***Shigella* species**
Colony morphology on blood agar		
Colony morphology on MacConkey agar		
Colony morphology on Salmonella Shigella agar		
Colony morphology on Xylose Lysine Deoxycholate agar		

Question 2

Note down the Gram stain characteristics and other biochemical reactions of each of them.

Species	***Salmonella* species**	***Shigella* species**
Gram stain characteristics		
Catalase test		
Oxidase test		
Motility test		
KIA pattern		
Indole		
MR		
VP		
Citrate		
PPA		
Urease		
ONPG		

Sero-typing of Salmonella species

*Salmonella s*pecies are classified according their somatic antigens (O) and flagellar antigens (H). This is known as the Kauffmann-White classification. This classification is used in the sero-typing of *Salmonella*.

Diagnostic antisera can be prepared in the laboratory by immunizing rabbits with the appropriate antigen.

Antisera to somatic antigens are available as polyvalent containing serogroups A to G or A to S and also as group A (O2), B (O4), C1 (O 6, 7), C2 (O8), D (O9), E (O3, 10, 15, 19), F (O11), and G (O13, 22). Antisera to flagellar antigens are also available as polyvalent and mono valent.

Antisera commercially available and should be kept in a refrigerator. Antisera should be at room temperature when they are used for the test.

After observing the KIA patterns, the isolates giving typical salmonella patterns are used for sero-typing.

Typical KIA pattern of *Salmonella* species – K/A, gas +, H_2S +

Other patterns that is given by *Salmonella* species – K/A, gas +, no H_2S – *S.* Patatyphi A

\- K/A, no gas, little H_2S – *S.* Typhi

Procedure of sero-typing

Confirm that you are handling an isolate of enterobacteriaceae by doing an oxidase (negative) from the KIA tube.

Take a clean glass slide and prepare two even suspensions of organisms taken from the KIA slant on a drop of sterile physiological saline.

Mix the polyvalent O anti-serum thoroughly and put a drop carefully on to one of the suspensions of organisms. Use the other suspension as a control to see whether there is any auto-agglutination.

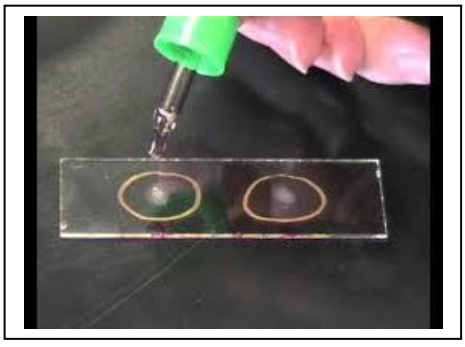
Using a sterile wire loop mix the contents gently and rotate for about 2 minutes.

Look for agglutination against a dark background in the suspension where the anti-serum was added.

If you see the agglutination, do similarly with the polyvalent H antiserum.

If no agglutination, no further testing with anti-sera.

If you see agglutination with that too, do the same procedure with the other O antisera groups according to the prevalence of the species in the country.



Sero-typing of Shigella species

Shigella species are divided in to 4 sero-groups depending on their somatic antigens. Sero-group A (*Shigella dysenteriae*), sero-group B (*Shigella flexneri*), sero-group C (*Shigella boydii*) and sero-group D (*Shigella sonnei*).

Diagnostic antisera can be prepared in the laboratory like for *Salmonella* by immunizing rabbits with the appropriate antigen.

Antisera to somatic antigens are available as polyvalent containing sero-groups A, B, C and D. Further monovalent antisera are available depending on the sero-group.

Procedure is similar to that of *Salmonella* sero-typing.

If the *Shigella* like organisms do not give a positive agglutination test with the anti-sera, suspect the presence of a masking K antigen. Make a suspension (about 10^9 bacteria/ml) of the culture in 0.5 ml of saline, heat at 100^0C for 1 hour, cool, centrifuge, re-suspend in fresh saline and re-test with the range of anti-sera.

Bacteriology
Practical - 18

Identification of Mycobacterial species

Question 1

Perform the Ziehl Neelsen stain on the provided slide of sputum. Follow safety precautions during the procedure. Note down the staining characteristics of the organisms and the background.

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Question 2

Perform the modified Ziehl Neelsen stain on the provided slide of slit skin smear. Follow safety precautions during the procedure. Note down the staining characteristics of the organisms and the background.

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Question 3

Note down the main differences in staining characteristics of *M. tuberculosis* and *M. leprae.*

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Bacteriology
Practical – 19 and 20

Bacterial Cell Counting

Read the questions and answers.

Question 01

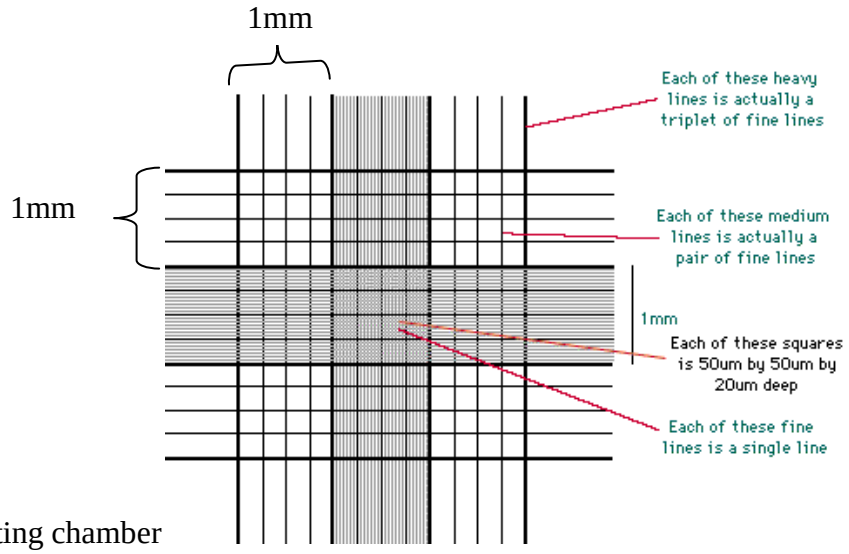
What do you mean by total bacterial count?

