MARRI LAXMAN REDDY INSTITUTE OF PHARMACY

(Approved by AICTE & PCI, New Delhi and Affiliated to J.N.T. U, Hyderabad)
Dundigal (M) Medchal (Dist) Hyderabad- 500043

SUBJECT: Pharmaceutical Microbiology

Year: II/I B.Pharm

About MLRIP

To be an educational Institute of par excellence and produce competent pharmacy professionals to serve the community through research and the ever-increasing needs of Industry.

1. Imparting quality education and innovative research for various career opportunities.
2. Creating conducive academic environment to produce competent pharmacy professionals.
3. Indoctrination of students adorned with high human values and make them aware of their responsibility as health care professionals.

PEO 1: To produce graduates with sound theoretical knowledge and technical skills required for their career opportunities in various domains.

PEO 2: To incite the students towards research and to address the challenges with their innovative contributions for the benefit of the mankind.

PEO 3: To instill the essence of professionalism, ethical commitment to become a health care professional with sound integrity and adherence to the core human values in the service of the society.

PROGRAM OUTCOMES

1. **Pharmacy Knowledge:** Possess knowledge and comprehension of the core and basic knowledge associated with the profession of pharmacy, including biomedical sciences; pharmaceutical sciences; behavioral, social, and administrative pharmacy sciences; and manufacturing practices.
2. **Planning Abilities:** Demonstrate effective planning abilities including time management, resource management, delegation skills and organizational skills. Develop and implement plans and organize work to meet deadlines.
3. **Problem analysis:** Utilize the principles of scientific enquiry, thinking analytically, clearly and critically, while solving problems and making decisions during daily practice. Find, analyze, evaluate and apply information systematically and shall make defensible decisions.
4. **Modern tool usage:** Learn, select, and apply appropriate methods and procedures, resources, and modern pharmacy-related computing tools with an understanding of the limitations.
5. **Leadership skills:** Understand and consider the human reaction to change, motivation issues, leadership and team-building when planning changes required for fulfillment of practice, professional and societal responsibilities. Assume participatory roles as responsible citizens or leadership roles when appropriate to facilitate improvement in health and well-being.
6. **Professional Identity:** Understand, analyze and communicate the value of their professional roles in society (e.g. health care professionals, promoters of health, educators, managers, employers, employees).
7. **Pharmaceutical Ethics:** Honour personal values and apply ethical principles in professional and social contexts. Demonstrate behavior that recognizes cultural and personal variability in values, communication and lifestyles. Use ethical frameworks; apply ethical principles while making decisions and take responsibility for the outcomes associated with the decisions.
8. **Communication:** Communicate effectively with the pharmacy community and with society at large, such as, being able to comprehend and write effective reports, make effective presentations and documentation, and give and receive clear instructions.
9. **The Pharmacist and society:** Apply reasoning informed by the contextual knowledge to assess societal, health, safety and legal issues and the consequent responsibilities relevant to the professional pharmacy practice.
10. **Environment and sustainability:** Understand the impact of the professional pharmacy solutions in societal and environmental contexts, and demonstrate the knowledge of, and need for sustainable development.
11. **Life-long learning:** Recognize the need for and have the preparation and ability to engage in independent and life-long learning in the broadest context of technological change. Self-assess and use feedback effectively from others to identify learning needs and to satisfy these needs on an ongoing basis.

RULES AND PRECAUTIONS FOR THE MICROBIOLOGICAL LABORARORY

1. Always wear apron before entering laboratory.
2. Clean the work bench before and after the laboratory work.
3. Never eat or drink in the laboratory.
4. Long hair should be tied back to minimize the contamination of culture and fire hazards.
5. Sterilize all the equipments before use.
6. All cultures and prepared solutions should be properly labeled or marked.
7. Before discarding any culture, the organism must be killed.
8. Put off the burners when not in use.
9. Broth cultures should never be pipette out.
10. Label all plates and tube cultures properly before starting the experiment.
11. Open the mouth of the culture tubes and Petri plates near the flame.
12. Aseptic conditions should be strictly followed at all the times.
13. It is advisable to wear gloves when work with chemicals, hazardous to eye and skin.
14. Protect yourself from exposure of eyes and skin to UV light by wearing goggles and clothing respectively.

EXPERIMENT-01

INTRODUCTION AND STUDY OF DIFFERENT EQUIPMENTS AND PROCESSING

BOD INCUBATOR:

An incubator is an instrument that consists of copper/steel chamber. The temperature of the incubator is kept constant due to its control by using thermostat.

A temperature less than ambient temperature cannot be maintained in an ordinary incubator. Sometimes, however, it becomes necessary to incubate the microbes like psychrotrophs much below the ambient temperature. BOD incubators are design to provide and maintain constant temperature much below the ambient temperature. An incubator is very similar to oven in construction however its low thermostats are designed in such a way to maintain low temperature (below 80°C). It is not used for sterilization purpose but for incubating and maintaining cultures at constant desired temperature above ambient temperature. Incubators are usually provided with double doors the inner one made of glass so that the contents of incubator may be viewed without disturbing temperature conditions of the cabinet as the incubators use dry heat for maintaining temperature there is possibility for dehydration and slow evaporation of culture medium, this can be prevented by keeping a breaker containing sterile water inside the incubator.

