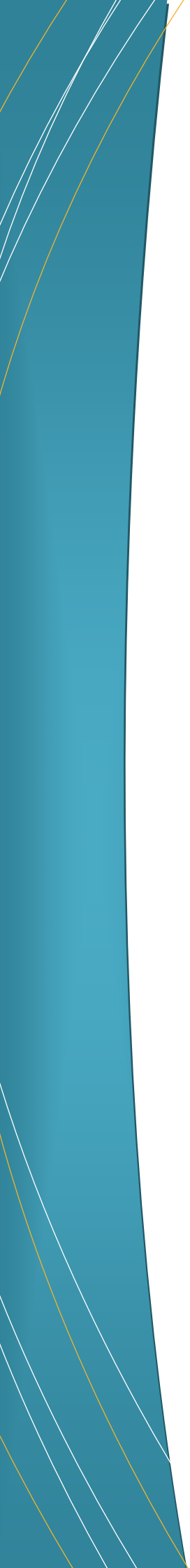
Laboratory Manual

VMC 211

GENERAL MICROBIOLOGY



LABORATORY MANUAL FOR GENRAL MICROBIOLOGY

GENERAL MICROBIOLOGY

VMC-211 (New Syllabus)

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LABORATORY MANUAL FOR GENERAL MICROBIOLOGY

CERTIFICATE

Certified that this is a bonafide record of practical work done in the laboratory for the course of **GENERAL MICROBIOLOGY** course No. VMC-211 during the year_____.

Name of the student: _____

Registration No.: _____

Exam seat No.: _____

Course Teacher

SEMESTER END EXAMINATION

Evaluated The Practical Record Submitted For The SEMESTER END Practical Examination Held On _____.

Course Teacher **Sectional Head**

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Practical 1

EQUIPMENTS, SETUP OF MICROBIOLOGY LABORATORY AND GENERAL INSTRUCTIONS

In this course, microorganisms isolated from infected animals and humans are handled. Certain rules and regulations are necessary to avoid the the possibility of zoonotic infection.

Any disregard to these rules or exhibits carelessness endangers the health of students handling/working in the laboratory.

Following rules are outlined for the safety of laboratory worker/students handling the microorganisms in the laboratory.

1.Microbiological procedures:

a. Reporting all spills and broken glasswares to laboratory technician.
b. Minimizing production of aerosols.
c. Washing hands prior to and following laboratories and at any time contamination is suspected
d. Never eat or drink in the laboratory
e. disinfecting lab benches prior to and at the conclusion of each lab session
f. identification and proper disposal of different types of waste
i. never applying cosmetics, including contact lenses, or placing objects (fingers, pencils) in the mouth or touching the face
j. reading and signing a laboratory safety agreement indicating that the student has read and understands the safety rules of the laboratory
k. good lab practice, including returning materials to proper locations, proper care and handling of equipment, and keeping the bench top clear of extraneous materials

2. Protective procedures: including a. tying long hair back, wearing personal protective equipment (eye protection, coats, closed shoes; glasses may be preferred to contact lenses), and using such equipment in appropriate situations b. always using appropriate pipetting devices and understanding that mouth pipetting is forbidden

3. Emergency procedures: including

a. locating and properly using emergency equipment (eye-wash stations, first-aid kits, fire extinguishers, chemical safety showers, telephones, and emergency numbers)
b. reporting all injuries immediately to the instructor
c. following proper steps in the event of an emergency

In addition, institutions where microbiology laboratories are taught will

1. train faculty and staff in proper waste stream management
2. provide and maintain necessary safety equipment and information resources
3. train faculty, staff, and students in the use of safety equipment and procedures
4. train faculty and staff in the use of MSDS. The Workplace Hazardous Materials Information System (WHMIS) requires that all hazardous substances, including microorganisms, be labelled in a specific manner. In addition, there must be a Material Safety Data Sheet (MSDS) available to accompany each hazardous substance. MSDS sheets

are now supplied with every chemical sold by supply houses. The person in charge of the microbiology laboratory should ensure that adherence to this law is enforced.

All laboratory work can be done more effectively and efficiently if the subject matter is understood before coming to the laboratory. To accomplish this, read the experiment several times before the laboratory begins.

Know how each exercise is to be done and what principle it is intended to convey. Also, read the appropriate sections in your textbook that pertain to the experiment being performed, this will save you much time and effort during the actual laboratory period. All laboratory experiments will begin with a brief discussion by your instructor of what is to be done, the location of the materials, and other important information.

Feel free to ask questions if you do not understand the instructor or the principle involved.

Much of the work in the laboratory is designed to be carried out in groups or with a partner. This is to aid in coverage of subject matter, to save time and expense, and to encourage discussion of data and results.

I have read the above rules and understand their meaning

_____

Signature

_____

Date

GENERAL PRECAUTIONS AND LABORATORY SAFETY PROCEDURES

General Precautions

1. All health-care workers should routinely use appropriate barrier precautions to prevent skin and mucous-membrane exposure when contact with blood or other body fluids of any patient is anticipated. Gloves should be worn for touching blood and body fluids, mucous membranes, or non-intact skin of all patients, for handling items or surfaces soiled with blood or body fluids, and for performing venipuncture and other vascular access procedures. Gloves should be changed after contact with each patient. Masks and protective eyewear or face shields should be worn during procedures that are likely to generate droplets of blood or other body fluids to prevent exposure of mucous membranes of the mouth, nose, and eyes. Gowns or aprons should be worn during procedures that are likely to generate splashes of blood or other body fluids.

2. Hands and other skin surfaces should be washed immediately and thoroughly if contaminated with blood or other body fluids. Hands should be washed immediately after gloves are removed.

3. All health-care workers should take precautions to prevent injuries caused by needles, scalpels, and other sharp instruments or devices during procedures; when cleaning used instruments; during disposal of used needles; and when handling sharp instruments after procedures. To prevent needlestick injuries, needles should not be recapped, purposely bent or broken by hand, removed from disposable syringes, or otherwise manipulated by hand. After they are used, disposable syringes and needles, scalpel blades, and other sharp items should be placed in puncture-resistant containers for disposal.

4. Health-care workers who have exudative lesions or weeping dermatitis should refrain from all direct patient care and from handling patient-care equipment.

5. The following procedure should be used to clean up spills of blood or blood-containing fluids:

(1) Put on gloves and any other necessary barriers.

(2) Wipe up excess material with disposable towels and place the towels in a container for sterilization.

(3) Disinfect the area with either a commercial or approved germicide or household bleach (sodium hypochlorite). The latter should be diluted from 1:100 (smooth surfaces) to 1:10 (porous or dirty surfaces); the dilution should be no more than 24 hours old. When dealing with large spills or those containing sharp objects such as broken glass, first cover the spill with disposable toweling. Then saturate the toweling with commercial germicide or a 1:10 bleach solution and allow it to stand for at least 10 minutes. Finally clean as described above.

Precautions for Laboratories

Blood and other body fluids from clinical cases should be considered infective.

1. All specimens of blood and body fluids should be put in a well-constructed container with a secure lid to prevent leaking during transport. Care should be taken when collecting each specimen to avoid contaminating the outside of the container and of the laboratory form accompanying the specimen.

2. All persons processing blood and body-fluid specimens should wear gloves. Masks and protective eyewear should be worn if mucous membrane contact with blood or body fluids is anticipated. Gloves should be changed and hands washed after completion of specimen processing.

3. For routine procedures, such as histologic and pathologic studies or microbiologic culturing, a biological safety cabinet is not necessary. However, biological safety cabinets should be used whenever procedures are conducted that have a high potential for generating droplets. These include activities such as blending, sonicating & vigorous mixing.

4. Mechanical pipetting devices should be used for manipulating all liquids in the laboratory. Mouth pipetting must not be done.

5. Use of needles and syringes should be limited to situations in which there is no alternative, and the recommendations for preventing injuries with needles outlined under universal precautions should be followed.

6. Laboratory work surfaces should be decontaminated with an appropriate chemical germicide after a spill of blood or other body fluids and when work activities are completed.

7. Contaminated materials used in laboratory tests should be decontaminated before reprocessing or be placed in bags and disposed of in accordance with institutional policies for disposal of infective waste.

8. Scientific equipment that has been contaminated with blood or other body fluids should be decontaminated and cleaned before being repaired in the laboratory or transported to the manufacturer.

9. All persons should wash their hands after completing laboratory activities and should remove protective clothing before leaving the laboratory.

10. There should be no eating, drinking, or smoking in the work area.

Exercise:

1. Enlist the equipments /instruments available in your microbiology laboratory along with its technical use.
2. What is GLP & GMP ?
3. Write the difference between Laminar Flow & Biosafety Cabinet, along with neat labelled diagram of air flow.

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Practical 2 & 3

STERILIZATION & DISINFECTION

Definition

According to the CDC (Centers for Disease Control and Prevention), ***"Sterilization means the use of a physical or chemical procedure to destroy all microbial life, including highly resistant bacterial endospores***."

Sterilization is an absolute term, i.e. the article must be sterile meaning the absence of all microorganisms.

Although the chemical or physical process used to destroy all pathogenic microorganisms including spores is not absolute, when all parameters of the sterilization process have been met, instruments, supplies and equipment are thought to be sterile.

Sterilization is categorised into:

1. Heat Sterilization
2. Filtration
3. Radiation sterilization
4. Chemical sterilization

The common sterilization methods within each category:

I. Heat Sterilization

i. Dry Heat- Incineration
Hot Air Oven

ii. Moist Heat
Temperature below 100^{O}C
Temperature at 100^{O}C
Temperature above 100^{O}C

II. Filtration

III. Chemical

i. Ethylene Oxide (EtO)
ii. Low Temperature Plasma Vapor
iii. Glutaraldehyde
iv. Formaldehyde
v. Ethanol
vi. Chlorine Dioxide
vii. Ozone

IV.Radiation

i.Gamma
ii. Electron Beam (E-Beam)
iii. X-rays
iv. Infrared rays
v. UV rays

Whatever method of sterilization is chosen, the procedure must be validated for each type of material, both with respect to the assurance of sterility and to ensure that no adverse change should take place.

Biological indicators are used to validate sterilization methods and sometimes for routine control of individual cycles. Periodic revalidation is recommended.

I. Heat Sterilization

i. Dry-heat sterilization

In dry-heat processes, the primary lethal process is considered to be oxidation of cell constituents. Dry-heat sterilization requires a higher temperature than moist heat and a longer exposure time.

Incineration

870^0C - 980^0C

Complete burning to ashes

Used for soiled dressings, animal carcasses, pathological material, disposables, non-reusable soiled bedding

Flaming

250^0C – 300^0C

Points of forceps & Inoculation loops – heat in bunsen flame till red hot

Slow passage through flame to destroy vegetative bacteria on surface of scalpel blade, glass slides, mouths of test tubes

Hot Air Oven

Holding temp & time: 160^0C for 1 hr

Used for glassware, forceps, swabs, water impermeable oils, waxes & powders

Before placing in hot air oven

- Dry glassware completely
- Plug test tubes with cotton wool
- Wrap glassware in Kraft papers
- Don’t over load the oven.
- Allow free circulation of air between the material.

The method is, therefore, more convenient for heat-stable, non-aqueous materials that cannot be sterilized by steam because of its deleterious effects or failure to penetrate.

Preparations to be sterilized by dry heat are filled in units that are either sealed or temporarily closed for sterilization. The entire content of each container is maintained in the oven for the time and at the temperature given in the table below. Other conditions may be necessary for different preparations to ensure the effective elimination of all undesirable microorganisms.

Temperature (°C)	Minimum sterilization time (min)
160	60 mins
180	30 mins

Specific conditions of temperature and time for certain preparations are stated in individual monographs.

The oven should normally be equipped with a forced air system to ensure even distribution of heat throughout all the materials processed. This should be controlled by monitoring the temperature. Containers that have been temporarily closed during the sterilization procedure are sealed after sterilization using aseptic techniques to prevent microbial recontamination.

The bioindicator strain proposed for validation of the sterilization process is: spores of ***Bacillus subtilis*** (e.g. *var. niger* ATCC 9372) for which the D-value is 5-10 minutes at 160 °C using about 10^6 spores per indicator.

ii. Moist Heat

Moist heat - Temp below 100^0C

Pasteurization

- 63^0C – 30 min (Holder method)
- 72^0C – 15-20 sec (Flash method)
- 132^0C – 1 sec (Ultra high temp)

Vaccine baths - 60^0C – 60 min

- For vaccines of non-sporing bacteria

Water bath - 56^0C – 60 min – 3 days

- For serum / body fluids containing coagulable proteins

Inspissation – 80-85^0C – 30 min – 3 days

- For media containing egg or serum – LJ

Moist Heat - Temp at 100^0C

Boiling - 100^0C for 10 min.
Kills all vegetative bacteria.
Water should be soft, deionized or distilled.
2% sodium bicarbonate promotes the process.
Kills vegetative bacteria, hepatitis virus & some spores
Steaming (free steam) – 30-60 min in Arnold /Koch steamer
For heat labile media – DCA, TCBS
Tyndallisation (intermittent sterilization) - 100^0C, 30 min, 3 days. On I^{st} day all vegetative bacteria are killed. On II^{nd} & III^{rd} day spores that germinate are killed.
Nutrient media & media containing sugars or gelatin.

Moist heat - Temperature above 100^OC

Autoclave (steam under pressure) - 121^0C, 15-20 min, 15 lbs
Used for rubber articles, dressings, sharp instruments, infectious medical waste, culture media.

Sterilization control

- **Browne's tube (red-green), Bowie & Dick tape (white-brown)**
- **10^6 spore of *Bacillus stearothermophilus*. Incubate at 55^0C for 5 days**

Steam autoclave is the oldest, safest, and most cost effective method of sterilization. The steam reaches 121-148^{O}C (250-300^{O}F) in the pressure chamber at 15 P.S.I. The sterilization period is dependent on the temperature and size of load and can range from 10-60 minutes. This method is not well suited for heat sensitive materials and instruments.

The common types of steam sterilization cycles are *gravity-displacement*, which removes air from the chamber by gravity displacement as steam-entering chamber exerts pressure on air; and the *pre-vacuum* cycle, which removes air by a vacuum pump while steam is simultaneously injected into the chamber.

Exposure of microorganisms to saturated steam under pressure in an autoclave achieves their destruction by the irreversible denaturation of enzymes and structural proteins. The temperature at which denaturation occurs varies inversely with the amount of water present. Sterilization in saturated steam thus requires precise control of time, temperature, and pressure. As displacement of the air by steam is unlikely to be readily achieved, the air should be evacuated from the autoclave before admission of steam. This method should be used whenever possible for aqueous preparations and for surgical dressings and medical devices.

In the steam autoclave process, microorganisms are killed by heat, and this is accelerated by the addition of moisture. Steam by itself is not sufficient for sterilization, and pressure that is greater than atmospheric is needed to increase the temperature of steam for thermal destruction of microbial life.

Steam, for a specified time at required temperature, must penetrate every fiber and reach every surface of items to be sterilized. When steam enters the sterilization chamber under pressure;

- It condenses upon contact with cold items.
- This condensation frees heat, simultaneously heating and wetting all items in the load, thus providing heat and moisture.

Any living thing will be killed when exposed to saturated steam at 120^{O}C (250^{O}F) longer than 15 minutes. As temperature is increased, time may be decreased.