- A count of the living and dead bacteria in a liquid culture or suspension.

To count bacteria we use an instrument called a counting chamber. A suitable chamber consists of a thin glass slide with a flat platform which is depressed exactly 0.02mm below the surface and surrounded by a deeper trench. On the platform, an area of 9 mm^2 is marked with engraved lines to give squares. When the counting is done, the chamber is closed with a thick, optically plane cover-glass. It permits to get a known volume of the suspension over each square when the space between the platform and the cover-glass is filled with a bacterial suspension.

A bacterial suspension is given to you. Using the counting chamber perform the total bacterial count. Write the procedure.

1. Fix the bacterial suspension by adding 2-3 drops of 40% formaldehyde per 10 ml of the suspension. Mix thoroughly.
2. Wash, rinse, drain and dry the counting chamber and cover glass and keep them covered until use without exposure to dust.
3. Apply the cover glass on the chamber by pressing it down until coloured 'Newton's rings' appear. With an aid of a pipette, a drop of the bacterial suspension is carefully placed between the chamber platform and the cover-glass. The size of the drop should be such that it will fill the space between the platform and the cover-slip but will not extend across the trench to float the cover-glass from the slide.
4. Examine using a phase contrast microscope, using high power dry objective; this shows unstained bacteria clearly. Otherwise you may use a dark ground microscope or normal microscope with the iris diaphragm closed or the condenser slightly defocused.
5. Count the bacteria in a sufficient number of squares to obtain a total of several hundred bacteria. Selecting the squares in a pre-arranged pattern. Focus at different levels for the bacteria that have not settled.
6. Calculate the average number of bacteria per ml by multiplying the number of bacteria in a known number of squares by the depth of the square and the dilution factor.



Slide counting chamber

If 341 bacterial cells were counted in 5 chambers how will you interpret the results? Write the formula for calculation.

- Number of bacteria counted in 5 mm^2 = 341
- Depth of the counting chamber = 0.2 mm
- dilution factor = a
- Number of bacteria in 1 mm^3 = $\frac{341}{5}$ x 0.2 x a

Question 02

a. What do you mean by viable count?

- The number of living bacteria.

b. How will you obtain a viable bacterial cell count of a given sample? What is the method?

- Pour plate method
- Surface spread method
- Miles and Misra method

c. Observe the pour plate method and perform the pour plate method for the given bacterial suspension.

Pour Plate Method

Culture the bacteria in a suspension.
Make the serial dilution.
Add the inoculums to the plate.
Pour media into each plate.
Gently rotate for thorough distribution of inoculums throughout the medium (5 times clockwise, 5 times anti-clockwise, 5 times vertically, 5 times laterally).
Incubate at 37^0C overnight (lid of the plate should face upwards).
Number of colonies counted on plate x dilution of the sample = number of bacteria per ml.

Question 03

a. Write two methods which you can perform to get the surface viable count of a given sample.

- Surface spread method.
- Miles and Misra method.

b. Observe the Miles and Misra counting method and write the correct procedure.

Materials required

- A calibrated dropping pipette / micro titer pipette, delivering drops of about 0.02 ml.
- Six plates of clear nutrient agar.
- Prepare serial dilutions of the bacterial suspension as required.
- The plates are divided into numbered sectors.
- The inoculum / suspension is deposited as drops of 0.02 ml from a height of 2.5 cm on to the culture medium to allow it spreads over an area of 1.5 – 2.0 cm diameter.
- Each of the 6 plates receives one drop of each dilution in separate numbered sectors.
- The plates are incubated for 18 – 24 hours and observed for growth.
- Sectors where more than 20 colonies are present without any confluence are utilized to make the viable counts.
- Viable count per 0.02 ml for a dilution is obtained by taking the average of counts for that dilution in all the six plates multiply by the dilution factor.
- Colony count/ ml=average number of colonies for that dilution x dilution factor.

C. Perform the Miles and Misra method for the given bacterial suspension and get the count for the given sample.

Question 04

a. Describe the use of MacFarlane standards in quantification of bacteria.

McFarland standards are barium sulphate standards against which the turbidity of the test and control inocula can be compared. When matched with the standard, the inocula should give confluent or almost confluent growth. Shake the standard immediately before use. The range of available standards are from 0.5 to 4. Commonly used standard is the 0.5.

Briefly state its applications;

- The McFarland standard is used to adjust the turbidity of the inoculum for the susceptibility tests.
- Visual comparison.

- Quality control and calibration for microbiological equipments e.g. spectrophotometer comparison, density meters, turbidimeters.

Preparation of McFarland turbidity standard

- Prepare a 1% v/v solution of sulphuric acid by adding 1 ml of concentrated sulphuric acid ***to*** 99 ml of water. Mix well.
- ***Caution*****:** Concentrated sulphuric acid is hygroscopic and highly corrosive, therefore do not mouth pipette, and never add the water to the acid.
- Prepare a 1% w/v solution of barium chloride by dissolving 0.5 g of dihydrate barium chloride ($BaCl_2.2H_2O$) in 50 ml of distilled water.
- Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution, and mix.
- Transfer a small volume of the turbid solution to a capped tube or screw-cap bottle of the same type as used for preparing the test and control inocula.
- When stored in a well-sealed container in the dark at room temperature (20–28 ^{0}C), the standard can be kept for up to 6 months.