LAMINAR AIR FLOW UNIT:

All the microbiological operations are to be carried out under aseptic conditions to prevent contamination. Similarly when we handle hazardous and pathogenic organisms, precautions should be taken. Laminar air flow cabinets provide aseptic working environment and also prevents spill over of pathogenic organisms. . There is a special filter system of high efficiency particulate air filter (HEPA) which can remove particles as small as 0.3mm. The laminar air flow is based on flow of air currents of uniform velocity along parallel flow lines which helps in transferring microbial cultures in aseptic condition. It is passed through the filters will not allow any kind of microbe to enter into the system.

Inside the chamber fluorescent tube and U.V tube is fitted. Due to the uniform velocity and flow of air current all microbial operations like transfer of cultures, pouring of media, plating etc can be carried without any contamination. Both horizontal and vertical laminar air flow cabinets are available.

OPERATION:

1. Open the front door of the laminar air flow cabinet and clean the platform with any disinfectant (preferably alcohol) with the help of smooth cloth.

2. Switch on the U.V light and continue for 3 min so as to decontaminate the chamber.
3. Switch off the U.V light and start work on platform.
4. All operations inside the cabinet should be carried our inflame zones of burner or spirit lamp.

PRECAUTIONS:

1. Don't get exposed to U.V light, wear black goggles.
2. Leave the shoes before entering to operate the apparatus.
3. Wash hands with detergent or soap before staring the work.
4. After finishing the work clean the platform with surgical spirit, switch on UV light and keep for 15 min before final switch off the cabinet.

AUTOCLAVE:

Autoclave is a common and the most essential instrument in every microbiology laboratory. It is used for sterilization of media (solid and liquid), heat stable liquids, heat resistant instruments, glass wares and rubber products. The autoclave is based on principle that saturated steam heats an object many times more efficiently than hot air at the same temperature. The increased pressure results in the evaluation of boiling point of water and produces steam with the high temperature. However, it is important to note that it is not the pressure that kills the organisms but the high temperature of the steam. The boiling point of water at 15 lbs pressure is 121^0C. Most of the organisms are killed at 121^0C (15 psi) in 15 minutes.

An autoclave is a double walled metallic vessel. The body is usually made up of steel. Both vertical and horizontal types of autoclaves are available, but for routine laboratory use vertical types are commonly used. Fully openable lid is provided with a pressure gauge for recording pressure, steam clock (exhaust valve) for air exhaustation, a safety valve to avoid explosions.

PRECAUTIONS:

1. Water level inside the autoclave should be well above the heating mantle.
2. Tighten the screw fasteners equally on all sides.
3. The required 15 psi pressure must be maintained constantly for the required period of time.
4. Over sterilization likely to change the composition of the medium. Agar may also lose the jellifying (solidifying) property.
5. Do not open the lid until the pressure gauge shoes 0 (The zero)

HOT AIR OVEN:

It is another common instrument of microbiology laboratory and it is used for sterilization of glass ware. It is based on the principle where sterilization is accomplished by dry heat or hot air. Hot air oven is most widely used instrument for sterilization of glass ware, dry heat is most reliable method for sterilization. Material that may be damaged by heat can be sterilized to protein denaturation and oxidative damage, toxic effect of elevated levels of electrolytes in a completely moisture free atmosphere. Bacterial spores are more resistant to heat. So they are killed with oxidation for coagulation. Flaming, incineration and hot air oven methods are based on dry heat principle. Hot air is bad conductor of heat and its penetrating power is low. The oven is usually heated electrically with heating elements in the wall of chamber it should not be overloaded and material should be arranged in such a way to allow free circulation of air between the objects. Glass ware should be preferably dried before placing in the oven. Test tube and flasks should be wrapped in foil. The oven must be allowed to cool slowly for about 2 hours before the door is opened because the glass ware may break due to sudden un even cooling. A holding period of 160^0C for 2 hours is used to sterilize the glass ware like Petri plates, test tubes, pipettes, metal instruments like forceps, spatula, glass syringes and some pharmaceutical products like dusting powders, liquid paraffin, mineral oils and grease, rubber materials except silica rubber can with stand high temperatures can also be sterilized by using hot air oven. Cutting instruments such as those used in ophthalmic surgeries should be sterilized for 2 hrs at 150^oC. Oils, dusting powders require 150^oC for 1 hr for sterilization.

S.No	Temperature (^{0}C)	Sterilization time (minutes)
1.	120	480
2.	140	180
3.	150	150
4.	160	120
5.	170	60
6.	180	20

COMPOUND MICROSCOPE:

Construction and Working:

Microscope is defined as an optical instrument comprising of lens or combination of lenses. It enables to magnify image. Microscope is of 2 types

1. Simple Microscope
2. Compound Microscope

SIMPLE MICROSCOPE: It is also called as dissecting microscope it consists of one set of lanes and gives lower magnification. It mainly helps to relieve the morphological characters of the compound.

COMPOUND MICROSCOPE: It consists of two sets of lenses 1. Eye piece 2.Objective.

Compound microscope mainly consists of 3 major systems

a. Support System
b. Illumination System
c. Magnification System

Support System: It consists of base, stage, body tube, mechanical stage inclination joint.

Illumination System: It consists of light source iris diaphragm and condenser; light source may be plane or concave mirror. Electrically illuminated by a tungsten filament lamp or halogen lamp.