The recommendations for sterilization in an autoclave are **20 minutes at 121-124 °C (200 kPa)/15lb pressure.** The temperature should be used to control and monitor the process; the pressure is mainly used to obtain the required steam temperature.

Minimum sterilization time should be measured from the moment when all the materials to be sterilized have reached the required temperature throughout. Monitoring the physical conditions within the autoclave during sterilization is essential. To provide the required information, temperature-monitoring probes should be inserted into representative containers, with additional probes placed in the load at the potentially coolest parts of the loaded chamber (as established in the course of the validation programme). Each cycle should be recorded on a time-temperature chart or by other suitable means.

Application:

1. Steam autoclave is used mostly for surgical instruments & sharp instruments.
2. In certain cases, glass, porcelain or metal articles, liquids in vented containers are sterilized at 121 - 124 °C for 20 minutes.
3. Fats and oils may be sterilized at 121 °C for 2 hours but, whenever possible, should be sterilized by dry heat.
4. Used for rubber articles, dressings, infectious medical waste, culture media

In certain cases (e.g. thermolabile substances), sterilization may be carried out at temperatures below 121 °C, provided that the chosen combination of time and temperature has been validated. Lower temperatures offer a different level of sterilization; if this is evaluated in combination with the known microbial burden of the material before sterilization, the lower temperatures may be satisfactory. Specific conditions of temperature and time for certain preparations are stated in individual monographs.

The bioindicator strain proposed for validation of this sterilization process is: spores of ***Bacillus stearothermophilus*** (e.g. ATCC 7953) for which the D-value (i.e. 90% reduction of the microbial population) is 1.5-2 minutes at 121 °C, using about 10^6 spores per indicator.

Filtration

Sterilization by filtration is employed mainly for **thermolabile solutions**. These may be sterilized by passage through sterile bacteria-retaining filters, e.g. membrane filters (cellulose derivatives, etc.), plastic, porous ceramic, or suitable sintered glass filters, or combinations of these. Asbestos-containing filters should not be used.

Appropriate measures should be taken to avoid loss of solute by adsorption onto the filter and to prevent the release of contaminants from the filter. Suitable filters will prevent the passage of microorganisms, but the filtration must be followed by an aseptic transfer of the sterilized solution to the final containers which are then immediately sealed with great care to exclude any recontamination.

Usually, membranes of not greater than 0.22 µm nominal pore size should be used. The effectiveness of the filtration method must be validated if larger pore sizes are employed.

To confirm the integrity of filters, both before and after filtration, a bubble point or similar test should be used, in accordance with the filter manufacturer's instructions. This test employs a prescribed pressure to force air bubbles through the intact membrane previously wetted with the product, with water, or with a hydrocarbon liquid.

All filters, tubes, and equipment used "downstream" must be sterile. Filters capable of withstanding heat may be sterilized in the assembly before use by autoclaving at 121^{O}C for 15 - 45 minutes depending on the size of the filter assembly. The effectiveness of this sterilization should be validated. For filtration of a liquid in which microbial growth is possible, the same filter should not be used for procedures lasting longer than one working day.

Sterilization of of tissue culture media / thermolabile liquids :

The various types of filters used for clarifying or to remove the bacteria, fungi from the thermolabile liquids,media,solutions & buffers are as follows:

1. Earthenware Candles e.g., Berkfeld, Chamberland filters.
2. Asbestos Paper Disks e.g., Seitz Filter.
3. Sintered Glass Filters.
4. Membrane Filters.

1.Earthenware Candles

Berkfeld Filters:

Made from kieselguhr, a fossil diatomaceous earth found in deposits in Germany.Filters are of coarse type owing to the size of the granules forming the substance the substance of filter.

Made in three grades of porosity:

V: Veil (the coarsest) do not allow the *Serratia marscens,* the test bacteria to pass through).

W: Wenig (the finest).

N: Normal (the intermediate)

Filters can be sterilized by steaming/autoclaving. Filters should be brushed with a stiff nailbrush and than boiled in distilled water. When clogged with organic matter heated to redness in a muffle furnace and allowed to cool slowly.

Chamberland Filters:

Made up of unglazed porcelain and are produced in various grades of porosity, the finest grade allows only small viruses such as FMD virus, Circovirus.

Most porous grades **L_{1a}, L_2,** and **L_3** are comparable with **V, N,** and **W** candles respectively.

2. Asbestos Paper Disk Filters. Seitz Filters:

Disk of Asbestos is inserted into a metal holder (14 cm in diameter-Large size). Various sizes are available.

Made in three grades of porosity:

K : Clarifying.

N : Normal.

EK: Special grade.

Do not allow *Serratia marscens*, the test bacteria to pass.

For sterilization the filter is loosely assembled wit h the asbestos disk in position and the delivery tube passed through a rubber bang when filtering flask if used. The whole assembly is wrapped in Kraft paper and sterilized in autoclave. Plug the filtration flask and the side arm is fitted with an air filter.

Before using flush the disk with sterile saline and then screw down tightly the metal holder.

3.Sintered Glass Filters:

Made up of finely ground glass fused sufficiently to make small particles adhere, giving uniform average pore diameter (APD).

Manufactured in three grades of porosity:

Grade 5 : Finest.
Grade 3 : Coarsest.
Grade5/3: Special grade

After use sintered glass filters are washed with running water in the reverse direction. They should be cleaned with warm sulphuric acid + potassium nitrate.

4. Membrane Filters:

Two types of cellulose acetate membrane filters are available:

-Older type (Gradocol membrane) is composed of cellulose nitrate whereas the
-Modern membrane filters in use nowadays are made up of cellulose acetate.

Gradocol membranes: Made in different grades with average pore diameter ranging from 3um to 10 nm. Used to determine the size of many viruses.

Modern membrane filters (Cellulose acetate): Developed by Millipore Filter Corporation in America.

Common Chemical Sterilization

A. Ethylene Oxide (EtO) Gas

Ethylene Oxide gas was introduced in the 1950's, and it is an effective, low temperature chemical sterilization method. It also takes *longer* than steam sterilization, typically, 16-18 hours for a complete cycle. Temperatures reached during sterilization are usually in the 50-60□Crange.

Ethylene Oxide (EtO) is an industrial chemical used in sterilizing medical items, fumigating spices, and manufacturing other chemicals.

Pure EtO is a colorless gas at room temperature and a mobile, colorless liquid at –47^{O}C. Sold as a mixture with either carbon dioxide or fluorocarbon 12.

Ethylene oxide kills microorganisms by denaturing their proteins and subsequently modifying their molecular structure.

The active agent of the gas sterilization process can be ethylene oxide or another highly volatile substance. The highly flammable and potentially explosive nature of such agents is a disadvantage unless they are mixed with suitable inert gases to reduce their highly toxic properties and the possibility of toxic residues remaining in treated materials. The whole process is difficult to control and should only be considered if no other sterilization procedure can be used. It must only be carried out under the supervision of highly skilled staff.

The sterilizing efficiency of ethylene oxide depends on the concentration of the gas, the humidity, the time of exposure, the temperature, and the nature of the load. In particular, it is necessary to ensure that the nature of the packaging is such that the gas exchange can take place. It is also important to maintain sufficient humidity during sterilization. Records of gas concentration and of temperature and humidity should be made for each cycle. Appropriate sterilization conditions must be determined experimentally for each type of load.

After sterilization, time should be allowed for the elimination of residual sterilizing agents and other volatile residues, which should be confirmed by specific tests.

Bioindicator strains: spores of *Bacillus subtilis* (e.g. *var. niger* ATCC 9372) or of *Bacillus stearothermophilus,* (e.g. ATCC 7953).

There are some hazards associated with EtO use. Acute inhalation of high levels of EtO has resulted in nausea, vomiting, neurological disorders, bronchitis, pulmonary edema, and emphysema. Skin and eye contact with solutions of EtO has caused irritation of the eyes and skin in humans. Tests involving acute exposure of animals have shown EtO to have relatively high toxicity from oral and inhalation exposures.

A short-term effect of EtO in humans is mainly central nervous system depression and irritation of the eyes and mucous membranes. Chronic (long-term) exposure to ethylene oxide in humans can cause irritation of the eyes, skin, and mucous membranes, and problems in the functioning of the brain and nerves. Some human data show an increase in the incidence of leukemia, stomach cancer, cancer of the pancreas, and Hodgkin's disease in workers exposed to EtO. EPA has classified EtO as a Group B1 hazard (probable human carcinogen).

EtO is not only present in sterilizers but also (in small concentrations) in the environment. Sources of environmental EtO include automobile exhaust and tobacco smoke.

Ethylene oxide (EtO) is a chemical agent that kills microorganisms, including spores. EtO gas must have direct contact with microorganisms on the items to be sterilized. Due to EtO being highly flammable and explosive in air, it must be used in an explosion-proof sterilizing chamber in a controlled environment.
Items sterilized by this process must be packaged with wraps and be aerated. The aeration time may be long and is needed to make sterilized items safe for
handling and patient use.

Note: There are also gas sterilizers available that use a mixture of EtO with carbon dioxide or chlorofluorocarbon (CFC) to represent it as nonflammable for use in healthcare facilities. In addition to safety concerns, this type of sterilization process requires an even *longer* aeration process compared to pure EtO sterilization.

In general, EtO gas is a reliable and safe agent for sterilization when handled properly.

Application:
EtO is used to sterilize items that are heat or moisture sensitive.

Disadvantages of EtO gas are that it can leave toxic residues on sterilized items and it possesses several physical and health hazards to personnel that merit special attention.

Since EtO poses several health hazards, the alternative technologies that is currently available: a plasma phase hydrogen peroxide-based sterilizing agent .

B. Low Temperature Hydrogen Peroxide Plasma
Low temperature plasma sterilization was introduced to fill the gap between autoclave: high temperature steam sterilization (safest, fastest and least expensive) and EtO gas sterilization, which leaves toxic residuals. It is a low temperature, non-toxic, but fairly expensive sterilization method.

In this process, hydrogen peroxide is activated to create a reactive plasma or vapor. Gaseous plasma is a new physical agent applied recently to sterilisation. High frequency energy initiates generation of the plasma from hydrogen peroxide vapours in a high vacuum and creates reactive species particles from the vapours that collide and kill microorganisms.

Note: Plasma is ionized gas made up of ions and electrons and is distinguishable from solid, liquid, or gas. Plasma is often referred to as the fourth state of matter. The Hydrogen Peroxide Gas Plasma Sterilization system with an operating temperature range of 45-50□C. Operating cycle times range from 45-70 minutes, depending on size of system.

This sterilization system uses a combination of hydrogen peroxide and low temperature as plasma to quickly sterilize most medical instruments and materials without leaving any toxic residues. Hydrogen peroxide is a known antimicrobial agent that is capable of inactivating resistant bacterial spores. Sterilization by this method occurs in a low moisture environment.

The Hydrogen Peroxide Plasma Process:
The process consists of *two* consecutive and equal sterilization phases.
Vacuum / Preplasma Stage:

When a low pressure is achieved in the vacuum stage, low temperature air plasma is generated. This helps in removing residual moisture from the chamber. The system is then vented to atmospheric pressure at the end of this stage.

Sterilization Stage:

Pressure in chamber is reduced and an aqueous solution of **hydrogen peroxide** is injected and **vaporized** into chamber.

The hydrogen peroxide diffuses throughout the chamber, surrounds the items to be sterilized, and starts the inactivation of the microorganisms.

After the pressure is reduced, applying **radio frequency (RF) energy** creates an electric field and thus **forms low temperature plasma**.

Free radicals are generated in the plasma by breaking apart the hydrogen peroxide vapor.

Once the activated components react with the organisms and kill them, they lose their high energy and re-combine to form oxygen, water vapor, and nontoxic by-products.

This is half of the total sterilization process. The other half of the cycle is completed by repeating the above sterilization steps.

At the completion of the second half cycle, the source of RF energy is turned off, vacuum is released, and chamber is returned back to atmospheric pressure by introduction of filtered air.

Application:

This system is best suited to sterilize heat sensitive medical equipment .

C. Chlorine Dioxide

Chlorine Dioxide is a chemical liquid sterilization process. The best operating temperature range for this process is 25-30□C, while using low concentrations of ClO2. The process requires 6 hours of contact time to achieve sterilization. The presence of organic matter reduces activity. A processor converts a compound of dilute chlorine gas with sodium chlorite to form ClO2 gas and this gas is then exposed to the equipment in a sterilizing chamber.

Note: This alternative may corrode some materials and must be generated onsite. Prehumidification of the ClO2 is also required.

D. Ozone

Ozone sterilizes by oxidation, a process that destroys organic and inorganic matter. It penetrates membrane of cells causing them to explode.

In this process, a generator is used to convert oxygen to ozone, as a 6 to 12 percent concentration of ozone continuously flows through the chamber. Ozone penetration is controlled by vacuum pressure or by adding humidity. After the process is complete, oxygen is allowed to flow through the chamber to purge the ozone. The cycle time may be up to 60 minutes depending on the size of the chamber or load of items to be sterilized.

Ozone is formed by applying electrical energy to the oxygen molecule, which splits some portion of those oxygen molecules in half, into singlets of O. Therefore ozone molecules contain three atoms of oxygen and are unstable. Due to ozone gas being corrosive, and it being able to damage moisture sensitive equipment, there has not been much use of it in the medical industry.

Radiation Sterilization

Non ionising radiations–

Infra Red radiation (rapid mass sterilization of syringes, etc)

Ultra Violet radiation (enclosed areas)

Ultraviolet rays with wavelengths shorter than 300 nm are extremely effective in killing microorganisms. The most effective sterilizing range for UV is within the C bandwidth (UVC). This range is called the germicidal bandwidth. UVC has been used in hospitals for decades to sterilize surgical instruments, water, and the air in operating rooms.

How UV Light Works

Germicidal ultraviolet (UVC) light kills cells by damaging their DNA. The light initiates a reaction between two molecules of thymine, one of the bases that make up DNA. The resulting thymine dimer is very stable, but repair of this kind of DNA damage–usually by excising or removing the two bases and filling in the gaps with new nucleotides–is fairly efficient. Even so, it breaks down when the damage is extensive.

The longer the exposure to UVC light, the more thymine dimers are formed in the DNA and the greater the risk of an incorrect repair or a "missed" dimer. If cellular processes are disrupted because of an incorrect repair or remaining damage, the cell cannot carry out its normal functions. If the damage is extensive and widespread, the cell will die.

Ionising – Gamma, X ray, cathode ray (plastics, syringes, oil, metal foils)

Gamma, Beta Sterilization

Mode of Action

Both, X rays and Gamma rays have wavelength shorter than the wavelength of ultraviolet light. X rays, which have wavelength of 0.1 to 40 nm, and gamma rays, which have even shorter wavelength, are forms of ionizing radiation, so named because it can dislodge electrons from atoms, creating ions. (Longer wavelengths comprise nonionizing radiation.) These forms of radiation also kill microorganisms and viruses and ionizing radiation damages DNA and produces peroxides, which act as powerful oxidizing agents in cells. This radiation can also kill or cause mutations in human cells if it reaches them.

Irradiation is an effective sterilization method, but it is limited to commercial use only. The product to be sterilized is exposed to radiation for 10 to 20 hours, depending on the strength of the source. The highest temperatures reached in gamma sterilization are usually 30-40□C.