McFarland Standard No.	0.5	1	2	3	4
Approx. cell density (1 x 10^8 CFU/ml)	1.5	3.0	6.0	9.0	12.0
Absorbance*	0.132	0.257	0.451	0.582	0.669

Bacteriology
Practical – 21 and 23

Identification of *Pseudomonas* species and *Burkholderia* species

Question 1
Observe the colony characteristics of the organisms on different culture media and note them down. Note down the Gram stain characteristics and other biochemical reactions of each of them.

Species	*Pseudomonas* species	*Burkholderia* species
Gram stain characteristics		
Colony morphology on blood agar		
Colony morphology on chocolate agar		
Colony morphology on Ceystine Lactose Electrolyte Deficient (CLED) medium		
Colony morphology on MacConkey agar		
Catalase test		
Oxidase test		
KIA pattern		
O/F test		

Bacteriology
Practical – 22 and 24

Antibiotic Susceptibility Testing and Techniques of Detection of Antibiotic Resistance of Organisms

Read the questions and answers.

Question No 1

a) What are the main methods available for antibiotic susceptibility testing in the laboratory?

1. Diffusion techniques
2. Dilution techniques (to get a minimum inhibitory concentration - MIC)
3. Automated methods (VITEK 1, VITEK 2)
4. Epsilometer testing (E-test)

b) Name the diffusion tests available for antibiotic susceptibility testing.

2. Same plate diffusion test (Joan Stokes)
3. Different plate diffusion [CLSI (Clinical Laboratory Standards Institute), EUCAST (The European Committee on Antimicrobial Susceptibility Testing), BSAC (British Society for Antimicrobial Chemotherapy)]

c) Name the dilution tests available for antibiotic susceptibility testing.

1. Agar dilution
2. Broth dilution (Macro broth dilution and micro broth dilution)

Question No 2

Write a short note on disc diffusion method.

Discs of blotting papers impregnated with a known volume and appropriate concentration of an antimicrobial is placed on a plate of sensitivity testing agar, inoculated with a standard inoculum of a test organism. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the discs where bacteria are not capable of growing (called a zone of inhibition). The size of the zone and the rate of antibiotic diffusion are used to estimate the bacteria's sensitivity to that particular antibiotic.

There are numbers of ways of performing these tests and 2 methods are widely accepted for disc diffusion test.

1. Joan Stokes method (same plate comparative disc diffusion technique) - Control organism is inoculated on the same plate. The zone of inhibition produced by the organism is compared directly with that of the control organism.

2. Kirby-Bauer (NCCLS / CLSI) antibiotic testing – Control strain is inoculated in a separate plate. The zone is measured and compared against a previously prepared scale.

Question No 3
What is the antibiotic test we are using in our laboratory?

- Clinical Laboratory Standards Institute (CLSI) method

Question No 4
What important features should be present in the culture media that is used for ABST?

- Better diffusion of antibiotics.
- The pH should be between 7.2 and 7.4. If the pH is too low, certain drugs loose potency (e.g. aminoglycosides, quinolones and macrolides) while certain others show excess activity (e.g. tetracycline). If the pH is too high then reverse reactions would occur. Excess moisture too affects the reliability of the result.
- Increased thymidine antagonises the inhibitory effect of sulphonamides and trimethoprim.
- Increased concentrations of Mg^{2+} and Ca^{2+} affects the results of aminoglycosides and tetracycline, also reduces zone size in case of *P. aeruginosa.*
- Decreased cation increases the zone size.
- Increased Zn would decrease the zone size of carbapenem group of antibiotics.

Question No 65
What is MIC and MBC?

- Minimum Inhibitory concentration (MIC) - The lowest concentration of the compound that will inhibit the visible growth of the test organism after appropriate incubation.
- Minimum bactericidal concentration (MBC) - The lowest concentration of the antimicrobial which kills at least 99.9% of the original test inoculums of the organism.

Perform the MIC and MBC of a given substance.

Kirby-Bauer CLSI modified disc diffusion technique

The validity of this carefully standardized technique depends on, for each defined species

- Using discs of correct antimicrobial content
- An inoculum which gives confluent growth
- A reliable Mueller Hinton agar

The test method must be followed exactly in every detail. After incubation at 35^0C for 16–18 hours, zone sizes are measured and interpreted using NCCLS standards. These are derived from the correlation which exists between zone sizes and MICs.

The test should only be used for well-evaluated bacterial species.
Not suitable for bacteria that are slow-growing, need special nutrients, or require CO2 or anaerobic incubation.

CLSI technique

1. Preparation of Mueller Hinton agar
Prepare and sterilize the medium as instructed by the manufacturer.
The pH of the medium should be 7.2–7.4.
Pour the medium into 90 mm diameter sterile petri dishes to a depth of 4 mm (about 25 ml per plate).
Care must be taken to pour the plates on a level surface so that the depth of the medium is uniform.

Note: If the medium is too thick the zones will be falsely small and if too thin the inhibition zones will be falsely large.

Unmodified Mueller Hinton agar is not suitable for susceptibility testing *H. influenzae*, *S. pneumoniae*, *N. gonorrhoeae*. The addition of lyzed blood will enable such organisms to be tested.