Magnification System: This includes the set of lenses aligned such a manner that a magnified real image can be viewed. The object is a set of lenses placed near the object. It partially magnifies the object which can be observed through eye piece in a more magnified form.

Base: It forms the foundation of microscope.

Stage: It holds the object or slide.

Body Tube: It holds the object at the bottom and eyepieces at top.

Mechanical Stage: For planar, forward, back ward, left and right movement of object with the help of knob.

Inclination Joint: This help to incline the microscope to avoid the strain on neck and back.

Eye piece: It is used to observe the magnified real image.

Draw Tube: It is used to fit eye piece inside.

Resolving Nose piece: It holds 2, 3 or 4 objectives which can be resolved the aligned the required objective.

Objective: It produces the first magnification showing real inverted image it may be 6, 10, 40, 45, 100x.

Mirror or Light Source: It reflects the light through the sub stage condenser and stage aperture.

Iris Diaphragm: Light illuminating object may be adjusted with this it may be opened or closed to diminish the light falling on the condenser and hence making the object more or less bright.

Condenser: It allows the parallel beam of light to pass through the stage aperture.

Knob: It is used for vertical movement of condenser and adjustment of knob. In case of compound microscope knob. It is of 2 types

A) Course adjustment knob
B) Fine adjustment knob

Course adjustment knob: It is used to focus on object

Fine adjustment knob: It helps to focus the specimen reveal the fine characteristics features.

Magnification System:

The objective lens is placed closed to the object to be viewed and the ocular lens is placed closed to the eye. The primary enlargement of the object is produced by the objective

lens. The image produced this is transmitted to ocular lens where final enlargement occurs. Therefore the magnifying capabilities of compound microscope are the product of magnification of objective and ocular lenses. For example using an objective lens of 40X ocular lens of 10X total magnification is 400X this magnification of microscope depends on following factors.

Optical tube length, focal length of objective, magnification of eye piece Primary magnification =optical length/focal length of objective.

The optical tube length of microscope is the d/w upper focal plane of objective and the lower focal plane of eyepiece or where the primacy image is formed the image produced by objective is real inverted image close of focal plane of eye piece. The eye piece is used in the compound microscope or simple magnifiers image produced by the objective. The image produced by the eyepiece is virtual inverted magnified image. It is fixed inside the draw it is used to observe more magnified real image. Eyepiece of 5x, 10x and 15x are available.

RESOLVING POWER OF OBJECTIVE:

An important property of microscope is its resolving power it is detained as its ability to distinguish 2 points that are closer together as distinct and separate. The greater the resolving power greater is efficiency of microscope it depend on the wave length of light optical property that is numerical aperture. This relationship can be expressed as

Resolving power= λ /NA where λ is wave length of light

NA is numerical aperture of objective lens.

WORKING DISTANCE:

It is the distance b/w the objective and the object. It decreases with increase in magnification.

FOCUSSING:

Focusing an objective is viewing through the eye piece that is adjusting of working distance this is done with coarse and fine adjustment of knob. Coarse adjustment knob is used to bring the object bin field of view fine adjustment knob is related to get the short image.

PRECAUTIONS:

1. Observe the slide with both eyes open to have less strain on eyes.
2. Always focus your slide slowly and carefully and with low power objective.
3. Keep the microscope and its parts clean and hands with care.

EXPERIMENT-02

STERILISATION OF GLASS WARE PREPARATION & STERILISATION OF MEDIA

AIM: To sterilize glassware, prepare media and sterilize the media prepared.

REQUIREMENTS: agar, peptone, beef extract or meat extract, NaCl, distilled water, Test tubes, measuring cylinder, conical flask, non absorbent cotton, pH meter , autoclave, Hot air oven.

PRINCIPLE:

Sterilization of equipment and materials is carried by:

Wire loop: Heat to redness in Bunsen burner flame.

Empty glassware: pipettes and Petri dishes :hot air oven at 160 °C for 2 hours, allowing additional time for items to come to temperature.

Culture media and solutions: Autoclave/pressure cooker.

Glass spreaders and metal forceps: Flaming in alcohol (70 % IDA).

Culture medium is a nutrient preparation which provides a balanced mixture of required nutrients that will permit good growth of microbes or other cells. Isolation, growth and, maintenance of microbes necessitates the preparation of suitable medium. Although all organisms need sources of energy, carbon, nitrogen, phosphorous, sulphur and various minerals, the composition of the medium will depend on the species to be isolated and cultured. Culture media are based on their components function and use . Depending on physical state; media are classified into 3 types.

1. **SOLID MEDIA:**

Solid media are needed for surface cultivation of microbes. Agar is widely used as a solidifying agent as it dissolves in boiling water and solidifies at about 40-42^0 C. Agar is a sulphated polymer composed of D-glucose, 3, 6-anhydro levo-galactose, D-galacturonic acid. It is prepared by addition of 105% agar to the corresponding liquid or broth medium. It is also an excellent hardening agent because most organisms cannot degraded it

2. **SEMI SOLID MEDIUM:**

It is prepared by the addition of 0.1-0.4% agar to the liquid medium. These media are used for demonstrating the motilities of the bacteria.