Gamma radiation is popular for sterilizing before shipment and it can be done through the packaging. A dose of 2.5 megarad is generally selected for many items. Ionizing radiation produces ions by knocking electrons out of atoms. These electrons are knocked out violently, and strike an adjacent atom and either attach themselves to it, or dislodge an electron from the second atom. The result is ionic energy that becomes converted to thermal and chemical energy.

This energy kills microorganisms by disruption of the DNA molecule, therefore preventing cellular division and propagation of biologic life.

The principal sources of ionizing radiation are **beta** particles and **gamma** rays.

Beta particles, free electrons, are transmitted through a high-voltage electron beam from a linear accelerator. These high-energy free electrons will penetrate into matter before being stopped by collisions with other atoms. This means their usefulness in sterilizing an object is limited by the density, thickness of the object and by the energy of the electrons. These free electrons produce their effect by ionizing the atoms they hit, producing secondary electrons that kill microorganisms.

Cobalt 60 is a radioactive isotope capable of breaking down to produce gamma rays. **Gamma rays** are electromagnetic waves that have the ability to **penetrate a much greater distance than beta rays** before losing their energy from collision. Because they travel with the speed of light, they must pass through a thickness measuring several feet before making sufficient collisions to lose all of their energy. Cobalt 60 is the most commonly used source for irradiation sterilization.

Gamma radiation and electron beams are used to effect ionization of the molecules in organisms. Mutations are thus formed in the DNA and these reactions alter replication. These processes are very dangerous and only well-trained and experienced staff should decide upon the desirability of their use and should ensure monitoring of the processes.

Application:

The radiation can change the properties of some materials like plastics and have adverse affects on glues or adhesives.

Sterilization controls:

Radiation doses should be monitored with specific dosimeters during the entire process. Dosimeters should be calibrated against a standard source on receipt from the supplier and at appropriate intervals thereafter. The radiation system should be reviewed and validated whenever the source material is changed and, in any case, at least once a year.

The bioindicator strains proposed for validation of this sterilization process are: spores of *Bacillus pumilus* (e.g. ATCC 27142) with 25 kGy (2.5 Mrad) for which the D-value is about 3 kGy (0.3 Mrad) using 10^7-10^8 spores per indicator; for higher doses, spores of *Bacillus cereus* (e.g. SSI C 1/1) or *Bacillus sphaericus* (e.g. SSl C_1A), *M. radiodurans* are used

E-Beam Radiation

In this process, the E-beam generator delivers a high dose of electrons in a narrow beam at the items to be sterilized. The electrons from the E-beam generator have limited penetrating power, less than gamma radiation. For example, a 10MeV Ebeam will penetrate about 5 cm of a unit-density material.

X-Ray Sterilization

This is a new developing process that is based on obtaining X-rays through conversion of electron beams. The X-rays produced have the same penetrating properties as the rays produced by Cobalt-60. But with this, treatment is faster, more flexible, and more environmentally friendly.

X-rays offer excellent product penetration in sterilization, thoroughly treating the surface and interior of a product.

Disinfection

Disinfection is the killing of many, but not all microorganisms. It is a process of reduction of number of contaminating organisms to a level that cannot cause infection, i.e. pathogens must be killed. Some organisms and bacterial spores may survive.

Disinfectants are chemicals that are used for disinfection. Disinfectants should be used only on inanimate objects.**Antiseptics** are mild forms of disinfectants that are used externally on living tissues to kill microorganisms, e.g. on the surface of skin and mucous membranes.

The common disinfectants used in the medical & veterinary laboratories and hospitals are as follows:

A. Glutaraldehyde

Glutaraldehyde, which has been a known *disinfectant* in the medical industry.

Glutaraldehyde is an organic compound with the formula $CH_2(CH_2CHO)_2$.
A pungent colorless oily liquid, glutaraldehyde is used to disinfect medical and dental equipment. It is also used for industrial water treatment and as a preservative. It is mainly available as an aqueous solution, and in these solutions the aldehyde groups are hydrated.

No carcinogenic properties.

3.4% alkaline glutaraldehyde solution, has tuberculocidal and highlevel disinfection capabilities. It achieves high-level disinfection in 20 minutes at 25 □C and ha
28-day reuse life.

2.4% alkaline glutaraldehyde solution, which has tuberculocidal and high-level disinfection capabilities. It achieves high-level disinfection in 45 minutes at 25 □C
and has up to a 14-day reuse life.

It is used to disinfect medical instruments and endoscopes. This solution can also be used in an automated reprocessor. (An automated reprocessor is the machine used to disinfect endoscopic and medical devices with a high level disinfectant solution.)

Both the concentrations have been used as a cold liquid high-level disinfectant for heat sensitive equipment.

*Note: G*lutaraldehyde products are being withdrawn from the European market due to concerns that it is toxic and harmful to health care staff in hospitals. Also, the U.S. market is requiring glutaraldehyde-free chemical solutions, which led to the formulation of the Cidex OPA solution. Cidex OPA solution is now known as the alternative to glutaraldehyde.

B. Ethanol

The effectivity of ethanol as e.g. desinfectant or antiseptic agent depends on the concentration of ethanol-water-mixture: An ethanol percentage of 50-80% destroys the cell wall/membrane of bacteria by denaturing their proteins and dissolving their lipids (effective against most bacteria, fungi and some viruses; ineffective against bacterial spores). Therefore, the ethanol has to pass the bacterial membrane/wall to get into the bacteria - if you use 100% ethanol instead, the bacteria get 'sealed' and they will survive... An other mechanism is the high osmotic pressure of ethanol/water-mixtures; and the 70% has the highest one.

C.Formaldehyde

Formaldehyde is used as a disinfectant and sterilant in both its liquid and gaseous states.
Formaldehyde is sold and used principally as a water-based solution called formalin, which is 37% formaldehyde by weight. The aqueous solution is a bactericide, tuberculocide, fungicide, virucide and sporicide.

It is indicated that formaldehyde should be handled in the workplace as a potential carcinogen and set an employee exposure standard for formaldehyde that limits an 8-hour time-weighted average exposure concentration of 0.75 ppm . The standard includes a second permissible exposure limit in the form of a short-term exposure limit (STEL) of 2 ppm that is the maximum exposure allowed during a 15-minute period .

Ingestion of formaldehyde can be fatal, and long-term exposure to low levels in the air or on the skin can cause asthma-like respiratory problems and skin irritation, such as dermatitis and itching. For these reasons, employees should have limited direct contact with formaldehyde, and these considerations limit its role in sterilization and disinfection processes.

Mode of Action. Formaldehyde inactivates microorganisms by alkylating the amino and sulfhydral groups of proteins and ring nitrogen atoms of purine bases .

Microbicidal Activity. Varying concentrations of aqueous formaldehyde solutions destroy a wide range of microorganisms.
Inactivation of poliovirus in 10 minutes required an 8% concentration of formalin, but all other viruses tested were inactivated with 2% formalin 72.
Four percent formaldehyde is a tuberculocidal agent, inactivating 104 M. tuberculosis in 2 minutes 82, and
2.5% formaldehyde inactivated about 107 Salmonella Typhi in 10 minutes in the presence of organic matter .

The sporicidal action of formaldehyde was slower than that of glutaraldehyde in comparative tests with 4% aqueous formaldehyde and 2% glutaraldehyde against the spores of B. anthracis. The formaldehyde solution required 2 hours of contact to achieve an inactivation factor of 104, whereas glutaraldehyde required only 15 minutes.
Uses. Although formaldehyde-alcohol is a chemical sterilant and formaldehyde is a high-level disinfectant, the health-care uses of formaldehyde are limited by its irritating fumes and its pungent odor even at very low levels (<1 ppm). For these reasons and others—such as its role as a suspected human carcinogen linked to nasal cancer and lung cancer . When it is used, direct exposure to employees generally is limited; however, excessive exposures to formaldehyde have been documented for employees of renal transplant units and students in a gross anatomy laboratory .
Formaldehyde is used in the health-care setting to prepare viral vaccines (e.g., poliovirus and influenza); as an embalming agent; and to preserve anatomic specimens; and historically has been used to sterilize surgical instruments, especially when mixed with ethanol. A 1997 survey found that formaldehyde was used for reprocessing hemodialyzers by hemodialysis centers.

If used at room temperature, a concentration of 4% with a minimum exposure of 24 hours is required to disinfect disposable hemodialyzers reused on the same patient .

Paraformaldehyde, a solid polymer of formaldehyde, can be vaporized by heat for the gaseous decontamination of laminar flow biologic safety cabinets when maintenance work or filter changes require access to the sealed portion of the cabinet.

Exercise:

1. Draw well labelled diagrams of: Autoclave, Hot Air Oven, Seitz Filter, Sintered Glass Filter, Membrane Filter Assembly, & Syringe Filter.
2. What is rectified spirit? Write its role in disinfection.
3. Write the grades of Membrane filter used for filtration to remove the viruses.

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Practical 4

BACTERIAL STAINING : SIMPLE & NEGATIVE STAINING

Bacteria consists of clear protoplasmic matter, differing but slightly in refractive index from the medium in which they are growing, it is difficult with the ordinary microscope except when special methods of illumination are used see them in unstained conditions.

Fixation and staining, therefore are of prime importance to increase visibility, accentuate specific morphological features and preserve them for future study.

Fixation:
Fixation is the process by which the internal and external structures of microorganisms and cells are preserved and fixed in position.

The stained cells should resemble living cells as closely as possible.
Fixation inactivates the enzymes that might disrupt cell morphology and toughens cell structures, so that they donot change during staining and observation.
During fixation microorganisms are usually killed and attached firmly to the microscope slide.
Fundamentally, there are two different types of fixation:

1. **Heat Fixation &**
2. **Chemical Fixation.**

1. **Heat Fixation**
   Routinely used to observe bacteria.
   Film of cells on a glass slide is gently heated by passing through a flame.
   Preserves overall morphology but not structures within the cell.

2. **Chemical Fixation**
   Used to protect fine cellular substructures and the morphology of larger or more delicate microorganisms or cells.

   Chemical fixative penetrate cells and react with cellular components, usually proteins and lipids and render them inactive, insoluble and immobile.
   Chemical fixative mixture contains ethanol, acetic acid, mercuric chloride and glutaraldehyde.

Dyes/Stain:
Many types of dyes are used to stain microorganisms have two features in common:

Chromophore groups, groups with conjugated double bonds that give the dye its colour, and bind with cesls by ionic, covalent, or hydrophobic bonding and stain the cells /microorganisms directly.

Some stains such as Indian Ink , Nogrosin Black stain the background instead of the cells, known as **Negative staining** in which the unstained cells appear bright against dark background.

Important terms:

Chromogen: The portion of the stain that is the colored molecule (often a benzene derivative).

Chromophore: The portion of the chromogen that gives it its color. A chromogen may have multiple chromophores, with each adding intensity to the color.

Auxochrome: The charged portion of the chromogen that allows it to act as a dye through ionic or covalent bonds between the chromogen and the cell.

Dyes that can bind cells by ionic interactions are probably the most commonly used dyes. These ionisable dyes are divided into two classes based on their nature of their charged group:

1. **Basic Dyes:** Methylene Blue, basic fuchsin, crystal violet, safranin and malachite green have positively charged groups (salts). Basic dyes bind to negatively charged molecules like nucleic acids, proteins and the surfaces of prokaryotic cells.
2. **Acidic Dyes:** Eosin, rose Bengal, and acid fuchsin-possesses negatively charged groups such as carboxyls (-COOH) and phenolic hydroxyls (-OH). Acidic dyes, because of the negative charge, bind to positively charged cell structures.

The staining effectiveness of ionisable dyes may be altered by pH, since the nature and degree of the charge on cell components change with pH. Thus acidic dyes stain best under acidic conditions when proteins and many other molecules carry a positive charge; basic dyes are most effective at higher pHs.

Dyes bind through covalent bonds or because their solubility e.g., Feulgen staining for DNA.

Simple Staining Procedure:

1. Begin with a heat-fixed emulsion
2. Cover the smear with stain- methylene blue (60 sec)
3. Rinse the slide with water
4. Gently blot dry with bibulous paper - do not rub
5. Observe under oil immersion

Negative Stain Procedure:

1. Begin with a drop of acidic stain at one end of a clean slide
2. Aseptically add organisms and emulsify with a loop. Do not over-inoculate and avoid spattering the mixture
3. Take a second clean slide, place it on the surface of the first slide, and draw it back into the drop
4. Do it until the drop flows across the width of the spreader slide
5. Then push the spreader slide to the other end and dispose of the spreader slide
6. Air dry and observe under the microscope

Why negative staining?

To determine morphology and cellular arrangement in bacteria that are too delicate to withstand heat-fixing. Also, where determining the accurate size is crucial, a negative stain can be used because it produces minimal cell shrinkage

Exercise:

1. Write the procedure to prepare bacterial smear.
2. Write the composition of simple stain & negative stains.

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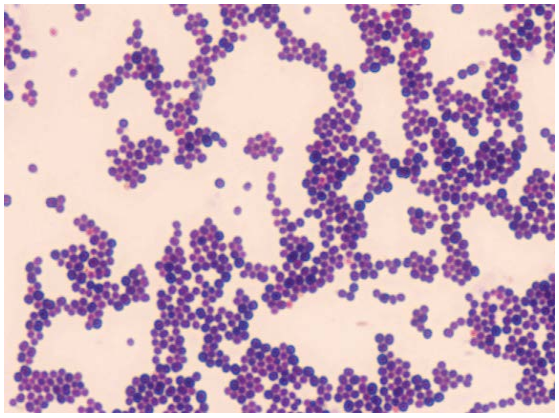
Practical 5

DIFFERENTIAL STAINING: GRAM'S STAINING

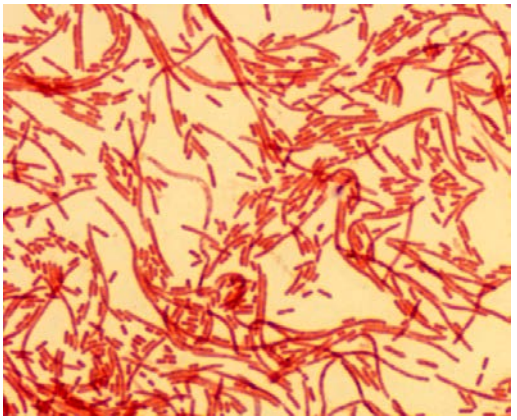
The Gram stain was first developed and used in 1884 by Hans Christian Gram. Gram was in search of a method to visualise cocci in tissue sections of lungs who had died of pneumonia.

In the original method of Christian Gram (1884), the smear was stained with aniline gentian violet treated with Lugol's iodine (Iodine 1g, KI 2g, water 300 ml), decolorized with absolute alcohol, and counterstained with Bismarck brown.

Certain bacteria when treated with basic para-rosaniline dyes such as methyl violet, Crystal violet (Hexamethyl-para-rosaniline 3 chloride) or gentian violet (Mixture of two preceding dyes), and then with iodine, 'fix' the stain so that subsequent treatment with a decolourizing agent - e.g. alcohol, acetone, does not remove the colour. Other organisms, however, are decolourized by this process. If a mixture of various organisms are thus stained and subjected to decolourization, it is found that some species retain the dye i.e., resist decolourization and theses bacteria are termed as Gram Positive, whereas others are completely decolourized and takes the counter stain and are termed as Gram Negative bacteria.