2. Selection of antimicrobial discs
The choice of antimicrobials to be included in susceptibility tests will depend on the pathogen, the specimen, range of locally available antimicrobials, and local prescribing policies.
Consultation between laboratory, medical, and pharmacy staff is required.
The range of first choice drugs should be limited and reviewed at regular intervals. Additional drugs should be included only by special request.
Where there is cross-resistance, only one member from each group of related antimicrobials should be selected.

Note: Paper antimicrobial discs are commercially available from most manufacturers of culture media. Most paper discs can be used for 1 year or longer from the date of manufacture providing they are stored correctly (-20^0C or working stock at $2–8^0$C) in an airtight container with an indicating desiccant. Discs that have expired should not be used.
Quality control of discs is essential.
About 1 hour before use, the working stock of discs should be allowed to warm to room temperature, protected from direct sunlight.

Important: Decreasing control zone size with a particular antimicrobial disc is often an indication of deterioration of the antimicrobial due to moisture or heat.

3. Control strains

Control strains are used to test the performance of the method. The following strains of bacterial species are recommended.

Staphylococcus aureus ATCC 25923.
Escherichia coli ATCC 25922.
Pseudomonas aeruginosa ATCC 27853
Haemophilus influenzae ATCC 49766
Enetrococcus faecalis ATCC 29212

Sources of control strains

Reference Laboratories should supply local laboratories.

Storage of control strains

For prolonged storage, maintain stock cultures at -20^{0}C or below or in liquid nitrogen in a suitable stabilizer (50% foetal calf serum, 10-15% glycerol in tryptic soy broth, defibrinated sheep blood or skim milk) or in the freeze- dried form.

Subculture frozen or freeze-dried stock cultures on appropriate media and incubate under appropriate conditions for the organisms. Subculture the frozen or freeze-dried stock cultures twice before use in the testing. The 2nd sub culture is referred to as Day 1 working culture. Prepare a new subculture each week to create a working culture. Prepare a new working culture each day.

Prepare new primary cultures from frozen or freeze-dried stock cultures at least once a month.

4. Method

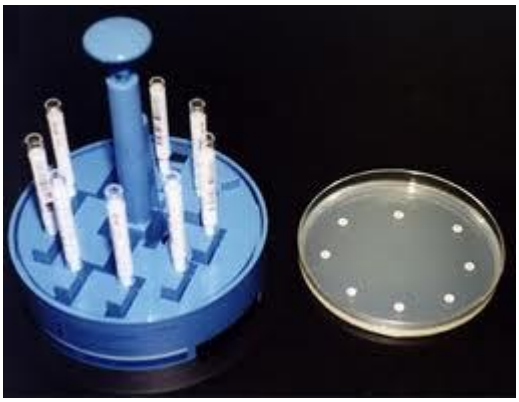
Direct colony suspension method is the most convenient method for inoculum preparation.

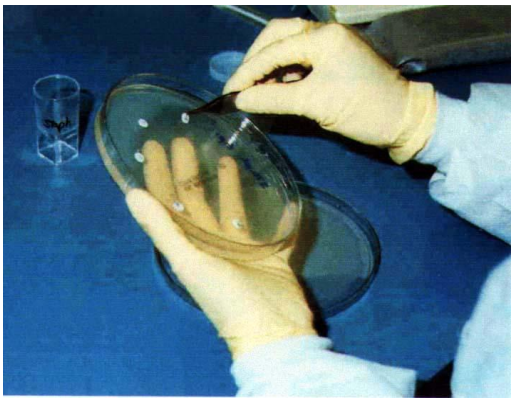
- Transfer 3–5 well-isolated colonies of similar appearance of the test organism and emulsify in 3–4 ml of sterile physiological saline or nutrient broth and vortex to mix.
- In a good light match the turbidity of the suspension to the 0.5 McFarland turbidity standard (vortex the standard immediately before use) by viewing against a printed card or sheet of paper.
- Use a sterile swab to get the organism in suspension. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension.
- Streak the swab evenly over the surface of the Muller Hinton agar in three directions, rotating the plate approximately 60^0 to ensure even distribution and finally take the swab round the edge of the agar surface.

- With the petri dish lid in place, allow 3–5 minutes (*no longer than 15 minutes*) for the surface of the agar to dry.

- Using sterile forceps, needle mounted in a holder, or a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate (no more than 5 discs on a 90 mm plate and no more than 12 discs on a 150 mm plate). Can use a template as to ensure the discs are correctly placed.
  Note: The discs should be about 15 mm from the edge of the plate and no closer than about 25 mm from disc to disc.

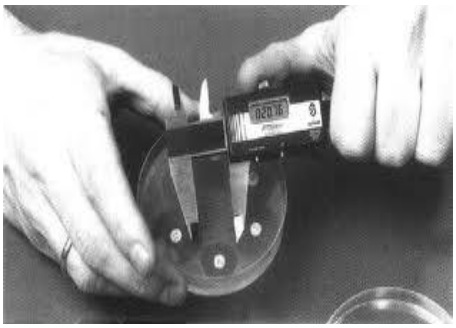
- Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once it is placed.

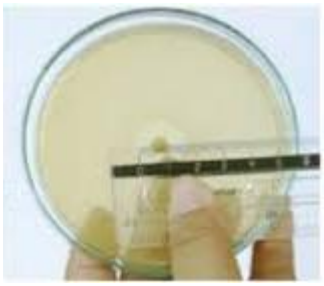




- Within 15 minutes of applying the discs, invert the plate and incubate it aerobically at 35^0C for 16–18 hours (temperatures over 35^0C invalidate results for oxacillin and cefoxitin).