3. **LIQUID OR BROTH MEDIUM:**

It is prepared without adding solidifying agent. These media are ideal for physiological and biochemical studies.

Nutrient agar and nutrient broth are routinely used in the laboratory hence they are referred as general purpose media. These media are essential in the isolation and identification of microbes. These media are frequently used in water and food analysis, industrial microbiology and also to find out antibiotic sensitivity.

PROCEDURE

COMPOSITION OF NUTRIENT AGAR

It is a solid medium and it's composition is

Agar-15 G
Peptone-5G
Beef extract-3G
NaCl-5G
Distilled water-1000ml

PREPARATION OF NUTRIENT AGAR

1. Dissolve required quantity of given chemicals in 1000ml distilled water.
2. Find out the pH of the solution by pH meter/pH paper.
3. Adjust pH (if required) by NaOH and HCl solution drop by drop to pH 7.0.
4. Now, put a cotton plug in the mouth of the conical flask and cover it with the aluminum foil.
5. Sterilize the medium in autoclave for 15-20 minutes at a pressure of 15 lbs and temperature of 121^0c.

PREPARATION OF AGAR SLANT

After allowing the tubes containing agar medium to cool down to $45\text{-}60^0$C keep them in slanting position by resting the plugged ends over a glass rod on the table and leave the test tube until the medium is cooled to room temperature.

COMPOSITION OF NUTRIENT BROTH

It is liquid medium and it's composition is
Peptone-5 G
Beef extract-3 G
NaCl-5 G
Distilled water-1000ml

PREPARATION OF NUTRIENT BROTH:

All steps are similar to the preparation of nutrient agar except the addition of agar. All ingredients are weighed and dissolved in water. Agar is not added in broth preparation and the sterilized liquid medium is directly distributed in to flask and culture tubes.

REPORT:

EXPERIMENT-03

SUBCULTURING OF BACTERIA & FUNGI
NUTRIENT STAB & SLANT PREPARATIONS

AIM: To learn the techniques of aseptic transfer of culture or a specimen in to a given nutrient media.

REQUIRMENTS: Inoculation needle, spirit lamp, cotton, culture

PRINCIPLE:

A pure culture consists of only one species of microbes. If another species of microbes is accidentally introduced in to the pure culture then the culture is contaminated and it is called mixed culture. To get a pure culture, culturing and sub culturing of bacteria should be done in a condition or environment which is free from all types of microbes (to avoid contamination). This condition is called aseptic condition and the procedure for obtaining and maintaining pure culture in this aseptic condition is called aseptic transfer technique.

Inoculation of culture in to media for the cultivation and identification of smears is both fundamental and important.

PROCEDURE:

A.BROTH CULTURE:

1. Clean with disinfectant.

2. Place the Bunsen burner in front of you, all tubes and other equipment in a suitable location which will allow you to reach them without any difficulty and without burning itself.

3. Take in one hand tube containing broth culture or one containing sterile nutrient broth.

4. Take inoculation loop with other hand and flame the entire wire to redness.

5. Remove the plugs from the tube by grasping them between the fingers of the hand holding the inoculation instrument.

6. Be careful not to bring plugs near to the Bunsen burner.

7. Flame the mouth of broth tubes, insert inoculating loop into the culture and obtaining loopful of inoculums.

8. Introduce the inoculum in to the tube of sterile medium by immersing the loop full of culture in broth.

9. In removing the inoculation loop touch it to the inner surface of tube in order to remove any left out inoculum.

10. Flame the mouth of the tube again and replace the plugs in the respective tubes.

11. Flame the inoculating loop again to redness to put the loop down and label the tube with microbes used, date your name and initials.

12. Incubate at room temperature for 48 hours or until next laboratory period.

B.AGAR SLANT TRANSFER:

1. Take in one hand tube containing agar slant, one tube containing sterile nutrient broth. Carry out preliminary steps flaming of inoculating needles, removing plugs and flaming the mouth of the tube as described in stage-A.

2. Insert the inoculating needle into the tube containing agar slant culture and obtaining inoculum by removing a small portion of surface growth.

3. Do not dig into the agar slant, immerse the inoculum into the tube of nutrient broth medium and gently shake the inoculating media to free the microbes adhering to it.

4. Flame the mouth of the tubes and replace the plugs in to the respective tubes.

5. Flame the inoculating loop again to redness to put the loop down and label the tube with microbes used, date your name and initials.

6. Incubate at room temperature for 48 hours or until next laboratory period.

C.LOOP TRANSFER:

1. Take one tube containing the broth culture and sterile nutrient agar slant grasp them as indicated previously in addition to have agar slant transfer upwards. So that you can see it clearly.

2. Carry out preliminary aseptic steps of flaming and plug removal as described previously.

3. Flame the inoculation loop as described previously and remove the inoculum from the culture tube.

4. Place the loop down on the surface of the agar slant at the bottom of the tube.

5. Flame the mouth of the tube and replace the cotton plugs.

6. Flame the inoculation loop and label the freshly inoculated tube as described in part-A and B.

7. Repeat this procedure and incubate the tubes at room temperature for 48 hours.

REPORT:

EXPERIMENT-4a

SIMPLE STAINING OF BACTERIA

AIM: To identify the given bacterial culture by simple staining.