Gram Positive Cocci



Gram Negative Rods

Grams staining was later on modified by Kupeloff and Beerman (1922- Decolourization with acetone), Burkes (1922- Decolourization with acetone), Jensen (Decolourization with alcohol), Weigert (Decolourization with aniline–xylol), Preston & Morrell (1962- Decolourization with iodine-acetone), (Gephardt et al., 1981- Use of Saffranin as counterstain) for better results.

Chemical Mechanism of Gram Reaction:

Many theories were putforth to explain why some bacteria resist decolourization and retain the primary dye and some bacteria get easily decolourized and take counter stain *i.e.,* Safranin. Theories includes differences in cytoplasmic pH (Gram positive bacteria-2pH and in case of Gram negative-3 pH), and presence of Magnesium ribonucleoprotein in Gram positive bacteria and its absence in Gram negative bacteria have been proposed. But the thickness of Gram positive cell wall due to thick peptidoglycan layer and presence of more lipids in Gram negative cell walls have been accounted for the Gram reaction.

The theory stands as positively charged crystal violet passes through the cell wall and cell membrane and binds to negatively charged components inside the cell. Addition of negatively charged iodine (in the mordant) binds to the positively charged dye and forms a large crystal violet-iodine complex within the cell. Crystal violet (Hexamethyl-para-rosaniline 3 chloride) interacts with aqueous Potassium iodide-Iodine via a simple anion exchange to produce a chemical precipitate. The small chloride anion is replaced by the bulkier iodide, and the complex thus formed becomes insoluble in water. During decolorization, alcohol dissolves the lipid present in the outer membrane of Gram negative bacteria and it leaches the dye-iodine complex out of the cell. A thin layer of peptidoglycan does not offer much resistance either. The dye-iodine complexes are washed from the Gram negative cell along with the outer membrane. Hence Gram negative cells readily get decolorized. On the other hand Gram positive cells become dehydrated from the ethanol treatment, closing the pores as the cell wall shrinks during dehydration. The dye-iodine complex gets trapped inside the thick peptidoglycan layer and does not get decolorized.

The mechanism of Gram reaction was determined in 1983 (Davies et al.,1983 and Beveridge and Davies, 1983). In aqueous solutions crystal violet dissociates into CV^+ and Cl^- ions which penetrates the cell wall and membrane of both Gram Positive and Gram Negative bacterial cells. The CV^+ interacts with negatively charged components of bacterial cells and stains bacterial cells purple. When Iodine is added Iodine (I^- or I_3^-) interacts with CV^+ to form large CVI complex within the cytoplasm and outer layers of the bacterial cell. The small Cl^- anion is replaced by the bulkier iodide, and the CVI complex thus formed becomes insoluble in water. On decolourization, decolourizer interacts with the lipid s of the membranes of both Gram Positive and Gram Negative bacteria. The outer membrane of the Gram Negative bacterial cell is lost , leaving the peptidoglycan layer exposed. Gram Negative bacteria do have very thin layer of peptidoglycan, as compared to one to three layered thick peptidoglycan layer of Gram Positive bacteria (Dmitriev, 2004).

Due to action of decolourizer (ethanol) Gram Negative bacterial cell wall becomes leaky (Lipids get dissolved) and allow the large CVI complexes to be washed away from the bacterial cell. Due to thick multilayered nature of peptidoglycan layer in Gram positive bacteria along with the dehydration effect (due to ethanol), the large CVI complex is trapped in the cell wall of Gram postivie bacteria. Hence, Gram positive bacteria resists decolourization and retains crystal violet-purple colour, whereas, Gram negative bacteria cell loses the purple colour and is only revealed when the counterstain, positively charged saffranin dye is added.

The length of the decolorization is critical in differentiating the gram-positive bacteria from the gram-negative bacteria. A prolonged exposure to the decolorizing agent will remove all the stain from both types of bacteria. Some Gram-positive bacteria may lose the stain easily, therefore appear as a mixture of Gram-positive and Gram-negative bacteria (Gram-variable).

The reagents for Gram's staining can be made or purchased commercially .

1. Primary Stain: Crystal Violet Staining Reagent.

Solution A

Crystal violet	2g
Ethanol, 95% (vol/vol)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Mix A and B to prepare 100ml **crystal violet staining reagent.** Store for 24 h, filter through paper & use.

2. Mordant: Gram's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 ml

Grind the iodine & potassium iodide in a mortar and add water slowly with continuous grinding to dissolve the iodine completly. Store in amber coloured bottles.

3. Decolorizing Agent

Acetone	50 ml
Ethanol (95%)	50 ml

4. Counterstain: Safranin

Stock solution:

Safranin O	2.5g
Ethanol (95%)	100 ml

Working Solution:

10 ml Stock Solution
90 ml Distilled water

*Freshly made staining reagents are recommended.

Procedure: (Gram staining modified by Gephardt et al, 1981)

1. Fix the air dried smear by passing over the flame 2-3 times.
2. Flood the fixed bacterial smear with crystal violet staining reagent for 1 minute.
3. Wash the slide gently with distilled water for 2 seconds.
4. Flood the slide with Gram's Iodine(mordant) and wait for 1 minute
5. Wash the slide gently with distilled water for 2 seconds.
6. Flood the smear with decolourizing agent - by adding drop by drop - decolourizer, untill the smear becomes clear (15 seconds).
7. Wash the slide gently with distilled water for 2 seconds.
8. Flood the smear with counterstain, safranin. Wait for 30 seconds to 1 minute.
9. Pour off the stain from smear and wash the smear with tap water.
10. Air dry the stained smear and observe under oil immersion using a Brightfield microscope.

Interpretation: Gram Positive bacteria will stain purple , whereas , Gram Negative bacteria stains pink red colour.

KOH string test :

KOH string test may be used as a confirmatory test for the Gram Stain (Arthi et al., 2003). The formation of a string (DNA) in 3% KOH indicates that the isolate is a Gram-negative organism.

Procedure:

1. Place a drop of 3% KOH onto a glass slide.
2. Emulsify in KOH a loopful of the culture from a Blood agar incubated for 18-24 hours.
3. Continue to mix the suspension for 60 sec and by slowly lifting the loop, observe for the formation of a string.

Interpretation:
Gram-negative cells form a string within 60 seconds.
Gram-positive cells are not affected.

Exercise:

1. Draw well labelled diagrams of Gram negative and Gram positive bacterial cell wall.
2. Prepare the smear, fix and stain with Grams staining. Write the results.

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Practical 6

DIFFERENTIAL STAINING: ACID FAST STAINING

Certain bacterial species have unusual lipids (**mycolic acid**) in their cell walls. Mycolic acid renders the cell wall very waxy and impenetrable by aqueous stain solutions.

In 1882 Robert Koch reported the discovery of the tubercle bacillus and described the appearance of the bacilli resulting from a complex staining procedure. Koch and Ehrlich simultaneously introduced a method for staining the previously undetectable *Mycobacterium tuberculosis*. Modifications by Ziehl and Neelsen produced the commonly used carbol-fuchsin solution which requires steaming to drive the stain in. (The melting point for mycolic acid is 56^{o}C).

Muller and Chermock modified carbol-fuchsin for use at room temperature by addition of a surfactant (wetting agent). Acid-alcohol removes stain from most cellular and tissue elements. The mycolic acid, however, resists penetration by this differentiating agent, leaving acid-fast bacteria red against a colorless background.

Methylene blue & malachite green is used as a counter stain to aid in the localization of cellular material on the specimen. The acid fast stain is used routinely on sputum samples for preliminary diagnosis of active tuberculosis.

The Ziehl-Neelsen method has endured as a reliable and effective way to demonstrate the acid-fast bacteria. In this method heat is used to help drive the primary stain into the waxy cell walls of these difficult-to-stain cells. The use of heat in this method has been the reason that this technique is called the “hot staining” method.

In 1915, Kinyoun published a method that has become known as the “cold staining” method because the heating step was removed in favor of using a higher concentration of the carbolfuchsin primary stain.

A. Ziehl-Neelsen method for acid-fast staining (6, 7)

Carbolfuchsin stain:

Basic fuchsin, 0.3 g
Ethanol, 95% (vol/vol), 10 ml
Phenol, heat-melted crystals, 5 ml
Distilled water, 95 ml
Dissolve the basic fuchsin in the ethanol; then add the phenol dissolved in the water.
Mix and let stand for several days. Filter before use.

Decolorizing solvent:

Ethanol, 95% (vol/vol), 97 ml
Hydrochloric acid (concentrated), 3 ml

Counterstain:

Methylene blue chloride, 0.3 g
Distilled water, 100 ml

B. Kinyoun method for acid-fast staining

Kinyoun carbolfuchsin solution:

Solution A. Dissolve 4 g of basic fuchsin in 20 ml of ethyl alcohol.
Solution B. Dissolve 8 g of phenol (melted) in 100 ml of distilled water.
Mix solutions A and B together and allow to stand for a few days.

Acid-alcohol decolorizing agent:

Ethanol, 95% (vol/vol), 97 ml
Hydrochloric acid (concentrated), 3 ml

Methylene blue counterstain:

Methylene blue chloride, 0.3 g
Distilled water, 100 ml
Dissolve by shaking.

A. Ziehl-Neelsen method for acid-fast staining

1. Films are made dried and fixed by flaming.
2. Cover the slide with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for 5 min, heat gently .The stain must not be allowed to evaporate and dry on the slide. If necessary pour on more carbol fuchsin to keep the whole slide covered.
   (The slide may be heated with a torch prepared by twisting a small piece of cotton wool on to the tip of an inoculating wire and soaking it in methylated spirit before lighting. when steam rises from the slide, remove and extinguish the torch. after about 1 minute recharge the torch with spirit, relight it and again heat the slide until the steam rises, continue this way for 5 min.
3. Wash with water.
4. Cover the slide with 20% sulphuric acid. Keep for 1` min. wash with water.
5. Treat with 95% alcohol for 2 min
6. Wash with water
7. Counterstained with Loeffler's methylene blue. Or dil. Malach. green for 15-20 sec.
8. Wash, blot dry and mount.

B. Kinyoun method for acid-fast staining

1. Heat fix an air dried smear at 80^{o}C for at least 15 minutes or for 2 hours on an electric hot plate at 65^{o}C – 70^{o}C
2. Flood slides with Kinyoun's carbol fuchsin reagent and allow to stain for 5 minutes at room temperature.
3. Rinse with deionized water and tilt slide to drain.
4. Decolorize with acid-alcohol for 3 minutes and rinse again with deionized water.*
5. Redecolorize with acid-alcohol for 1-2 minutes or until no more red color runs from the smear.
6. Rinse with deionized water and drain standing water from the slide surface by tipping the slide.

7. Flood slide with methylene blue counterstain and allow to stain for 4 minutes.
8. Rinse with distilled water and allow to air dry.
9. Examine under high dry (400X) magnification, and confirm acid-fast structures under oil immersion (1000X).

Interpretation

Acid-fast bacilli take pink red colour against blue background.

Exercise

Q1.Draw well labelled diagram of cell wall of Acid Fast bacteria.
Q2. Enlist the names of acid fast bacteria.

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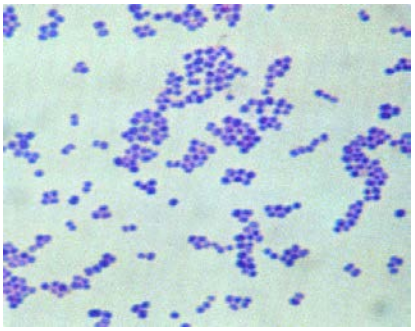
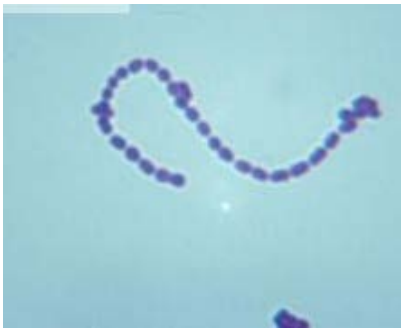
COLOUR PLATE 1

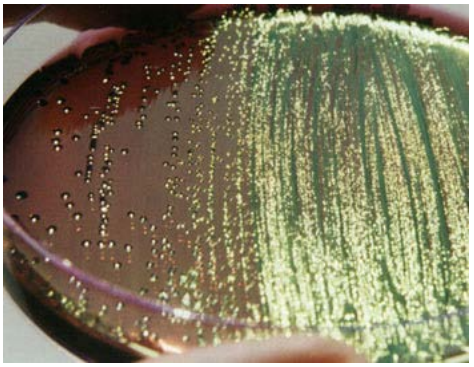


1.Microscopic picture of *Staphylococcus*

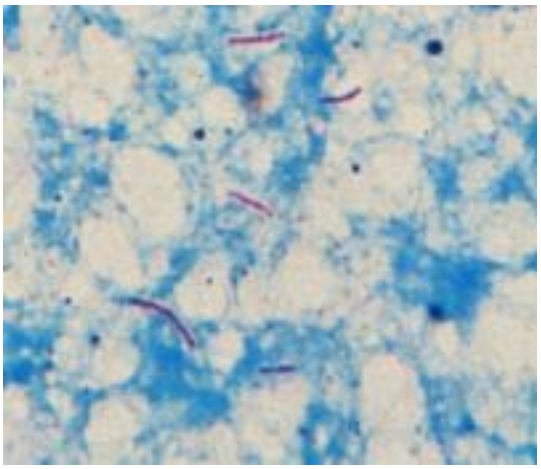


2.Microscopic picture of *Streptococcus*

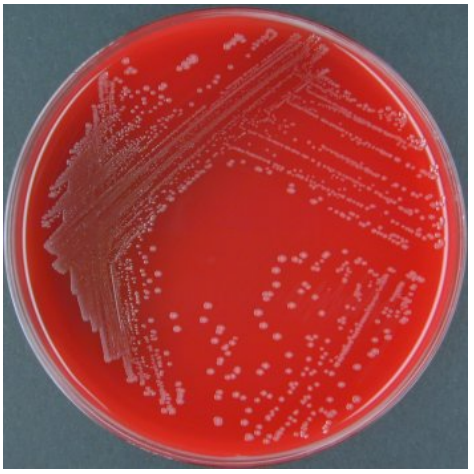
3. E. coli on MacConkeys Agar
Lactose Fermenting Pink Red Colonies



4.E. coli on EMB: Characteristic "**Metallic sheen''**



5. Acid Fast Bacteria
Mycobacterium tuberculosis



6. Quadrant Streak PLate

Practical 7

SPECIAL STAINING

(Endospore / Capsule / Flagella / Metachromatic Granules)

Spore Staining

If spore bearing organisms are stained with ordinary dyes, or by Gram's stain, the body of the bacillus is deeply coloured, whereas the spore remains unstained. The vegetative bacterial cells are stainable with aqueous dyes, but endospores possess permeability barrier that prevents stain/dyes from entering the spore coat unless the barrier is destroyed by heating, UV light, mechanical rupture or by treatment of acid. The tough spore coat is formed to protect the bacterial cells DNA and important proteins from adverse environmental conditions (excessive heating, short of nutrients, drying etc.)Spore coat is a complex multilayered structure containing high calcium ions and dipicolinic acid which makes the structure more tough .
Below spore coat, lies the peptidoglycan. Once the protective tough spore coat is penetrated, the stain/dye interacts with peptidoglycan to produce the desired effect of staining.