- After overnight incubation, examine the test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate or using a vernier calliper, measure the diameter of each zone of inhibition in milimeters. The endpoint of inhibition is where growth starts.



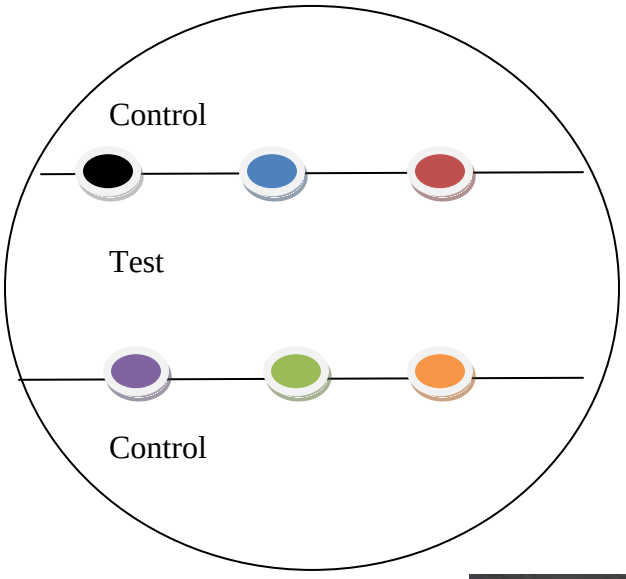


- Using the interpretative chart, interpret the zones sizes of each antimicrobial, reporting the organism as 'Resistant', 'Intermediate/Moderately susceptible', 'Susceptible'.
- Quality control should be done to monitor disc potency and culture media.

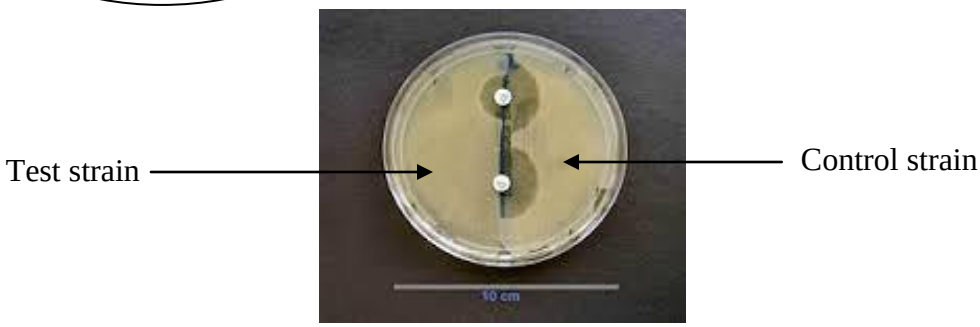
Discs place on a 90 mm plate

Discs placed on a 150 mm plate

Joan Strokes Method



Stokes disc diffusion showing the positioning of antimicrobial discs between the test and control organisms



Medium

Any antibiotic sensitivity test medium which sustain the growth of the test organism can be used (MHA, Iso-Sensitest agar, lysed blood can be added into those media for the growth of fastidious organisms).

Thickness of the medium is same as for CLSI.

Inoculum

This should be a semi-confluent growth after overnight incubation. Inoculating directly from agar cultures to antibiotic sensitivity test plates results in a too heavy growth.

Emulsify 3-5 similar colonies to make suspension equivalent in opacity to an overnight broth culture.

Control strains

Staphylococcus aureus NCTC 6571
Escherichia coli NCTC 10418
Pseudomonas aeruginosa NCTC 10662
Haemophilus influenzae NCTC 11931
Neisseria gonorrhoeae and *Neisseria meningitidis* Fully sensitive clinical isolates

Both the test and control organisms are inoculated on the same plate.
The zone sizes of the test organism are compared directly with that of the control.

Not as highly standardized as CLSI technique and is used in laboratories particularly when the correct concentration of antimicrobial in a disc cannot be guaranteed due to difficulties in obtaining discs and storing them correctly or when the other conditions required for the CLSI technique cannot be met.

Antimicrobial disc strengths are different to the CLSI for some antibiotics.

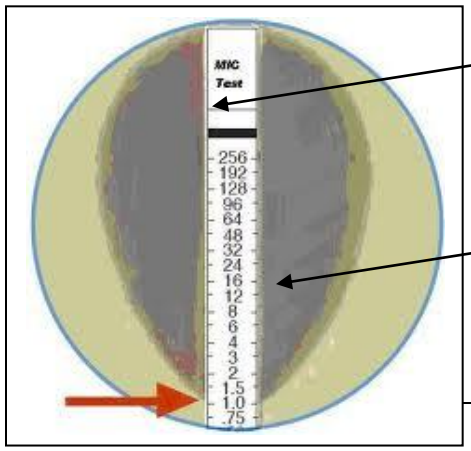
Question No 6
How do you report and record the results of ABST according to the Joan Stokes method.

- Sensitive – Zone radius is wider than, equal to or not more than 3 mm smaller than the control.
- Intermediate – Zone radius greater than 2 mm but smaller than the control by more than 3mm.
- Resistant – Zone radius measures 2 mm or less.

Epsilometer test (E-test)

E-test is a method for measuring MICs of antimicrobial agents against bacteria and is based on diffusion of a pre-formed antibiotic gradient from a plastic strip.