PRINCIPLE: Simple staining requires only one type of stain. Basic dyes such as methylene blue, crystal violet or safranin are used for simple staining. By this staining it is easy to study gross morphology of the organisms. Basic dyes like crystal violet are positively charged and negatively charged cell wall of bacteria can easily absorb such dyes.

REQUIRNMENTS: Sprit lamp, inoculation needle, glass slide, bacterial culture, glass rod, distilled water, immersion oil, microscope with 10X, 40X and 100X objectives, dyes such as methylene blue, crystal violet or safranin.

PROCEDURE:

1. Prepare the smear of given bacteria.
2. Spread the staining solution over the smear and keep it for about 30seconds.
3. Wash off the stain using distilled water and dry the slide with blotting paper.
4. Clean the underside of the slide and observe under 10X, 40X and 100X objectives of the microscope.

OBSERVATIONS AND RESULTS: Cells stain uniformly with sample staining. Bacterial cells stained with different staining solutions take the following colours methylene blue- blue, crystal violet-violet, safranin- pink.

REPORT:

EXPERIMENT-4b

DIFFERENTIAL STAINING (GRAM'S STAINING)

AIM: To identify the given bacterial culture by gram staining method.

PRINCIPLE: The gram staining, developed in 1884 by Christian gram, is the most widely employed staining method in bacteriology. In this staining two stains are used, the first stain (Crystal violet) is called primary stain and the second one is called counter stain (safranin). Iodine solution functions as mordant i.e the iodine increase the interaction between the cell wall and the dye so that the cell is stained more strongly. The cell wall of gram negative bacteria consists of more lipopolysaccharides and hence the primary stain is decolourised with alcohol treatment. In case of gram positive bacteria cell wall consists of more peptidoglycans and hence the primary stain the crystal violet is retained even after alcohol treatment. Thus gram positive bacteria retain violet colour and gram negative bacteria appears pink upon safranin treatment.

S.No	Cell component/treatmentWith grams staining reagent	Gram positive	Gram negative
1	Peptidoglycans in cell wall	90%	10%
2	Lipids	Less	More
3	Teichoic acid	Present	Absent
4	Crystal violet	Violet	Violet
5	Iodine	Mordant	Mordant
6	Alcohol	Dehydration	Decolourisation
7	Saffronin	Violet	Pink

REQUIRMENTS: Young culture of Bacillus cereus (Gram +ve), Escherichia coli (Gram-ve), gram stain(Crystal violet, gram's iodine solution and 95% of ethyl alcohol) distilled water, dropper, inoculating needle, blotting paper, sprit lamp, microscope with oil immersion objective.

PROCEDURE:

1. Prepare the smears of the two bacteria on two separate slides.
2. Spread crystal violet over both the smears for 30 seconds and then wash off.

3. Then spread Gram's iodine solution over the stained smears for about 30 seconds and when it takes colour wash off iodine. Decolourise the smear using 95% ethyl alcohol, later wash with distilled water and then dry.
4. Safranin which acts as a counter stain is spread over the decolourised smear for about 30 seconds and then wash the slide.
5. Allow the slide to dry.

PRECAUTIONS:

1. Always use young cultures not old than 24 hours.
2. Avoid excess heating during fixing of smears.
3. Decolourisation should be stopped when most of the colour is removed.

OBSERVATION AND RESULTS: Examine the slides microscopically using oil immersion objective. Identify and classify both the bacteria culture with gram's reaction. Make sketches of the morphology of culture and describe the morphology and arrangement of cells. Bacteria which appear as purple rods are referred as gram+ve and those appearing as pink rods are referred as gram-ve bacteria.

REPORT:

EXPERIMENT-4c

ACID FAST STAINING

AIM: Acid Fast Staining of Bacteria (Mycobacterium species)

PRINCIPLE:

The acid fast staining was first developed by Ehrlich in 1882. He found that *Tuberculi bacilli* retained a dye reagent composed of crystal violet and aniline in water even after a wash treatment with an acidified ethanol solution. Acid fastness is due to the high lipid content of cell walls, in particular, mycolic acid-a group of branched chain hydroxyl lipids.

In the staining procedure, the primary dye, acid fuchsine is formulated with phenol to allow permeation through the wax-like cell walls of the mycobacteria. The slide is usually heated in order to facilitate permeation. Ethanol is employed as the decolourizer. However, the reagent is prepared with hydrochloric acid in order to aid in the decolourization of non-acid cells.

REQUIREMENTS: *Mycobacterium smegmatis,* 48 hours Trypticase Soy Agar (TSA) slant, *Staphylococcus aureus,* 24 hours Trypticase Soy Agar (TSA) slant, sputum of TB patient, acid fast reagent set Zeil-Neelson reagent, staining rack etc.

PROCEDURE:

1. Prepare separate air dried and heat fixed smears of *Mycobacterium smegmatis, Staphylococcus aureus,* and sputum.
2. Place the slides on the staining rack.
3. Cover the smear with carbolfuchsin. Allow to stand for 30-60 seconds before heating.
4. Heat the preparations gently by passing over the Bunsen burner under the slides. Continue heating until steaming is observed when the flame is removed.
5. Maintain steaming of five minutes. Add more dye as needed to prevent drying out of the smear.
6. Allow slides to cool on the staining rack, and then rinse them gently in flowing tap water.
7. Decolorize with acid alcohol, using the same procedure as in the gram stain method. Rinse as above.
8. Cover smears with methylene blue reagent for one minute.
9. Rinse as above, blot dry and observe with the oil immersion lens.