There are other staining methods to introduce dye into the substance of the spore. When thus stained, the spore tends to retain the dye and resist decolourization. Several methods such as Acid Fast Staining Method for Spores (Spores stained bright and protoplasm of the bacillus stains blue), Hansen's Method, Dorner's method and Schaeffer and Fulton's Method are widely applied methods for staining spores in proper.

Commonly used staining methods for endospores includes:

1. The Schaeffer-Fulton method - Most common method used to stain endospores.
2. Dorner method
3. Hansens method

1. Schaeffer-Fulton method for staining endospores

Malachite green stain (0.5% (wt/vol) aqueous solution)
0.5 g of malachite green
100 ml of distilled water

Decolorizer
Tap water,

Safranin counterstain
Stock solution (2.5% (wt/vol) alcoholic solution)
2.5 g of safranin O
100 ml of 95% ethanol
Working solution
10 ml of stock solution
90 ml of distilled water

Procedure:

Schaeffer-Fulton method:

1. Fix the air dried smear by passing over the flame 2-3 times.
2. Flood smear with malachite green and heat for 5 min. Do not boil.
3. Allow to cool and wash with water.
4. Counter stain with dilute safranin (working solution) for 1 min.
5. Wash the smear and air dry it.

Spore stains: Endospore takes bright green and bacterial cells are brownish red to pink.

Dorner method for staining endospores –

Spore stains: Bacterial cells are colorless, endospores are red, and the background is black. Carbol Fuchsin – primary stain and counterstain Nigrosin .

Hansen's Method

Material: Bacterial smear, Cocn. carbol fuchsin, 5% Acetic acid, Loeffer's methylene blue.

Procedure:

1. Fix the air dried smear by passing over the flame 2-3 times.
2. Stain the smear as follows,
3. Flood smear with Conc. carbol fuchsin and heat for 5 min. Do not boil.
4. Allow to cool and wash with water.
5. Decolorize with 5% Acetic acid for 1 min. and wash with water.
6. Counter stain with Loeffer's methylene blue for 3 min.
7. Wash the smear and air dry the smear and observe under microscope.

Spore stains: spores stain red and the vegetative cells blue.

Capsular Staining

The best way to demonstrate capsules of bacterial cells is to stain them by some procedure, which differentiates them from the bacterial cell itself. Anthony's method (with Tyler's modification) to stain capsule is the simplest method.

Anthony's method (With Tyler's modification)

Staining solution:

Acetic crystal violet

Crystal violet (35% dye content)--------- 0.1 g

Glacial acetic acid ---------- 0.25 ml

Distilled water ----------100 ml

Procedure:

Anthony's method (With Tyler's modification)

1. Prepare a smear of bacterial culture on the slide.
2. Dry it in the air.
3. Stain for 4-7 minutes in the 'acetic crystal-violet' solution.
4. Wash with 20% aqueous copper sulphate ($CuSO_4$, $5H_2O$)
5. Dry with blotting paper and examine.

Capsules stains blue violet; Bacterial cell stains dark blue.

Flagellar Staining

Robert Koch published staining procedure for bacterial flagella in 1877. Subsequently several modifications and methods were developed for staining flagella were developed. In 1930, Leifson published a simple flagella stain. Many modifications or alternative methods includes a wet-mount procedure of Mayfield and Innis and a more traditional dried-smear preparation ,combination of the wet-mount technique of Mayfield and Innis and the stain of Ryu suggested by Kodaka et al. ,overcame most difficulties in staining flagella. **Presque Isle Cultures flagella stain** – ready made staining method available commercially.

A silver-plating stain for flagella was developed in 1958 and simplified in 1977. Recently a fluorescent protein stain, NanoOrange from Molecular Probes (Eugene, OR), is being applied to screen for bacteria possessing flagella by light microscopy .

Materials

12-16 Hrs incubated bacterial culture, Microscope slides, 95% ethanol
Micorpipette with sterile disposable tips, Distilled water.

Leifson flagella stain

Solution A:	
Sodium chloride	1.5 g
Distilled water	100 ml
Solution B:	
Tannic acid	3.0 g
Distilled water	100 ml
Solution C:	
Pararosaniline acetate	0.9 g
Paraosaniline hydrochloride	0.3 g
Ethanol, 95% (vol/vol)	100 ml

Take equal volumes of solutions A and B and then add 2 volumes of the mixture to 1 volume of solution C. The resulting solution may be kept refrigerated for 1 to 2 months. (Ryu method - reagent stable at room temperature)

Preparation:

Bacterial cultures incubated for 12-16 Hrs can be used for flagellar staining. Collect small quantity of growth from agar medium and emulsify in 100 ml of distilled water. Take in a micro-centrifuge tube mix by gentle vortexing. Avoid too much of inoculums.
If culture is used from incubated broth, centrifuge the culture, remove spent medium.

Resuspend in 100 ml of distilled water by gently vortexing, again centrifuge, and remove supernatant. Finally, resuspend in 200 ml of distilled water and prepare slightly cloudy emulsion to be used for staining.

Preparation of smear:

1. Take ethanol treated clean new microscope slide and flame to dry before use.
2. Cool the slide, place 5 to 10 ml of the culture emulsion on one end of the slide and spread it with the help of pipette.
3. Dry at room temperature. Do not heat fix.(Heating destroys the flagella)

Staining procedure: Leifson flagella staining method

1.Take a prepared slide and mark an area of 1x1.5 inch^2 with grease pencil.
2. Flood Leifson dye solution on the slide within the marked area.
3. Incubate at room temperature for 7 to 15 minutes or allow to act till formation of fine precipitate.(Golden film develops on the dye surface).
4. Remove the stain by gentle wash with water steam and air dry.
5. Observe under oil immersion.

Bacterial body and flagella will stain red

Staining of Metachromatic Granules - Albert's Method

Metachromatic granules:

Special stains (Albert, Neisser) pick out the volutin granules and give the bacilli a beaded or barred appearance; the granules are polar in short bacilli. Volutin staining reactions are best seen in young cultures .

Albert staining :

Albert's stain

Toluidine blue	1.5 g
Malachite green	2.0 g
Glacial acetic acid	10 ml
Alcohol (95% ethanol)	20ml
Distilled water	1000ml

Dissolve the dyes in the alcohol and add to the water and acetic acid. Allow to stand for one day and then filter.

Albert's Iodine

Iodine	6 g
Potassium iodide	9 g
Distilled water	900ml.

Procedure:

Albert staining:

1.Make the film dry and fix by heat.
2.Cover slide with Albert's stain and allow to act for 3-5 min.
3.Wash in water and blot dry.
4.Cover slide with Albert's iodine and allow to act for 1 minute.
5.Wash and blot dry.

Metachromatic granules stain bluish black; the protoplasm green and other organisms mostly light green.

Exercise:

1. Explain why spore coat is impermeable for routine dyes.
2. What is the role of tannic acid in staining flagella..
3. Difference between capsule & slime layer

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Practical 8

BACTERIAL MOTILITY : HANGING DROP METHOD

Material Required:

Cavity slide, cover slip, petroleum jelly, overnight broth culture of motile bacteria, bacteriological loop, compound microscope with low power and high power lens.

Procedure:

1.With the help of matchstick dipped in a Vaseline/petroleum jelly, a ring is outlined round the concavity of the slide.

2.Take a clean grease free cover slip, lay it on the table and place a drop of the liquid culture of bacteria at the center.

3.Invert the cavity slide over the cover slip, allowing the glass to adhere to the petroleum jelly and quickly turn round the slide, so that the cover slip is uppermost. The drop should then be hanging from the cover slip in the center of the concavity.

4.Place the slide on the microscope; rack down the condenser slightly and partially close the diaphragm. (**Excessive illumination renders the organism invisible**)

5.With the low power objective, focus the edge of the drop so that it appears across the center of the field.

6.Turn the high power lens into position and focus the edge of the drop. Obtain the illumination by lowering or raising the condenser and secure sharp definition by reducing the aperture of the iris diaphragm.

7.Away from the edge of the drop motile bacteria will be visible on slight fine adjustment.

Exercise

Q1. Draw a well-labeled diagram of hanging drop preparation.
Q2. Differentiate between bacterial motility and Brownian movement.
Q3. Draw diagram of different flagellar arrangements of the bacteria with examples.

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Practical 9

PREPARATION OF CULTURE MEDIA

Culture Media

Types of culture media

1. Basic /Simple / All purpose media

It is a media that supports the growth of micro-organisms that do not require special nutrients.

Uses :

. To prepare enriched media
. To maintain stock cultures of control bacterial strains
. To subculture pathogenic bacteria from selective/differential medium prior to performing biochemical or serological tests.

Eg. Nutrient Broth
Nutrient Agar

2. Enriched media

Media that are enriched with whole blood, lyzed blood, serum,special extracts or vitamins to support the growth of pathogenic bacteria.

Eg. Blood Agar
Chocolate Agar

3. Enrichment media

Fluid media that increases the numbers of a pathogen by containing enrichments and/or substances that discourage the multiplication of unwanted bacteria.

Eg. Selenite F broth media
Alkaline peptone water

4. Selective media

Media which contain substances (Eg. Antibiotics) that prevent or slow down the growth of bacteria other than pathogens for which the media are intended.

Eg. Modified Thayer –Martin Agar
Salmonella-Shigella(SS) agar

5. Differential media

Media to which indicator substances are added to differentiate bacteria.

Eg. TCBS Agar differentiates sucrose fermenting yellow colonies of *Vibrio cholerae* to non-sucrose fermenting blue colonies other Vibrio species.

Note: Most differential media distinguish between bacteria by an indicator which changes color when acid is produced following carbohydrate fermentation.

6. Transport media

Media containing ingredients to prevent the overgrowth of commensals and ensure the survival of pathogenic bacteria when specimens can not be cultured soon after collection.

EG. Amies transport media
Stuart media
Kelly-Blair media

Choice of culture media

The selection culture media will depend on:

1. The major pathogens to be isolated, their growth requirements and the features by which they are recognized.
2. Whether the specimens being cultured are from sterile sites or from sites having normal microbial flora.
3. The cost, availability and stability of media.
4. The training and experience of laboratory staff in preparing, using and controlling culture media.

Forms of culture media

1. Solid culture media
2. Semisolid culture media
3. Fluid culture media

1. Solid culture media

Plate cultures in petri dishes. Stab/slope cultures in tubes and bottles

Uses: Description of bacterial colonies

- Size : diameter in mm
- Out line : circular, entire, wavy, indented
- Elevation: flat, raised, low convex and dome shaped.
- Transparency: transparent, opaque, and translucent.
- Surface: smooth (mucoid) and shiny, rough and dull.
- Color: colorless, white, pink, and pigmented
- changes in medium

Eg. Hemolysis in Blood Agar

Blackening of medium due to hydrogen sulfide production.

2. Semisolid culture media

Uses:

. as an enrichment media

. as motility media

3. Fluid culture media

Bacterial growth in fluid media is shown by a turbidity in the medium.

Uses :

. as an enrichment media

. as biochemical testing media

. as blood culture media

Common ingredients of culture media

Peptone
Meat/Beef extract
Yeast extract
Mineral salts
Carbohydrates
Agar & Water

1.Peptone: Hydrolyzed product of animal and plant proteins: Free amino acids, peptides and proteoses(large sized peptides). It provides nitrogen; as well carbohydrates, nucleic acid fractions,minerals and vitamins.

Peptone is an enzymatic digest of animal protein. Peptone was first introduced in 1914 and became the standard Peptone for the preparation of bacteriological culture media. The nutritive value of Peptone is largely dependent on the amino acid content that supplies essential nitrogen. Peptone contains only a negligible quantity of proteoses and more complex constituents.

Applications

Peptone is used as an organic nitrogen source in microbiological culture media for cultivation of a variety of bacteria and fungi. Peptone has also been utilized as a nitrogen source in cell culture media formulations.

2.Meat/Beef extract:

Beef Extract is a nutritive ingredient in many classical culture media, including Antibiotic Assay media, and several media recommended for standard methods applications.

Beef Extract is a mixture of peptides and amino acids, nucleotide fractions, organic acids, minerals and some vitamins. "Its function can therefore be described as complementing the nutritive properties of peptone by contributing minerals, phosphates, energy sources and those essential factors missing from peptone. Beef Extract is not exposed to the harsh treatment used for protein hydrolysis, so it can provide some of the nutrients lost during peptone manufacture.

Beef Extract is derived from infusion of beef and provides an undefined source of nutrients.

Applications

Beef Extract is intended to replace aqueous infusion of meat in microbiological culture media.

Beef Extract is frequently used at a concentration of 0.3 to 1.0% in culture media, although concentrations may vary depending on the nutritional requirements for the medium formulation.

Beef Extract was used in media for early studies of non-sporulating anaerobes of the intestinal tract and as a stock broth in the study of nutritional needs of streptococci.

3.Yeast extract:

Yeast extract is a complex and widely used hydrolysate of yeasts. It provides nitrogenous compounds, carbon, sulfur, trace nutrients, vitamin B complex and other important growth factors, which are essential for the growth of diverse microorganisms.

There are two different types of yeast extracts - the hydrolyzed yeast extract, also called yeast peptone, and the autolyzed yeast. The hydrolyzed yeast extract is produced by digestion of exogeneous enzymes or acid to hydrolyze the proteins. A yeast autolysate or yeast autolysate extract is made by fermentation of yeast to a concentration level where the yeast dies and the cells walls break. The proteases from the yeast itself start the digestion of the proteins and split them into peptides and amino acids. The insoluble portion is removed.

The yeast extract contains glutamate. The “glutamate” refers to the content of the amino acid glutamate – which is in fact naturally present in yeast and yeast extract, but also occurs in many other foodstuffs. Glutamate represents approximately 5% of yeast extract. In a product with yeast extract, where the ingredient is only present in small quantities, the average glutamate content is less than 1%.

4.Mineral salts: these are: Sulfates as a source of sulfur.
Phosphates as a source of phosphorus.
Sodium chloride
Other elements.

5.Carbohydrates: Simple and complex sugars are a source of carbon and energy.
.Assist in the differentiation of bacteria.
Eg. Sucrose in TCBS agar differentiates vibro species.
Lactose in MacConkey agar differentiates enterobacteria.
Preparation of commonly used Media:

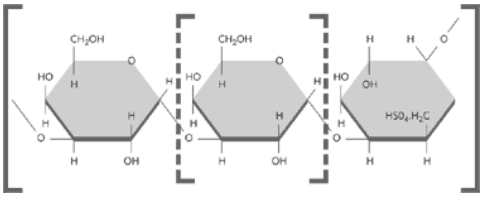
6.Agar Agar
Agar is an inert polysaccharide of seaweed.It is not metabolized by micro-organism.
Throughout history into modern times, agar has been chiefly used as an ingredient in desserts throughout Asia and also as a solid substrate to contain culture media for microbiological work. The agar was discovered in the late 1650s or early 1660s by Minoya Tarozaemon in Japan, where it is called **Kanten.**

In the natural state, agar occurs as structural carbohydrate in the cell walls of agarophytes algae, probably existing in the form of its calcium salt or a mixture of calcium and magnesium salts. It is a complex mixture of polysaccharides composed of two major fractions:

Agarose, a neutral polymer, and
Agaropectin, a charged, sulfated polymer.