Method
The E-test utilizes a rectangular strip that has been impregnated with a concentration gradient of the antibiotic to be studied. Bacteria are inoculated onto the surface of an agar plate and the E-test strip is placed on the loan of bacteria; the drug diffuses out into the agar, producing gradient of the drug to be tested. There is a scale printed on the strip. After 24 hours of incubation, an elliptical zone of inhibition is produced and the point at which the ellipse meets the strip gives a reading for the minimum inhibitory concentration (MIC) of the drug.



Chromogenic cephalosporin method (nitrocefin test) to detect beta-lactamase production

Direct betalactamase detection is mostly used for *Haemophilus influenzae*, *Neisseria* species and *Moraxella caterrhalis*.

Procedure

Place a nitrocefin disc or a strip on a clean glass slide and moisten it with a drop of sterile distilled water.

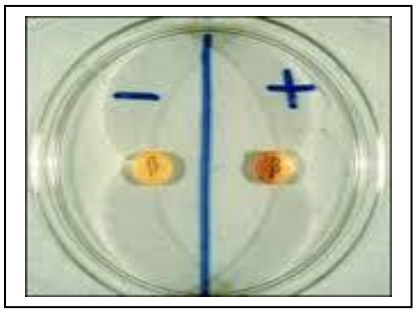
Using a sterile wire loop, sterile glass rod or a sterile wooden stick, take several well isolate colonies of similar morphology and smear onto the disc.

Observe a colour change on the disc within 5 minutes.

Results

Yellow to red – Positive for beta lactamase

No colour change - Negative for beta lactamase



Apple plate technique to detect beta-lactamase production

Procedure

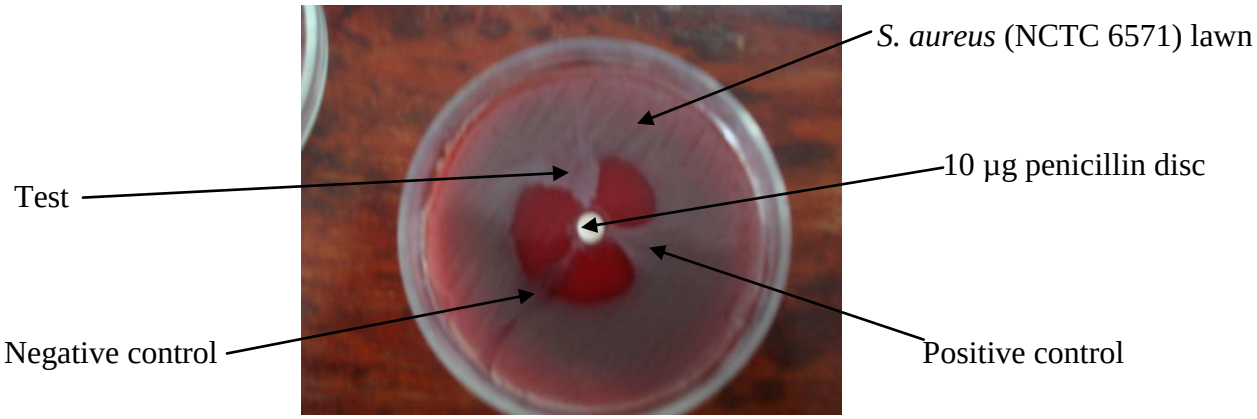
Spread *S. aureus* (NCTC 6571) on blood agar to create a lawn.

Streak the test organism and the positive and negative control organisms as shown in the diagram.

Place a 10 µg penicillin disc in the center as shown.

Incubate at 35^0C overnight and observe the changes.

Inoculum of *S. aureus* growing towards the penicillin disc adjacent to the positive control and the test organism streak indicates the production of betalactamase.



Controls

Positive control – Staphylococcus aureus ATCC 29213

Negative control – Haemophilus influenzae ATCC 10211

Detection of extended spectrum beta-lactamase (ESBL) production

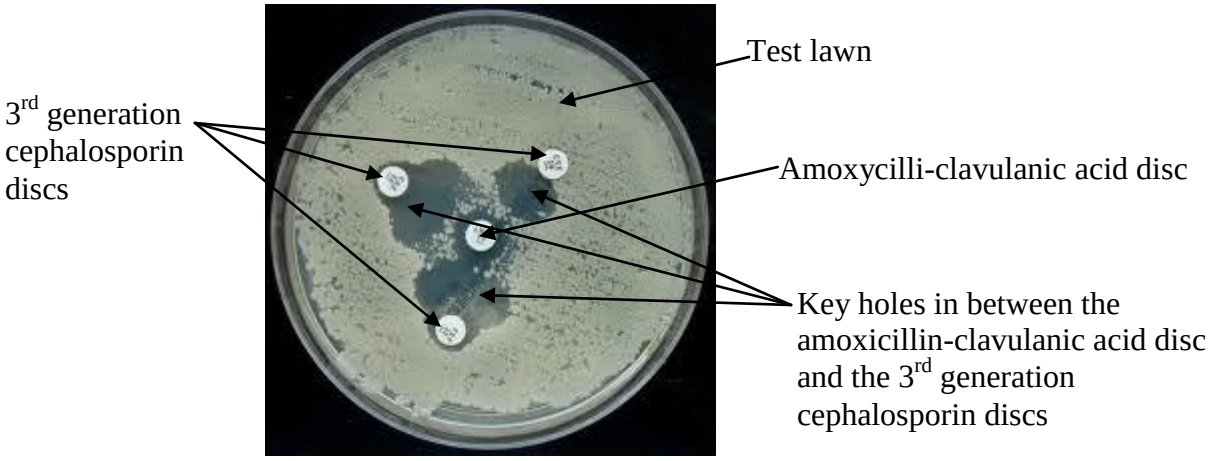
ESBL are produced by *E. coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Proteus mirabilis*. Hence, before performing the test, you should do speciation of the isolate.