OBSERVATION AND RESULTS:

Organisms positive for this stain appear in chains of **red or pink** coloured rods and negative for this stain appear as chain of **blue** coloured rods.

REPORT:

EXPERIMENT-5

ISOLATION OF PURE CULTURE BY MULTIPLE STREAK AND OTHER TECHNIQUES

AIM: Isolation of pure culture by the use of plate, streak plate and spread plate techniques.

REQUIREMENTS: 24 hours nutrient broth culture of bacillus, E.coli, sterile nutrient agar, Sterile Petri plates, marking pencil.

PRINCIPLE:

Pure culture is the culture which contains descendants obtained from single patent cell. The microbial population in environment (air, water, soil) includes many species of bacteria, fungi, algae which usually exists in mixed cultures. To study these microbes pure culture is required. Generally two techniques that is streak and pour plate methods are used to isolate pure culture. In streak plate method a loop full of culture is streaked across an agar medium. The streaking is done in different directions. This thins out bacteria and the individuals are separated from each other. In case of pour plate technique the mixed culture is diluted in the tubes of agar and cooled to the temperature of 40^0C. After the dilution process the agar culture are poured into Petri plates and kept for incubation and the separated colonies will be observed. Pour plate method was first developed by Robert Koch. The streak plate procedure is another example of isolation technique and it was developed by loeffer and gaffey.

PROCEDURE:

POUR PLATE TECHNIQUE:

1. Take sterile Petri plate and write your name with marking pencil and microorganism used and temperature of incubation on the bottom of the plate.
2. Place the agar tube into the boiling water bath for melting
3. Wipe the tube to remove any superficial moisture
4. Inoculate the melted agar deep with bacillus and E. coli and mix the contents of the tube by placing the tubes between the palms and rotating it several times
5. After mixing remove the cotton plug. Flame the mouth of agar deep tube and raise the top of the Petri plate just enough to empty the inoculated agar into the Petri dish
6. Rotate the Petri plate gently to distribute the agar evenly
7. After the agar has cooled and solidified incubate the Petri dish

STREAK PLATE TECHNIQUE:

1. Melt and cool a nutrient agar deep in a manner similar to the above method.
2. Wipe the outside of the tube to remove any water present.
3. Remove the cotton plug, flame the mouth of the tube and pour the content into the Petri plate.

4. Rotate the Petri plate and allow the agar to cool and solidify.
5. Remove the loop full of growth from the tube of bacillus and E.coli using aseptic techniques.
6. Take the agar plate in the left hand so that the Petri plate bottom rests on the palm of the hand and the top can be manipulated up and down with the thumb.
7. Lift the Petri plate cover and place the inoculums at the edge of agar farthest from you. Streak the inoculums from side to side lines covering approximately one quarter of the plate.
8. Lower the top of the plate and flame the inoculating loop.
9. Rotate the Petri plate on quarter of a full turn. Raise the top and cool the inoculating loop by touching the agar surface away from the set of streaks.
10. Lower the top of the Petri plate and follow the same steps once again with another plate. Incubate the Petri plates for 24 - 48 hours.

SPREAD PLATE TECHNIQUE:

PRINCIPLE:

It is helpful in the separation of organism which is in a low population density in a mixed population of microorganisms. In this technique microorganisms spread on the solidifying agar medium with a sterile L shape glass rod.

REQUIREMENTS:

Nutrient broth culture of bacillus, E.coli, Nutrient agar plate, L shaped glass rod, 95% alcohol.

PROCEDURE:

1. Inoculate nutrient agar plates with bacillus and E.coli.
2. Sterilize the bent glass rod by dipping in 95% alcohol.
3. Aseptically transfer loop full of culture in the centre of the appropriately labeled nutrient agar plates.
4. Place the inoculated plate on the turn table. Remove the cover of Petri dish and spin the turn table. Lightly touch the sterile bent rod to agar surface and move it back and froth while the turn table is spinning for spreading the culture over the agar surface.
5. Replace the Petri dish cover when the turn table stops spinning. Immerse the bent rod in alcohol and re - flame to sterilize.

OBSERVATION:

REPORT :

EXPERIMENT-6

MICROBIAL ASSAY OF PENCILLIN (CUP PLATE METHOD)

AIM: To determine the potency of given sample of pencillin and to construct a standard graph.

REQUIREMENTS: Sterile petri plates, sterile pipettes, test tubes, boiling tubes and nutrient agar.

COMPOSITION:

Meat extract – 0.3%, peptone – 0.5%,Agar – 2%, Sodium chloride – 0.5%,distilled water up to 100 ml.

TEST ORGANISM: *Staphylococcus aureus.*

PRINCIPLE:

The inhibition of microbial growth under the standard condition may be utilized for demonstrating the therapeutic efficiency of an antibiotic. Any subsequent change in the antibiotic molecule which may not be detected by chemical methods can be revealed by microbial activity. Here, microbial assays are very useful to know the possible loss of antibiotic activity. There are also routinely employed to determine the potency of all antibiotic preparations at various stages of development from their crude forms to finished product. The microbial assay is based upon the comparision of inhibition of bacterial growth by measured amount of antibiotic to be examined with that produced by the known concentration of standard preparation of the antibiotic having known activity.