Agarose - gelling fraction, is a neutral linear molecule essentially free of sulfates, consisting of chains of repeating alternate units of β-1,3-linked- D-galactose and α-1,4-linked 3,6-anhydro-L-galactose.

Agaropectin- non gelling fraction, is a sulfated polysaccharide (3% to 10% sulfate), composed of agarose and varying percentages of ester sulfate, D-glucuronic acid, **and** small amounts of pyruvic acid. The proportion of these two polymers varies according to the species of seaweed. Agarose normally represents at least two-thirds of the natural agar-agar.



Properties

Agar-agar may come in several forms: powdered, flakes, bars and threads. Available as yellowish powder.

Agar-agar is insoluble in cold water, but soluble in water and other solvents at temperatures between 95^{O} and 100^{O} C. It dissolves readily in boiling water and sets to a firm gel at concentrations as low as 0.50%.

The melting point of agar is 85-90 ^{O} C, whereas the solidifying point is in between 32-45 ^{O} C depending upon the concentration.

Specifications

Appearance	Yellowish Powder
Particle size	100 Mesh
Moisture content	Max. 18%
Water absorption	Max. 75 cc
Acid insoluble ash	Max. 0.5%
Total Ash	Max. 6.5%
Foreign organic material	Max. 1%
Foreign insoluble material	Max. 1%
pH	6.8-7.0
Gelatin	Negative
Gel Strength 1.5% sol at 20^{O}C	700-1000gm/cm^2
Viscosity 1.5% solution at 60^{O}C	10-100cps
Melting Point	85-95 ^{O}C
Setting Point	32-45 ^{O}C
Solubility	Boiling water
Arsenic	Max. 3ppm
Lead	Max. 10 ppm
Heavy metals	Max. 10 ppm
Starch	Negative

7. Water: Deionized or distilled water must be used in the preparation of culture media.

Commonly used culture media in microbiological laboratory:

Nutrient Broth:

Peptic digest of animal tissue	5gm
Sodium chloride	5gm
Beef extract	1.5 gm
Yeast Extract	1.5 gm
Distilled water	1000ml
pH	7.4

Mix the contents boil to dissolve. Autoclave at 15 lb pressure at 121^{O}C for 20 minutes. Dispense in sterile test tubes and use.

Nutrient Agar:

Peptone	10gm
Sodium chloride	5gm
Beef extract	4gm
Agar	20 gm
Distilled water	1000ml
pH	7.4

Mix the contents boil to dissolve. Autoclave at 15lb pressure at 121^{O}C for 20 minutes. Dispense in sterile petridish and use.

Blood Agar:

Prepare nutrient agar. Cool it to 40-50^{O}C. And add 5-10% defibrinated blood collected in sterilized flask.

MacConkey's Agar:

Peptone	5gm
Bile salt (Sodium taurocholate)	5gm
Sodium chloride	5gm
Neutral Red (2% in 50% ethanol)	3.5ml
Crystal violet	0.001gm
Lactose	10gm
Agar	20gm
Distilled water	1000ml
pH	7.4

Mix the contents boil to dissolve. Autoclave at 15 lb pressure at 121^{O}C for 20 minutes. Dispense in sterile petridish and use.

Brilliant Green Agar:

Yeast Extract	3gm
Peptone	10gm
Sodium chloride	5gm
Lactose	10gm
Sucrose	10gm
Phenol Red	0.08gm
Brilliant Green	0.125gm
Agar	20gm
Distilled water	1000ml
pH	7.4

Mix the contents boil to dissolve. Autoclave at 15 lb pressure at 115^{O}C for 15 minutes. Dispense in sterile petridish and use.

Eosin Methylene Blue Agar:

Peptone	10gm
Sucrose	5gm
Lactose	5gm
Di-potassium phosphate	2gm
Eosin Y	0.4gm
Methylene Blue	0.065gm
Agar	13.5gm
Distilled water	1000ml
pH	7.2

Mix the contents boil to dissolve the medium completely. Cool to 50OC and shake the medium in order to oxidize the methylene blue (i.e., to restore it's blue colour) and to suspend the flocculent precipitate, which is an essential part of the medium. Dispense and sterilize by autoclaving at 15 lb pressure at 121^{O}C for 15 minutes.

Exercise:

1. Why lactose fermenting bacterial colonies appear pink red on MacConkey's agar?
2. How the metallic sheen is developed by growth of *Escherichia coli* on EMB agar.
3. What makes MacConkey's agar selective for enteric bacteria.
4. What concentration of NaCl is required for the growth of bacteria of veterinary & medical importance.
5. What is halophillic bacteria?
6. What do you understand by fastidious bacteria?
7. Name the reducing agents used in anaerobic media for development of anaerobiosis.

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http://www.yeastextract.info/yeast-extract

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Practical 10

CULTIVATION OF BACTERIA: AEROBIC BACTERIA

Important steps in Isolation of Bacteria in pure culture:

First - It is required to isolate and grow the bacteria from natural environment to laboratory medium. It requires the knowledge of growth requirements of bacteria of interest.

Second- Bacteria of interest should be separated from other bacteria to obtain a pure culture of one type of bacteria.

Third- Once a pure culture is achieved, maintenance of pure culture without contamination is a task again.

Pure Culture: Population of cells arising from a single bacterial cell, to study characteristics in detail.

Pure Culture Technique- Developed by Robert Koch.

In natural habitat i.e., clinical sample, bacteria of interest usually grow in complex mixed population. It is required to separate the bacteria of interest from the mixed population, as pure culture to study the characteristics of the bacteria in detail.

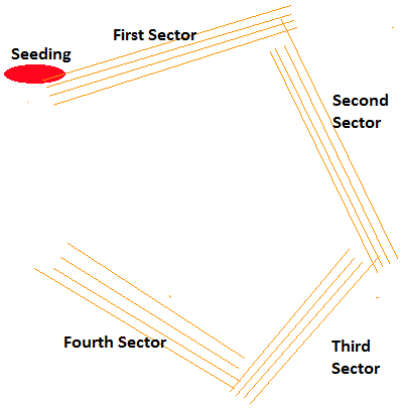
Generally, initial incubation of clinical sample is done using liquid broth i.e., Nutrient Broth for growing the bacteria or increasing the number of bacteria in the given clinical sample, in laboratory using artificial culture media.

1. **Inoculation of Nutrient Broth** : The clinical samples should be added to the nutrient broth aseptically to avoid contamination and incubate the nutrient broth for 24 Hrs at $37^{O}C$.
   In nutrient broth after incubation:
   Turbidity : Indicates the growth of bacteria
   Transparency: Indicates no growth of bacteria.

2. **Streak Plate Method:**
   To obtain a pure culture , it is required to obtain separate , single bacterial colony. To attain this, well known 'Quadrant streaking method' can be used.
   The microbial mixture is seeded on the edge of an agar plate with inoculating loop and than streaked out over the surface.

   After seeding the culture, first sector is streaked originating from seeding area. After first sector is streaked, inoculum loop is sterilized and second sector is streaked using inoculum from the first sector. Similarly, Third sector is streaked .
   In this way, the number of bacteria in seeding area is diluted in first sector, second sector .
   Eventually, very few cells remain on the loop in third or fourth sector and single cells dropped from inoculation loop will develop into separate colonies.

   Separate single colony is picked up , streaked on fresh plate to get a pure culture.



Quadrant Streaking

3. **Pour Plate Method**

   The sample is diluted several times to reduce the microbial count sufficiently to obtain separate colonies. When plating , small volumes of several diluted samples are mixed with molten agar that has been cooled to 45^{O}C before pouring into sterile plates.

   Bacteria and fungi are not killed at 45^{O}C for a short exposure. After solidification of agar each bacterial cell is fixed in a place and form an individual colony.

   Like the spread plate, pour plate can also be used to determine the number of cells in a population. Plates containing 30-300 clonies are counted. The total number of colonies equals the number of viable bacterial cells.The count of bacterial cells should be multiplied by dilution factor to work. Can also yield isolated colonies. Surface colonies can be used to prepare the pure culture.

4. **Spread Plate/Lawn Culture**

   In this method, small volume of dilute microbial mixture containing around 30-300 bacterial cells is transferred to the centre of agar plate and with the help of spreader, spread evenly over the surface of agar medium. The dispersed cells develops into a single colony.

   Numbers of colonies are equal to the number of viable organisms in the sample.

   This type of method is used in Antibiotic Sensitivity /Drug Sensitivity Testing.
   Spread plate method can be used to count microbial population.

5. **Slant and stab culture**

   Slants of agar in a test tube are generally used for maintaining the bacteria in pure culture. The bacterial colonies are streaked on the surface of slants. Slant culture is also used for various chemical tests such as Citrate utilization test, Urease test and TSI test. In TS test, the culture is streaked on agar surface as well as the culture is stabbed into the butt.

Exercise:

1.Process the clinical sample and isolate in Pure Culture

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Practical 11

CULTIVATION OF BACTERIA: ANAEROBIC BACTERIA

A variety of anaerobic culture methods are available for the culture of anaerobic organisms on the laboratory.

I. **Exclusion of oxygen from the medium** is the simplest method. And is effected by growing the organisms in freshly steamed liquid medium or deep stab in nutrient agar with 0.5% glucose / 1% ascorbic acid / 0.1% Cysteine / 0.1% sodium thioglycollate or particles of meat in cooked meat broth e.g. Robertson cooked meat medium. With minimal shaking and solidifying rapidly by placing the tubes in cold water.

Robertson's Cooked Meat Medium (RCM)

Preparation of RCM

1. Procure 500 gm bullock heart, mince it (shredded into small freckles) and add 1N NaOH 1.5 ml. Also add 500ml-distilled water.
2. Simmer the above for 20 minutes in boiling water.
3. Drain off the liquid. (Collect the liquid in another sterile container-, which can be used later for the preparation of Peptone Infusion Broth)
4. Minced boiled (cooked) meat is placed in sterile test tubes.
5. For the preparation of Peptone Infusion broth use the drained liquid (as above) i.e.,500ml,add peptone 2.56 gm and Nacl 1.25 gm.
6. Steam for 20 mins ($100^{O}C$) and cool immediately.
7. Add 1ml of pure HCl and filter.
8. Adjust pH 8.2 and steam for 30 mins at $100^{O}C$.
9. Adjust pH 7.8. (Peptone Infusion Broth).
10. Add the peptone infusion broth to the tubes with minced cooked meat (as above) in such a way that the level of Peptone infusion broth should be 2.5 cm above the level of cooked meat.
11. Adjust the pH 7.8 and autoclave.

Robertson Cooked Meat Medium

II. Anaerobic jars (McIntosh Fildes' anaerobic jar)

Material Required

McIntosh Fildes' anaerobic jar, Catalyst (Asbestos/Palladium), Resazurin indicator, 10 ml tap water.

Method

1. Clean the McIntosh Fildes' anaerobic jar with spirit.
2. Pick up the stock culture of anaerobic bacteria maintained in the Robertson's cooked meat media and draw approximately 0.1ml of inoculums in sterilized Pasteur pipette.
3. Discharge the inoculums at a corner of blood agar plate and spread by inoculating loop following quadrant pattern.
4. Place the blood agar plate along with charged catalyst.(Charging is done by pre-heating at 100 ^{O}C and Risazurin indicator in the jar.
5. Cut open the 'Gas-Pack' at the corner with a scissor. Put 10 ml of tap water in the pack and immediately place in upright position in the jar. And tighten the lid.
6. Place the jar in incubator at 37^{O}C for 48-72 hrs.
   (Maintenance of anaerobic condition in the jar will be shown by risazurin indicator turning white from its original pink colour.
7. Open the jar after 72 hrs and examine the plate for bacterial growth.
8. Prepare the smear of a suggestive colony, stain with Grams method and observe under microscope.

Exercise

Q1. Explain the principle of 'Gas-Pack' system.
Q2.Explain the role of superoxide dismutase and catalase in destruction of superoxide radical and H_2O_2 in aerobes and facultative anaerobes.
Q3. Exposure to oxygen proves toxic for strict anaerobes.

*****

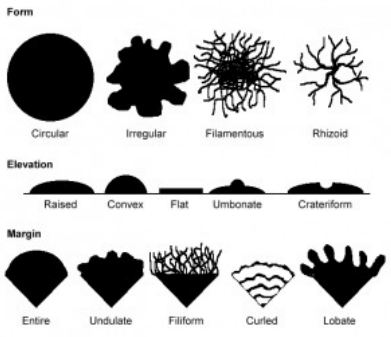
Practical 12

BACTERIAL CULTURAL CHARACTERISTICS

Cultural characteristic of bacteria is one of the important aspects applied in identification of bacteria. Cultural characteristics describe various features of growth of bacteria on medium.

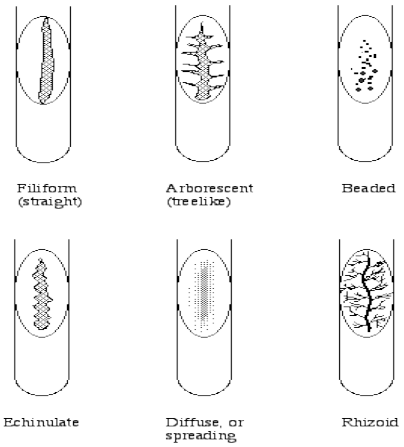
Growth on solid media

Size	- in mm
Shape	- Round, irregular, rhizoid, spindle
Elevation	- Flat, raised, convex, umbonate
Surface	- Smooth, rough, papillate, ringed, fried egg, medusa head
Margins	- Entire, wavy, lobate, erose, curled, filamentous
Opacity	- Opaque, translucent, transparent
Consistancy	- Butyrous, friable, viscid
Emulsifiability	- Easy, difficult, forms homogenous or granular suspension.



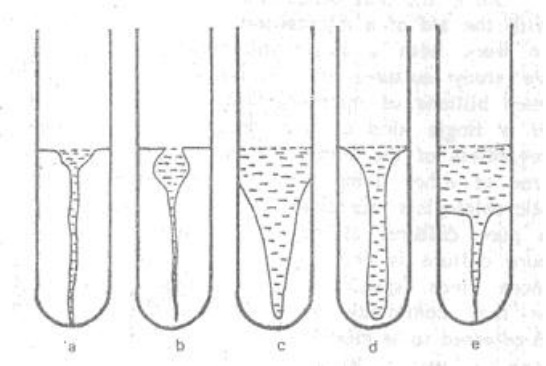
Growth on slant

Amount	- Scanty, moderate, abundant
Form	- Spreading, filiform, rhizoid



Growth in stab

Amount	- Scanty, moderate, abundant
Form	- Papillate, filiform, beaded, arborescent
Surface growth	- Absent, present, colouration
Liquification	- forming layer, like cup, like turnip, like funnel, tubular



Gelatin stab cultures: a.crateriform; b.napiform; c.infundibuliform; d. snatiform

Growth in liquid media

Amount	- Scanty, moderate, abundant
Surface growth	- Absent, present, ring, pellicle (thin, thick, smooth, granular)
Deposit	-Absent, present(slight, moderate, abundant, powdery, viscid)
Turbidity	- Absent, present
Odour	- Absent, present

Exercise

1. Describe the colony characteristics of *Escherichia coli* on MacConkey's agar medium.
2. Describe cultural characters of *Staphylococcus aureus* in Nutrient Broth.