Screening test

Procedure

Prepare a 0.5 McFarland equivalent suspension of the test organism and inoculate on Muller Hinton agar to get a confluent growth. Place a cefotaxime, ceftriaxone or ceftazidime disc and amoxicillin-clavulanic acid or ticarcillin-clavulanic acid disc with their centers 20 mm apart on test lawn. Presence of ESBL is indicated by an enhanced clear inhibitory zone resembling a key hole or a clear elliptical area between the discs.

On routine antibiotic sensitivity plates, zones of inhibition ≤ 17 mm with cefpodoxime 10 µg disc, ≤ 22 mm with ceftazidime 30 µg disc, ≤ 27 mm with cefotaxime 30 µg disc, ≤ 25 mm with ceftriaxone 30 µg disc and ≤ 27 mm with aztreonam 30 µg disc is suggestive of ESBL production.



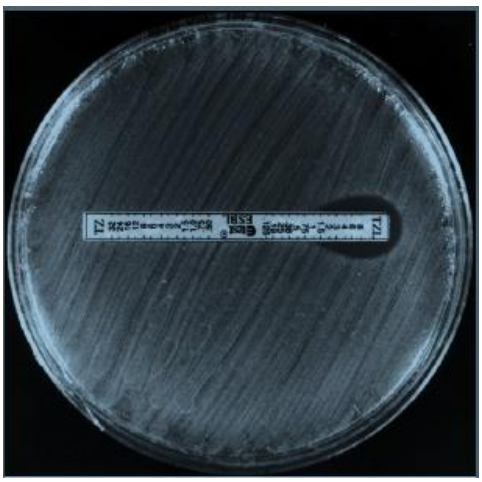
Positive screening tests should be confirmed with confirmatory test.

Confirmatory test

Procedure

Place ceftazidime 30 µg and ceftazidime-clavulanic acid 30/10 µg and cefotaxime 30 µg and cefotaxime-clavulanic acid 30/10 µg discs on the same plate of the test lawn. A ≥ 5 mm increase in diameter with the combined disc confirms ESBL.

Same is available as an E-strip where one end contains the 3rd generation cephalosporin gradient and the other end containing 3rd generation cephalosporin combined with beta lactamase inhibitor. If the ratio of MIC of 3rd generation cephalosporin to the MIC of cephalosporin-betalactamase inhibitor combination is ≥ 8, that confirms the ESBL production.



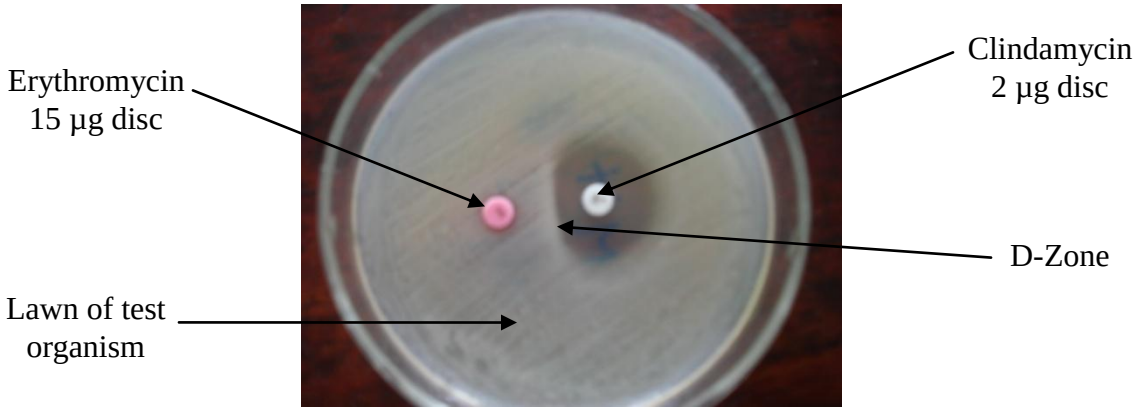
Detection of inducible clindamycin resistance in *S. aureus*

Procedure

Prepare a 0.5 McFarland equivalent suspension of the test organism and inoculate on Muller Hinton agar to get a confluent growth. Place a 15 µg erythromycin disc and 2 µg clindamycin disc 15-26 mm apart. Incubate at 35^0C overnight and observe the pattern.

Flattening of the zone of inhibition of clindamycin adjacent to erythromycin disc (raffered to as a D-zone) indicates inducible clindamycin resistance.

Hazy growth within the zone of inhibition f clindamycin indicates clindamycin resistance even in the absence of a D-zone.



Detection of inducible chromosomal betalactamases (AmpC betalactamase)

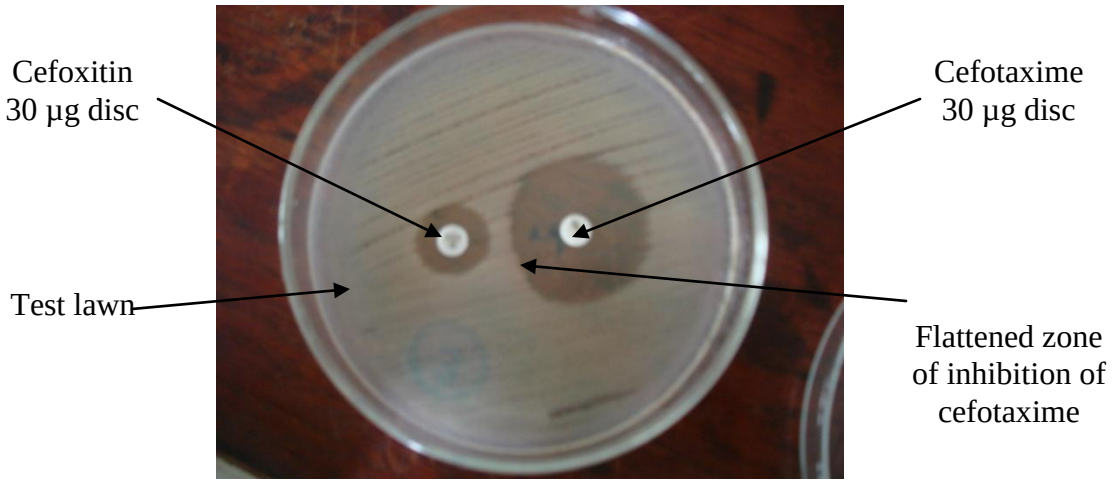
AmpC enzymes are chromosomal and inducible in most *Enterobacter* spp, *Citrobacter freundii*, *Serratia* spp, *Morganella morganii*, *Providencia* spp and *Pseudomonas aeruginosa*.

First generation cephalosporins, ampicillin and amoxicillin induce AmpC enzymes and are destroyed by them. Hence, AmpC inducible strains are resistant to those antibiotics and cannot be used in their treatment.

Cefuroxime, third generation cephalosporin, aztreonam and piperacillin are also liable but induce AmpC weakly.

Procedure

Prepare the lawn of the test organism on Muller Hinton Agar and place a cefotaxime 30 µg disc and a cefoxitin 30 µg disc 25 mm apart. Flattening of the zone of inhibition of cefotaxime adjacent to cefoxitin disc indicates inducible AmpC betalactamase production.



Confirmation of carbapenemase production by Modified Hodge Test (MHT)

Species of Enterobacteriaceae may produce carbapenemases. Once you detect intermediate resistance or resistance to a carbapenem using routine methods, this can be confirmed using MHT.

Procedure

Prepare a 0.5 McFarland suspension of *E.coli* ATCC 25922 – indicator organism in broth or saline and prepare the lawn on Muller Hinton agar plate as for routine disc diffusion procedure.

Allow the plate to dry for 3 -10 minutes.

Apply ertapenem 10 µg discs or meropenem 10 µg discs as appropriate as shown in the following 2 diagrams.

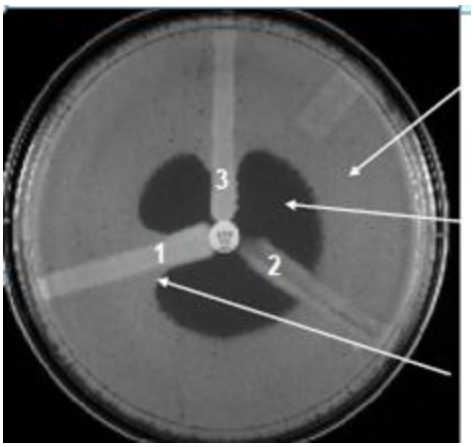
Using a sterile wire loop or a swab, pick 3-5 colonies of the positive control, negative control and test isolates separately and inoculate as a straight line on the lawn starting from the edge of the carbapenem disc without touching on it.

Following chart can be used to decide on test isolates, QC isolates and discs per plate.

	Small plate (90 mm)	Large plate (150 mm)
Test isolates	1	1-6
QC isolates	2	2
Number of carbapenem discs	1	1-4

Incubate at 35^0C for 16-18 hours.

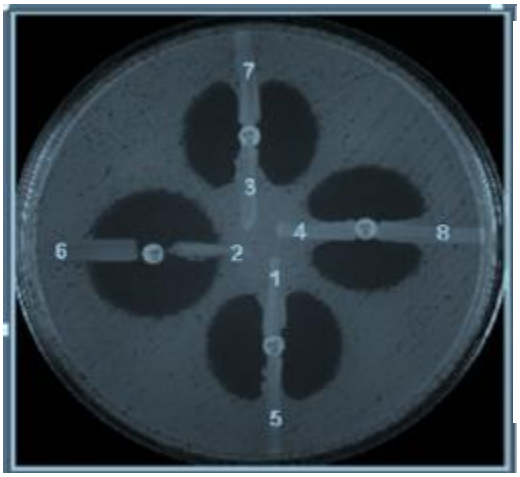
Examine the plates for an enhanced growth around the test or positive control organism at the intersection of the streak and the zone of inhibition.



E.coli ATCC 25992

Inhibition of *E.coli* ATCC 25992 by ertapenem

Enhanced growth of E.coli ATCC 25992. Carbapenemase produced by *K.pneumoniae* ATCC BAA-1705 inactivated ertapenem that diffused into the medium. Thus there is no longer sufficient ertapenem here to inhibit E. coli ATCC 25922 and an indentation of the zone is noted



The MHT performed on a large MHA plate with ertapenem.

1. *K.pneumoniae* ATCC BAA-1705 – Positive results
2. *K.pneumoniae* ATCC BAA-1706 – Negative results

3-8. Clinical isolates

6 – Negative results

3, 4, 5, 7, 8 – Positive results

Results

Enhanced growth – Positive for carbapenemase production

No enhanced growth – Negative for carbapenemase production

Controls

Klebsiella pneumoniae ATCC BAA 1705 – MHT positive

Klebsiella pneumoniae ATCC BAA 1706 – MHT negative

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