PROCEDURE:

For the microbial assay of penicillin, cup plate method is employed. The melted agar medium which is previously inoculated with the sensitive organism is poured into a petridish of uniform depth of 3.4mm. 2 or 3 plates are prepared for each concentration of penicillin upon solidification of medium, 4 cavities are made at equal distances with a cup borer aseptically.

Penicillin stock solution of 100ug/ml was prepared by dissolving 20mg of penicillin in 33.4ml of sterile water. From the stock solution, different concentrations of 5ug/ml, 10ug/ml, 15ug/ml, 20ug/ml, 25ug/ml of penicillin were prepared. They are designated as S_1, S_2, S_3, S_4 and S_5 respectively. S_3 concentration is taken as reference concentration of penicillin (suppose S_1) and reference concentration (S_3) are poured aseptically in alternate petridish in cups.

The same is followed for other concentrations replacing with S_2, S_4 and S_5. In each plate 2 cups are filled with one concentration and two cups with another concentration (reference).

The petri plates are kept for sometime at room temperature to allow the antibiotic to diffuse in agar medium. Then the plates are incubated at 37ºC for 18hrs. After incubation, the diameter of zone of inhibition is measured.

Standard graph is obtained by taking log concentration on x-axis and zone of inhibition on y-axis.

REPORT: the concentration of unknown antibiotic sample was found to be…..

MICROBIAL ASSAY OF ANTIBIOTIC (DISC PLATE METHOD)

AIM: To determine microbial assay of penicillin by disc plate method.

APPARATUS: Sterile petriplates, petri dishes, forceps, boiling tubes and nutrient agar medium.

TEST ORGANISM: *Staphylococcus aureus*.

PRINCIPLE: The inhibition of microbial growth under the standard condition may be utilized for demonstrating the therapeutic efficiency of an antibiotic. Any subsequent change in the antibiotic molecule which may not be detected by chemical methods can be revealed by microbial activity. Here, microbial assays are very useful to know the possible loss of antibiotic activit

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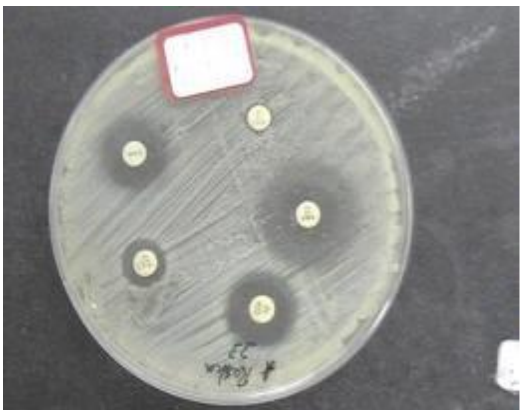
PROCEDURE:

The required materials were weighed and nutrient agar medium was prepared. It was sterilized by autoclaving at 121°C at 15lb pressure per inch for 15-20 min. after sterilization it was inoculated with *Staphylococcus aureus* and then the medium was transferred into petridish which was sterilized.

Different concentrations of penicillin (5, 10, 15, 20, 25 ug/ml) were prepared. Take the sterilized paper discs and dip in different concentrations of penicillin with forceps. In each plate, 2 paper discs were placed with one concentration and 2 paper discs with reference concentrations. Then the petriplates were kept aside for 45min for diffusion of antibiotic into agar. Then they are kept for incubation at 37°C for 18-24 hrs in an inverted position.

After incubation, the zone of inhibition was measured and standard graph was plotted on graph by taking log concentration on x-axis and inhibition zone on y-axis.



OBSERVATION:

REPORT: The concentration of unknown antibiotic sample was found to be…

EXPERIMENT-7

MOTILITY DETERMINATION BY HANGING DROP METHOD

AIM: To observe the motility of bacteria in a given bacterial culture by hanging drop method.

REQUIREMENTS: Sprit lamp, inoculation needle, cavity slides, cover slip, Vaseline, young bacterial broth culture, microscope with oil immersion object.

PROCEDURE:

1. Take a clean glass cavity slide and apply Vaseline around the cavity with the help of needle.
2. Using the sterile inoculation loop, place a drop of culture in the centre of cover-slip.
3. Invert the cavity slide gently over the cover-slip and se that the drop of culture is in the centre.
4. Press down on the edges of cover-slip and see that the Vaseline makes a seal. See that the drop does not touch the slide.
5. Quickly and carefully turn the slide right side up, so that the cover-slip is on the upper side and the drop is suspended from cover-slip.

OBSERVATION: Motility is much observed along the edges of the drop.

REPORT:

EXPERIMENT-9

BACTERIOLOGICAL ANALYSIS OF WATER

AIM: To determine the microbiological water Quality.

THEORY:

Natural water supplies such as river, lakes and streams contain sufficient nutrients to support growth of various microorganisms. Microorganisms enter in the water by domestic waste. Hence it is necessary to test the water quality. Water quality can be tested by the presence of some indicator organism. The most frequently used indicator organism is the coliform bacterium, *E.Coli*. But its presence does not prove the presence of pathogenic bacteria. But this establishes the possibility of the presence of such pathogenic bacteria. Thus presence of coliform bacteria in water is regarded as warning signal.