******

Practical 13

MORPHOLOGICAL STUDIES ON BACTERIA OF VETERINARY IMPORTANCE

Staphylococcus aureus

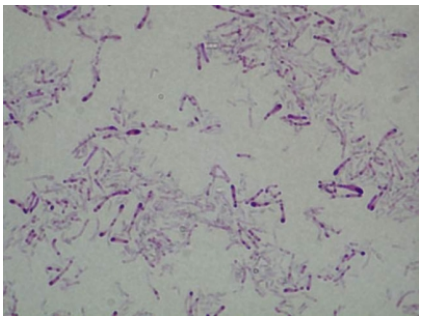
- Gram positive, Spherical cocci .
- Measuring 0.8 to 1.0 um in diameter.
- Arranged characteristically in 'Grape like' clusters. Cluster formation is due to cell division occurring in more than one plane with daughter cells remaining closer together.
- Non-spore forming,
- Non-motile,
- Non-capsulated (Exceptions rare strains)

Streptococcus pyogenes

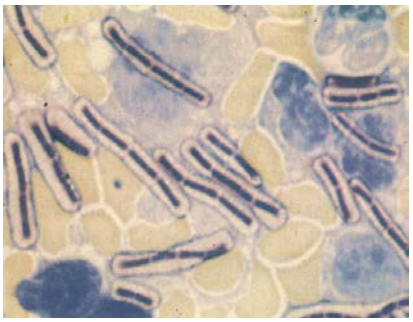
- Gram-positive cocci (In older cultures decolorized).
- *Streptococcus pyogenes* is a coccus 0.5 um to 1 um in size arranged in chains, chains made up of many diplococci. (Division in one plane.)
- Chains are shorter in artificial medium.
- Length of chain is increased by the presence of specific antibodies and is decreased in the absence of abs.
- Non-motile, Non- sporulating.
- Capsule producing strain - Streptococcus epidemicus.

Corynebacterium bovis

- Gram-positive slender rods with a tendency to clubbing at one or both ends;
- Non-sporing,
- Non-motile,
- Non-capsulated,
- Non-acid fast,
- The granules are more strongly Gram positive than the rest of the bacterial cell.
- Stained with Loeffler's methylene blue, the granules take up a reddish purple color and hence they are called metachromatic granules. They are called as **volutin or Babes Ernst Granules**, often situated at the poles of the bacilli and are called polar bodies.
- Special stains, such as Albert's, Neisser's and Ponder's have been devised for demonstrating the granules.
- Stained smears from animal tissues often reveal groups of cells in parallel (Palisades) or cells at sharp angles to each other (Chinese letter or Cuneiform arrangement).



Corynebacterium bovis



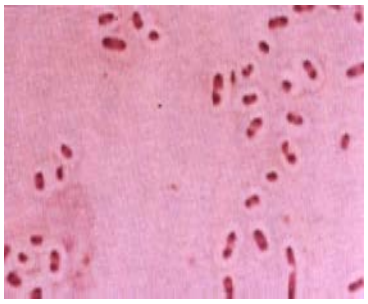
Bacillus anthracis

Bacillus anthracis

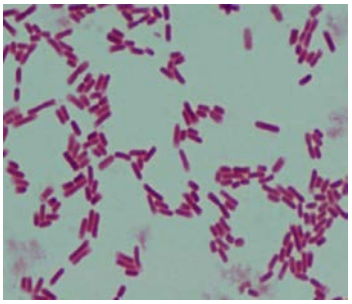
- Largest pathogenic Bacteria, 1-1.5 X4-8 u in size.
- Rod shaped bacilli with truncated ends , Arranged in chain 'Bamboo' like appearance
- Gram Positive,
- Non-motile,
- Capsulated(D-glutamic acid-polypeptide),
- McFadyeans reaction,
- Spore forming.

Pasteurella multocida

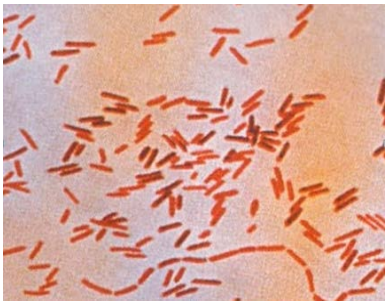
- Small coccoid rod .
- Measuring 0.25-0.4 u by 0.6-2.6u.
- Pleomorphic.
- Gram negative rods with characteristic **'Bipolar'** appearance in the stained smears.
- Possess capsule (Hyaluronic acid) when recently isolated from the disease process.On subculturing looses the capsule.
- Non-sporulating,Non-motile.



Pasteurella multocida



Escherichia coli



Salmonella Pullorum

Escherichia coli

- Gram negative rods, measuring 1-3um x 0.4-0.7um in size varying from coccid bipolar to filamentous forms, occurs singly or in short chains.
- Fimbriae are present in 80% of the strains,
- Motile by means of peritrichous flagella,
- Non-capsulated, few extra-intestinal strains possess polysaccharide capsule,
- Non-spore forming.

Salmonella Pullorum

- Gram-negative short plump rods, measuring 0.4-0.6um x 0.8-1.6um in size.
- Occur singly or in short chains,
- Non-motile,Non-spore forming,
- Non-capsulated, Long filamentous forms occur.

Mycobacterium tuberculosis

- *Mycobacterium tuberculosis: Slender* rod, 0.2-0.6um X 1.5-4.0um in size.
- Non-spore forming.
- Non-motile.
- Extremely pleomorphic.
- Acid fast (resist acid fast decolourization-due to waxy substances which prevents the ready absorption of dyes.)

Exercise

Q1. Prepare the smear stain with Gram's staining and write the shape, size, arrangement, and staining reaction of the given bacteria.

*****

Practical 14

BIOCHEMICAL & SUGAR FERMENTATION TEST

Indole Test

Some bacteria have the ability to break down tryptophan for nutritional needs using the enzyme tryptophanase. When tryptophan is broken down, the presence of indole can be detected by a colorimetric reaction with Kovac's Reagent (p-dimethyl–aminobenzaldehyde).

Kovac's Reagent

Amyl alcohol or iso-amyl alcohol	150ml
p-Dimethy-bezaldehyde	10g
Conc. Hydrochloric acid	50ml

Dissolve the aldehyde in the alcohol and slowly add the acid.

Prepare in small quantities and store in the refrigerator. Shake gently before use .

Method

1.Inoculate one tube of peptone water with bacterial isolate under test.
2.Incubate at 37 ^{O}C for 48 h (Sometimes a period of 96 hr at 37 ^{O}C).
3.Add 0.5 ml Kovac's reagent and shake gently.

Interpretation

Red colour ring in the alcohol layer indicates a positive reaction. Yellow colour ring (colour of Kovac's reagent) indicates negative test.

$$\text{Tryptophan} \xrightarrow{\text{Tryptophanase}} \text{Indole + Pyruvic Acid + Ammonia}$$

Methyl Red Test

Detects the production of acid due to fermentation of glucose.

MR-VP Medium (Glucose phosphate peptone water)

Peptone	5gm
Di-potassium hydrogen phosphateK_2HPO_4	5g
Distilled Water	1000ml
Glucose 10% solution (sterilized separately)	50ml

Dissolve the peptone and phosphate, adjust the pH to 7.6, filter, dispense in 5 ml amounts and sterilize at 121 ^{O}C for 15 minutes. Sterilize the glucose solution by filtration and add 0.25ml to each tube. (Final concentration 0.5 %)

Methyl red indicator solution

Methyl red	0.1gm
Ethanol	300ml
Distilled water	200ml

Method:

1.Inoculate MR-VP medium lightly from a young agar slope of bacterial isolate under test.
2. Incubate at 37 ^{O}C for 48 h.
3. Add 4 -5 drops of methyl red reagent.
4. Mix and read immediately.

Interpretation

Bright red colour indicates positive test and negative are yellow.

Voges –Proskauer Test

Detects the production of acetoin (acetyl methyl carbinol) which is produced by the fermentation of CHO by many bacteria.

MR-VP Medium (Glucose phosphate peptone water)

Peptone	5gm
Di-potassium hydrogen phosphateK2HPO4	5g
Distilled Water	1000ml
Glucose 10% solution (sterilized separately)	50ml

Dissolve the peptone and phosphate, adjust the pH to 7.6,filter, dispense in 5 ml amounts and sterilize at 121 ^{O}C for 15 minutes. Sterilize the glucose solution by filtration and add 0.25ml to each tube. (Final concentration 0.5 %)

Method

1. Inoculate the MR-VP medium lightly from a young agar slope of bacterial isolate under test.
2.Incubate at 37 ^{O}C for 48 h.
3.Add 1ml of potassium hydroxide and 3ml of 5% solution of *α*- napthol in absolute alcohol.

Interperetation

A positive reaction is indicated by the development of pink colour in 2-5 minutes and crimson in 30 minutes.

[Generally Members of Family Enterobacteraceae are either MR positive and VP negative or MR Negative and VP Positive]

Citrate Utilization Test

Test detects the ability of an organism to utilize citrate as the sole source of carbon and energy for growth and ammonium salt as the sole source of nitrogen.
Koser's Liquid citrate medium or Simmon's citrate agar may be used.

Koser's Medium

Sodium chloride	5.0g
Magnesium sulphate	0.2g
Ammonium di-hydrogen phosphate	1.0g
Potassium di-hydrogen phosphate	1.0g
Sodium citrate	5.0g
Distilled water	1000ml

The pH should be 6.8.
The medium is dispensed &sterilized by autoclaving at 121 ^{O}C for 15 min.

Simmon's Medium

(Modification of Koser's medium with agar and indicator added.)

Koser's medium	1000ml
Agar	20g
Bromothymol blue (0.2%)	40ml

Dispense autoclave at 121 ^{O}C for 15 min and allow to set as slopes.

Method
1.Inoculate the suspension of the organism to be tested.
2. Incubate for 96 hours at 37 ^{O}C
3.Read the results as follows

Interpretation
Koser's medium
Positive = Turbidity i.e., Growth
Negative = No turbidity

Simmon's citrate medium
Positive = Blue colour and streak of growth
Negative = Original green colour and no growth.

Dead Organisms can act as a source of carbon and may produce false positive test .

Oxidase Test

The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzyme.The test is used as an aid for the differentiation of *Neisseria, Moraxella, Campylobacter and*
Pasteurella species (oxidase-positive).

Principle
Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein).Both catalyse the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen).The test reagent*, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride* acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue.

Reagent
1% N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride in distilled water or impregnated oxidase test strips

[The test solution auto-oxidises rapidly- use a fresh solution or add 1% ascorbic acid to retard oxidation. Do not use if the solution is blue.]

Method
Direct Plate Method (do not use on colonies intended for sub-culture)

Add 2 drops of reagent to suspect colonies on an agar plate. Do not flood the plate. Examine for blue colour within 10 seconds.

Filter Paper Method
Soak a piece of filter paper in the reagent solution.
Scrape some fresh growth from the plate with a disposable loop or
stick and rub onto the filter paper or touch a colony with edge of paper.
Examine for blue colour within 10 seconds.

Interpretation
Positive result : development of a blue colour indicates oxidase production
Negative result : No blue colour

Do not use nichrome inoculating loops or wires. False positive reactions may occur due to surface oxidation products formed during flame sterilisation .

Nitrate Reduction Test

This test detects the production of enzyme nitrate reductase which reduces nitrate to nitrite
e.g.,*Enterobacteriaceae* family members are positive for the test.

Medium

Potassium Nitrate (KNO3) 0.2 G
Peptone 0.5 G
Distilled Water 1 L
Tube in 5 ml amount and autoclave 121 ^{O}C for 15 minutes.

Reagents

Reagent A Disssolve 8.0 G of Sulphanilic acid in 1 L of 5N acetic acid.
Reagent B Dissolve 5.0G of alpha –Napthylamine in 1L of 5N acetic acid.
Immediately before use mix equal volumes of solution A & B.

Method

Inoculate test organisms in 5ml medium containing potassium nitrate,peptone and distilled water.

Incubate at 37^{O}C for 96 Hrs.

[Ad 0.1 ml test reagent which consists of equal volumes of 0.8% Sulphanilic acid and 0.5 % alpha napthylamine in 5N acetic acid mixed just before use.]

Interpretation

A red colour develops within few minutes indicating the presence of nitrite and indicating the ability of test organism to reduce nitrate to nitrites.

Medium +	Nitrate	Sulphanilic acid
0.02 % Potassium nitrate & 0.55 peptone	---------- Nitrite ---------- Reductase	---------- Diazo Red Dye ---------- Alpha Naphtylamine

If no colour develops this may indicate that either nitrate has not been reduced or that nitrate has been reduced beyond nitrite to nitrogen gas, nitric oxide or nitrous oxide, which the reagents will not be able to detect. To detect this add Zinc dust to the test. Metallic zinc reduces nitrate to nitrite, and red colour develops following addition of zinc dust means that the organism was unable to reduce the nitrate to nitrite.

Phenylalanine Deaminase Test

This test indicates the ability of an organism to deaminate phenylalanine with the production of phenylpyruvic acid which will react with ferric acid to give a green colour.

Medium

Yeast extract	3g
DL-Phenylalanine	2g
Na2HPO4	1g
Sodium Chloride	5g
Agar	12g
Distilled Water	1L

Adjust the pH to 7.4, distribute and sterilize by autoclaving at 121^{O}C for 15 minutes. Allow to solidify in tubes as long slopes.

Method

- Inoculate with a fairly heavy inoculum. Incubate for 4 Hrs or if desired for up to 24 Hrs at 37^{O}C.Allow few drops of a 10% solution of ferric chloride to run down over the growth on the slope.
- If the test is positive, a green colour will develop in the fluid and in the slope.
- 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.
- 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.
- Production of gas is determined with a Durham tube ,a small inverted vial filled with the carbohydrate fermentation broth.
- If gas is produced during fermentation of the sugar, it is trapped at the top of the Durham tube and appears as a bubble.
- Slow fermenters may take a week or more to cause color changes detectable by the human eye.

Interpretation

Positive (yellow color or yellow color with gas bubble) and negative results (red color, no gas bubble).

Sugar Fermentation Test

Used to differentiate bacteria on the basis of CHO fermentation abilities.

Ability of an organism to ferment a specific carbohydrate added in basal medium results in the production of acid or acid and gas. This ability has been used to characterize a specific species of bacteria which helps in differentiation between genera and aid in the differentiation between genera and aid in the differentiation of species as well.

Principle of sugar fermentation test

When CHO is added to a culture medium. On incubation, it is fermented by microorganisms, the acid (or acid and gas) produced lowers the pH and the indicator in the basal medium changes the colour e.g., Phenol red changes from red to orange to yellow and the gas produced if any, collects in the Durhams tube.

Media and Reagents

Sugars are used as 1% solutions in peptone water to test fermentative reactions of bacteria. Beef extract is also added to the medium. Small inverted tube (Durhams tube) is placed in the medium to detect the formation of gas and one of the indicators such as Phenol Red, Andrades Indicator etc., as shown in the table given below is added to detect formation of acid.

Interpretation

A positive result for acid is yellow after indicator is added (indicating sugar fermentation)
A positive result for gas is a bubble in the Durhams tube.
A completely negative result has no color change or reddish color & no bubble.

Sugars used

Pentoses	Arabinose, Rhamnose, Xylose.
Hexoses	Glucose(Dextrose), Fructose(Laevulose), Galactose, Mannose, Trehalose.
Disaccharides	Sucrose(Saccharose), Lactose, Maltose, Trehalose.
Trisaccharides	Raffinose.
Polysaccharides	Starch,Dextrin,Inulin,Glycogen.
Glucosides	Salicin,Aesculin
Alcohols	Glycerol, Erythritol, Adonitol, Dulcitol, Mannitol, Sorbitol, Inositol.

Reactions of Indicators at different pH ranges

Indicators	Conc. used in the medium	Colour Change	pH Range
Andrade	1 N NaOH in 0.5% acid fuchsin (until colour becomes yellow)	Pink –-Yellow	5.0-8.0
Phenol Red	5% of 0.2% Solution	Yellow –-Red	6.8-8.4
Bromothymol Blue	1% of 0.2% solution	Yellow–-Blue	6.0-7.6
Bromocresol Purple	1% of 0.4% solution	Yellow–-Blue	5.2-6.8

Exercise

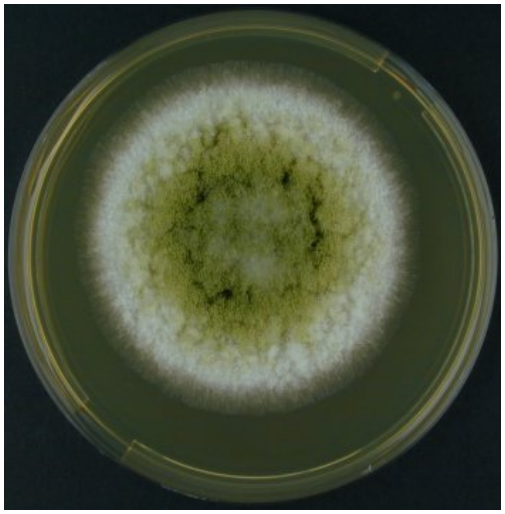
Q1. Enlist the steps in identification of the bacteria.

Q2. Write the biochemical tests & Sugar fermentation test results for the bacteria mentioned below:

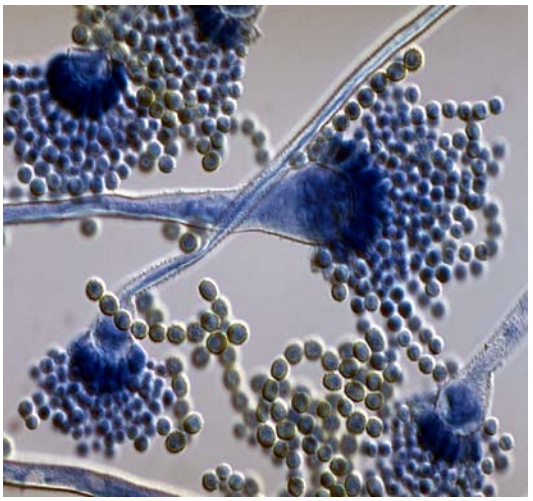
a.*Escherichia coli*
b.Salmonella Pullorum
c.*Proteus mirabilis*
d.*Klebsiella pneumoniae*
e.*Shigella dysentriae*

*****

COLOUR PLATE 2



1. *Aspergillus flavus*

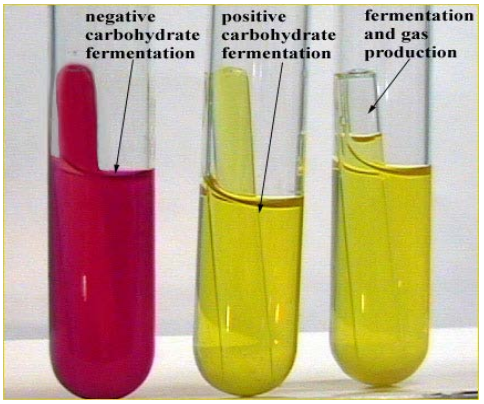


2.*Aspergillus flavus*- Microscopic

3. Urease Test

4.Indole Test

5. Methyl Red Test



6. Quadrant Streak Plate

Practical 15

ANTIBIOTIC SENSITIVITY TEST

On the advent of Penicillin by Alexander Fleming in 1928, proved to be wonder drug in treating the infections. Many antimicrobial compounds were discovered and was predicted that infectious diseases would be eliminated through the use of antimicrobials. But the indiscriminate use of antimicrobials led to the development of bacterial resistance to antimicrobials, diminished the optimism and resulted in the need for physicians to request the microbiology laboratory to test a pathogen against various available and widely used array of antibiotics in medical and veterinary treatment.

Initially broth dilution method was used, later on modified to disc diffusion method in which the antimicrobials are impregnated onto the 6 mm size paper disc used for assessing the antimicrobial activity on lawn culture of pathogen on specific solid gel media incubated for specific time at $37^{O}C$ for 12-24 Hrs.

Antibiotic sensitivity test is carried out to find out sensitivity of organisms to particular antibiotic/antimicrobial drug. This test has great importance to direct the clinicians for employing antibiotic therapy in patients.

In 1950's many laboratories modified the disc diffusion method to suit their requirement. Changes in the media used, concentrations of drug impregnated on disc, number of bacteria in inoculums, time and temperature combination, led to mass confusion and variations in results.

The present day method, described by W. M. M. Kirby and A. W. Bauer (1966) is the result of extensive review of literature, known as **Kirby-Bauer Disc Diffusion method** and put forth one standard protocol published and accepted by all (WHO) to test the sensitivity of an organisms towards antimicrobial drugs/antibiotic.

To bring the uniformity and reproducibility, the Clinical Laboratory Standards Institute (CLSI) is authorized (USA) for updating and modifying the original procedure of Kirby and Bauer. The zone of inhibition for a particular antibiotic and pathogen should be as per the guidelines of CLSI.

Principle of AST:

When disk impregnated with antimicrobial, is placed on Mueller Hinton Agar medium, water is absorbed in to the disk immediately and the antimicrobial begins to diffuse into the surrounding agar medium. The rate of diffusion is not rapid, so the concentration of antimicrobial is highest closer to the disc and a logarithmic reduction in concentration occurs as the distance from the disc increases. The rate of diffusion depends upon the solubility property of drug in agar.

If the agar plate is seeded with pathogen prior to the placement of discs, simultaneous growth of organism and antimicrobial diffusion through agar medium occurs. The antibiotic will diffuse in agar medium and sensitive organism will not grow in the vicinity of such antibiotic while the resistant strain will show either very less or no zone of inhibition depending upon the antibiotic concentration in the agar medium near to the disc.

Zone of inhibition of any size observed in a disk diffusion test has no meaning as such. The interpretation of resistance and susceptibility to antimicrobials is determined by correlating with zone sizes resulting in the interpretive standards (CLSI).

Materials:

Muller and Hinton Agar plates, Antibiotic discs, Scale, Zone of inhibition interpretation chart, Sterile swabs, Clinical Sample/Fresh Culture, Peptone water, Inoculation loop, Forcep, Spirit lamp.

Preparation of media

MH agar plates from dehydrated media, should be prepared with a depth of 4 mm (25 ml of liquid agar for 100-mm plates). Too shallow plates will produce false susceptible results as the antimicrobial compound will diffuse further than it should, creating larger zones of inhibition. Conversely, plates poured to a depth of more than 4 mm will result in false resistant results.

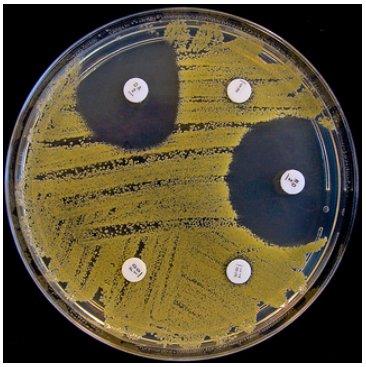
Preparation of inoculums

1. Using a sterile inoculating loop or needle, touch four or five isolated colonies of the organism to be tested.
2. Suspend the organism in 2 ml of sterile saline.
3. Vortex the saline tube to create a homogenous suspension.
4. Adjust the turbidity of this suspension to a 0.5 McFarland standard by adding more colonies if the suspension is too thin or diluting with sterile saline if the suspension is too thick. Use this suspension within 15 minutes of preparation.

Procedure:

1. With the help of sterile loop inoculate the sample in peptone water tube and incubate at 37 ^{0}C for 24 hrs.
2. Inoculate the culture on Muller and Hinton Agar plate by spread plate method using sterile swab. Allow the plate to absorb the culture.
3. With the help of sterile forcep place the antibiotic discs on agar surface keeping 2 cm distance between two adjacent discs. **Do not move a disk once it has contacted the agar surface even if the disk is not in the proper location, because some of the drug begins to diffuse immediately upon contact with the agar.**
4. These antibiotic discs shall be chosen depending upon the type of sample and history of symptoms provided by clinicians.
5. Incubate the plate at 37 ^{0}C for 18-24 hrs.
6. Measure the diameter of zones of inhibition of bacterial growth in mm with the help of scale.
7. The diameter is measured vertically as well as horizontally and average of both is taken as reading.
8. Record the observations and compare with their respective prescribed zones of inhibition mentioned in interpretation chart.
9. The suitable antibiotic shall be chosen for treatment amongst effective antibiotics.

Interpretation



Zone Interpretation Chart for *Staphylococcus aureus* **(Zone diameter in mm)**

Antibiotics	Susceptible (mm or more)	Intermediate (mm)	Resistant (mm or less)
Amikacin (30mcg)	17	15-16	14
Penicillin (10 Units)	29	-	28
Tetracycline (30 mcg)	19	15-18	14
Ciprofloxacin (5 mcg)	26	22-25	21
Ceftriaxone (30 mcg)	21	20-22	19
Cefotaxime (30 mcg)	23	15-22	14

Zone Interpretation Chart for *Enterobacteriaceae* **(Zone diameter in mm)**

Antibiotics	Susceptible (mm or more)	Intermediate (mm)	Resistant (mm or less)
Amikacin (30mcg)	17	15-16	14
Streptomicin (10mcg)	15	12-14	11
Gentamicin (10mcg)	15	13-14	12
Ciprofloxacin (5mcg)	21	16-20	15
Ceftriaxone (30mcg)	23	20-22	19
Cefotaxime (30 mcg)	26	23-25	22
Tetracycline (30mcg)	15	12-14	11

Exercise:

1. Write the Zone of Inhibition Chart for other commonly used antibiotics.

References:

1. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol.36:493-496.

2. www.microbelibrary/Kirby-Bauer Disk Diffusion Susceptibility Test Protocol.htm

For interpretive Zones of Inhibition log on to the links:

http://www.himedialabs.com/intl/en/products/Microbiology/Antimicrobial-Susceptibility- Sensitivty-Discs-Antibacterial-As-per-CLSI/100000449

Clinical Laboratory Standards Institute

http://www.clsi.org/blog/2012/01/13/clsi-publishes-2012-antimicrobial-susceptibility- testing-standards/

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Practical 16

SLIDE CULTURE TECHNIQUE AND LACTOPHENOL COTTON BLUE STAINING FOR FUNGUS EXAMINATION

Fungus are eukaryotic organism and they are classified into two main groups that is yeast and molds. Its cell wall is made up of chitin. The fungal structures include mycelium, sporangiospore, spores etc. The Lactophenol Cotton Blue wet mount is simple and widely used staining method for fungi.

Lactophenol Cotton Blue Stain (LCB)

Cotton Blue	0.05g
Phenol Crystals	20g
Glycerol	40ml
Lactic Acid	20ml
Distilled water	20ml

Method

Preparation of staining requires two days.

1. Dissolve the Cotton Blue in distilled water and leave overnight to eliminate insoluble dye.
2. Next day, add phenol crystals to the lactic acid in a glass beaker and stir it on magnetic stirrer until the phenol is dissolved.
3. Add the glycerol and filter the cotton blue solution into the Phenol + Glycerol + lactic acid solution.
4. Mix and store at room temperature.

The main components of LCB staining are :

1. Phenol: Fungicidal in nature
2. Lactic Acid :Preserves fungal structures
3. Cotton Blue: Stains the chitin in the fungal cell walls & the cytoplasm (in light blue).

Staining of Clinical Specimens (Non-keratinized) Procedure (LCB Staining)

1. Place a drop of 70% alcohol on the slide.
2. Add the specimen to the drop of alcohol.
3. Add one or two drops of Lactophenol Cotton Blue Stain before alcohol gets off.
4. Place the coverslip on the drop avoiding air bubbles to be trapped.
5. Examine under Microscope using 10X and 40X objective.

Staining of fungus from culture

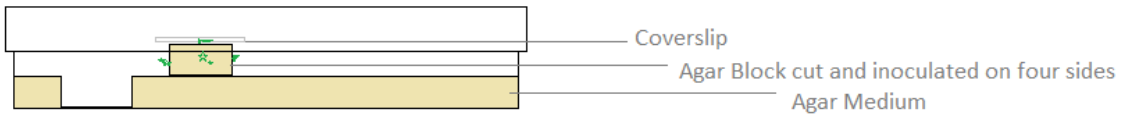
1. Take a grease free slide.
2. Add a drop of lactophenol cotton blue solution on a slide.
3. Sterilize the inoculation loop or needle and cool it then transfer mycellial growth onto the LCB stain and press it gently so that it easily mix with the stain.
4. Take a clean cover slip and with the help of a forcep place the cover slip on mycellial gowth + LCB.
5. With the help of blotting paper, wipe the excess stain .
6. Observe the preparation under low & high power objectives of the microscope.

Slide Culture Technique

For accurate identification of fungi, it is required that the precise arrangement of the conidiophores and the way in which the spores are produced is essential. The simple method of slide culturing used widely is described here, which permits fungi to be studied virtually *in-situ* with as little disturbance as possible.

Procedure:

1. With the help of a sterile blade cut out an agar block (6 x 6 mm) enough to fit under the coverslip.
2. Flip the block up onto the surface of the agar.
3. Inoculate the sides of the agar block with spores or mycelia of the fungus to be grown.
4. Flame the coverslip and place it on agar block.
5. Incubate at 26^{O}C until growth and sporulation take place.
6. After attaining the growth, remove the cover slip from the agar block.
7. Apply a drop of 95% alcohol as a wetting agent and gently lower the coverslip onto a small drop of Lactophenol cotton blue on a grease free glass slide.
8. The slide can be left overnight to dry and later sealed with nail polish.
9. When sealing with nail polish use a coat of clear polish followed by one coat of red coloured polish.



Slide Culture Technique

Exercise

Q1. Draw a well labelled diagram of *Aspergillus niger and Candida albicans*

References

Leck Astrid, 1999. Preparation of Lactophenol Cotton Blue Slide Mounts, *Community Eye Health*. 1999; 12(30): 24

http://www.generalmicroscience.com/microbial-laboratory-techniques/staining-fungus-using-lactophenol-cotton-blue/

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General Microbiology

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