REQUIREMENT:

Nutrient agar medium, Water sample, Sterilized petriplate, conical flask, Glass spreader.

PROCEDURE:

1. Put 0.1ml of water sample on the nutrient agar medium.
2. Spread it with the help of spreader.
3. Now, incubate the plates at 37^0C for 18-24hrs.
4. Count the colonies appeared.

OBSERVATION:

Bacterial colonies appear on the surface of the medium. Count the colonies by Colony counter and record the average number of colonies of all plates.

No. of bacteria/100ml= No. of colonies x 1000

RESULT:

EXPERIMENT-10

BIOCHEMICAL TEST

IMVIC tests includes four tests i.e., Indole test, Methyl red, Voges-Proskauer and citrate tests. These tests are meant to differentiate the Gram-negative intestinal bacilli (enteric bacilli)

AIM: To study the characterization of microbes through biochemical reactions.

PRINCIPLE: Some bacteria oxidise tryptophan resulting in the formation of indole, pyruvic acid and ammonia. Indole thus formed reacts with Kovac's reagent (Para dimethyl amino benzaldehyde) resulting in the formation of cherry red coloured complex.

Tryptophan→Indole+Pyruvic acid+ammonia

Indole→P-dimethylamino benzaldehyde→Quinoidal red-voilet complex

MATERIALS REQUIRED:

Nutrient broth culture of *Escherichia coli, Proteus vulgaris, Enterobacter aerogenes*: SIM agar deep tubes, Kovac's reagent, bunsen burner, inoculating needle, marking pencil.

PROCEDURE:

1. Inoculate each experimental organism in to its appropriately labeled deep tube by stab inoculation. Maintain one as control (Uninoculated)
2. Incubate the tubes at 37^0c for 24 hours
3. After incubation add 10 drops of Kovac's reagent to all deep tube cultures and agitate the cultures gently.

OBSERVATION AND RESULTS:

Development of cherry red colour on the top layer of the tube is positive for indole test, while absence of such red colouration is negative. For *E.coli* this is positive, while for *E.aerogenes* it is negative.

REPORT:

MR-VP TEST

AIM: To study the characterization of microbes through biochemical reactions.

MATERIALS REQUIRED: Nutrient broth, culture of *E.coli, Enterobacter aerogenes.* MR-VP broth tubes, methyl red, pH indicator, Barrit's reagent

PRINCIPLE:

Methyl red and voges- proskauer tests are performed simultaneously on the same medium and they are used to distinguish bacteria that produce large amount of acid and those that produce neutral product acetone as the end product. Opposite results are usually obtained for the Methyl red and voges- proskauer test. If organisms produce large amount of organic acids from the glucose, the medium turns acidic and methyl red remains red, while other organisms which do not produce acids, the pH of medium will remain above 6.0 and methyl red turns yellow.

MR-VP test is of value in the separation of *E.coli, Enterobacter aerogenes* which are identical except these characters. This test is also useful as an indicator of sanitary quality of water.

PROCEDURE:

1. Take 5 ml of MR-VP broth in each tube and sterilize at 15 lbs pressure for 15 minutes.
2. Inoculate two MR-VP tubes with *E.coli* and another two with *Enterobacter aerogenes* and incubate at 35^0c for 48 hours
3. Uninoculated tube serves as control.
4. At the end of incubation period, add 1-2 drops of methyl red and 2-3 drops of reagent II to each tube and shake well after removing the caps so as to expose optimum amount of oxygen.
5. Allow the reaction to complete for 15-30 minutes
6. Observe the colour changes in microbial culture broth.

OBSERVATIONS AND RESULTS:

Methyl red remains as red in *E.coli* inoculated broth is a positive for MR test, while *Enterobacter aerogenes* methyl red gets decolorized and turn yellow indicating negative for MR test.Appearance of crimson to ruby pink colour is a positive for VP test in *E.coli* culture broth, while no colour change is indicative of negative in *Enterobacter aerogenes*.

REPORT:

CITRATE TEST

AIM: To study the characterization of microbes through biochemical reactions.

MATERIALS REQUIRED: Cultures of E.coli, E.aerogenes, Simmons citrate agar.

PRINCIPLE:

This test is useful in differentiating enteric bacteria on the basis of ability to use citrate as the sole carbon source.

1. Citric acid → Oxalo acetic acid+ Acetic acid → Pyruvic acid + excess $C0_2$
2. $C0_2 + 2Na^+ \rightarrow Na_2CO_3$ → Alkaline PH →colour change from green to blue

   The organism capable of utilizing sodium citrate turns the medium alkaline which is indicated by the use of bromomethyl blue in the medium. The organism which is positive for this test changes medium colour from green to blue.

PROCEDURE:

1. Prepare the Simmons citrate agar medium and adjust the PH to 6.9 and sterilize at 15 lbs pressure for 15 minutes and prepare the slants.
2. Inoculate one set of slant with *E.coli* and other with *E.aerogenes* and incubate for 48 hours at 37^0c.

OBSERVATIONS AND RESULTS:

At the end of incubation period, observe for growth and coloration of the medium. *E.coli* is a citrate positive as colour of the medium turns to blue, while in *E.aerogenes* slants there will be no growth and no change in the colour in the medium and it is citrate negative.

REPORT: