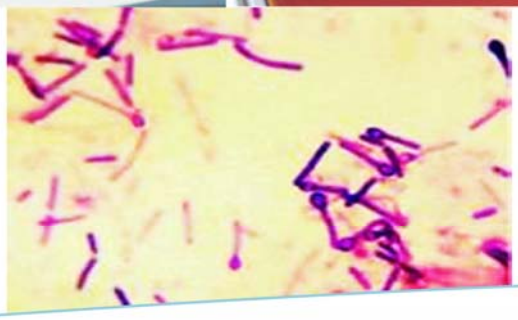
Laboratory Manual

VETERINARY MICROBIOLOGY

VMC Unit-I



Department of Veterinary Microbiology

College of Veterinary & Animal Sciences, Udgir

MAHARASHTRA ANIMAL & FISHERY SCIENCES UNIVERSITY, NAGPUR

LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY

VMC

(New Syllabus As Per MSVE 2016)

Unit – I

Veterinary Microbiology

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LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY

CERTIFICATE

Certified that this is a bonafide record of practical work done in the laboratory for the course of **VETERINARY MICROBIOLOGY (VMC) Unit I** during the year .

Name of the student: _____

Registration No.: _____

Exam seat No.: _____

Course Teacher

ANNUAL EXAMINATION

Evaluated the practical record submitted for the Annual Practical Examination held on_____.

Course Teacher **Sectional Head**

Examiner

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Practical 1

ORIENTATION TO MICROBIOLOGY LABORATORY AND GENERAL INSTRUCTIONS

In this course, microorganisms isolated from infected animals and humans are studied. Certain rules and regulations are necessary to avoid 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 glassware to laboratory technician.
b. Minimizing production of aerosols.
c. Washing hands prior to and following lab & 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

Exercise

1. Enlist the equipment /instruments available in the microbiology laboratory & write its use.
2. What is GLP & GMP ?
3. Explain the difference between Laminar Flow & Biosafety Cabinet, with neat labelled diagram of air flow.
4. Explain the setup of BSL2 laboratory along with diagram.

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Practical 2

STERILIZATION AND DISINFECTION

Definition

"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 as:

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. X-rays
iii. Infrared rays
iv. 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.

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.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^{O}C

Autoclave (steam under pressure) - 121^{0}C, 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^{0}C 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.

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.

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.

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

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. 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 same filter should not be used for procedures lasting longer than one working day.

Sterilization 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. i.e., Seitz Filter:

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

EK: Special grade(Do not allow *Serratia marscens,* the test bacteria to pass).

For sterilization the filter is loosely assembled with 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^{O}C range.

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

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. It must only be carried out under the supervision of highly skilled staff.

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

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

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.

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.

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^{O}C, while using low concentrations of ClO_2. 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 ClO_2 gas and this gas is then exposed to the equipment in a sterilizing chamber.

D. Ozone

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

The cycle time may be up to 60 minutes depending on the size of the chamber or load of items to be sterilized.

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.

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.
The highest temperatures reached in gamma sterilization are usually 30-40^{O}C.

Gamma radiation is popular for sterilizing through the packaging. A dose of 2.5 megarad is generally selected for many items.
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.

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 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

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 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.

4% alkaline glutaraldehyde solution, has tuberculocidal and highlevel disinfection capabilities. It achieves high-level disinfection in 20 minutes at 25□C and has up to a 28-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.**

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 available 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.

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 .
Four percent formaldehyde is a tuberculocidal agent, inactivating M. tuberculosis in 2 minutes .
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*.

Exercise

1. Draw well labelled diagrams of: Autoclave, Hot Air Oven, Seitz Filter, Sintered Glass Filter, Membrane Filter Assembly and Syringe Filter.
2. What is rectified spirit? Write its role in disinfection.
3. Write the various grades of membrane filter?

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Practical 3

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.

e.g., 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.

e.g., 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.

e.g., 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.

e.g., Modified Thayer –Martin Agar
Salmonella-Shigella(SS) agar

5. Differential media

Media to which indicator substances are added to differentiate bacteria.

e.g., 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.

e.g., 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
e.g., 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.

Application

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.

1. 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 exogenous 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.
e.g., 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.

Properties

Agar-agar is vailable 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.

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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Practical 4

CULTIVATION OF BACTERIA: AEROBIC & ANAEROBIC BACTERIA

Cultivation of 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 in 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 of agar.

Eventually, very few cells remain on the loop in third or fourth sector streaking and single cells dropped at the end of streaking from inoculation loop will develop into separate colonies. Separate single colony is picked up , streaked on fresh plate to get a pure culture.

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.

Cultivation of 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.

Reinforced 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 and adjust pH 8.2 and steam for 30 mins at $100^{O}C$.
8. Adjust pH 7.8. (Peptone Infusion Broth).
9. 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.
10. Adjust the pH 7.8 and autoclave.

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

1. Explain the principle of 'Gas-Pack' system.
2. Explain : Exposure to oxygen proves toxic for strict anaerobes.

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Practical 5

ISOLATION OF BACTERIA IN PURE CULTURE

Pure Culture: The bacterial culture originating from a single colony and having single type of bacterial colonies.

To obtain a pure culture , it is required to obtain separate , single bacterial colony. To attain this, well known 'Quadrant streaking method' is commonly used. IN this method, the microbial mixture is seeded on the edge of an agar plate with inoculating loop and than streaked out over the surface.

Procedure:

1. Sterilize the inoculating loop on the bunsen burner by flaming until it is red hot. Allow it to cool.

2. Pick an isolated colony from the agar plate culture and seed the culture over the first quadrant (approximately 1/4 of the plate).

3. Flame the loop and cool it.

4. Turn the plate 90°, first sector is streaked originating from seeding area.

5. Flame the loop.

6. Turn the plate 90°, touch the previous streaks, and streak into the next quadrant as in step 4.

7. Flame the loop and cool it.

8. Repeat #6, streaking the remainder third and fourth sector/quadrant of the plate.

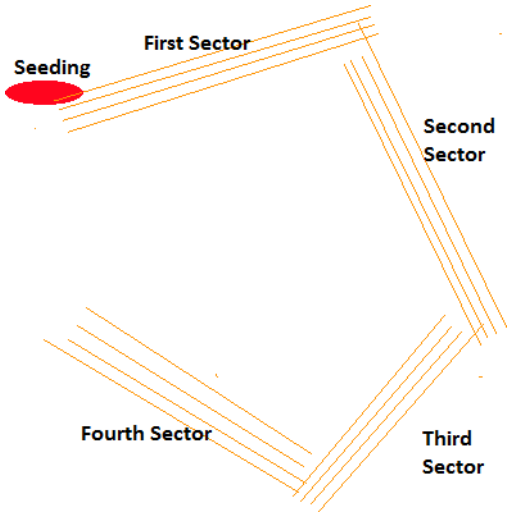
9. Invert the plate and incubate at 37°C for 24 hr. The reason the plate is inverted is the fact that the air space between the dish lid and the agar surface is saturated with moisture; during incubation, the moisture condenses on the upper lid as droplets. As these droplets collect into a large drop, the water drips onto the agar surface causing the spread and mixing of colonies. Inversion of the plate eliminates this problem.

10. Pick up single colony and streak it on fresh plate to get a pure culture.

After each sector streaking loop is sterilized. Flame your loop and cool. And to start new sector/quadrant , loop is touched to last streaked line to carry forward the culture to new streaking. In this way, the number of bacteria in seeding area is diluted in first sector, second sector, third sector and fourth sector.

Eventually, very few cells remain on the loop in third or fourth sector and single cells dropped from inoculation loop will develop into new distinct colony. One bacterial cell can give rise to one

bacterial colony hence bacterial growth is measured in CFU/ml i.e., Colony Forming Units/ml for broth culture.



Quadrant Streaking

Exercise

1. Enlist the different streaking methods and applications.

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Practical 6

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 cells by ionic, covalent, or hydrophobic bonding and stain the cells /microorganisms directly. Some stains such as Indian Ink , Nigrosin 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 pH.

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 7

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.

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.

Chemical Mechanism of Gram Reaction:
Many theories were put forth 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 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 completely. 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 8

DIFFERNTIAL 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).

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 carbol fuchsin 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

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.

Interpretation

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

Exercise

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

Reference

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Practical 9

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.

Albert staining:

Procedure:

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 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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8. Schaeffer, A. B., and M. Fulton. 1933. A simplified method of staining endospores.
9. Science77:194.
10. www.microbelibrary.org

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Practical 10

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 centre.

3. Invert the cavity slide over the cover slip, allowing the glass to adhere to the Vaseline/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 centre 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

1. Draw a well-labelled diagram of hanging drop preparation.
2. Differentiate between bacterial motility and Brownian movement.
3. Draw diagram of different flagellar arrangements of the bacteria with examples.

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Practical 11

CULTURE 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.

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 Culture Sensitivity Test:

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 diameter of zones of inhibition of bact. 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 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

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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/

Practical 12

OUTLINES OF COLLECTION, TRANSPORTATION AND PROCESSING OF SAMPLES FOR BACTERIAL DISEASES

Collection, Preservation and Dispatch of Material For Laboratory Examination.

Most of the infectious diseases cannot be diagnosed by the clinical symptoms alone .The help of a bacteriological laboratory is absolutely necessary for correct diagnosis. As the clinicians always do not find such laboratories at hand, the specimens have to be sending either through special messengers or by post parcels so as to reach the laboratory within a short period of time. The specimens should be received in the laboratory with minimum or no alterations. Materials for the bacteriological isolation should be placed in convenient sized watertight glass or plastic containers. Plastic bags of screw capped plastic tubes and jars are now available which are unbreakable and very handy for the dispatch of the material by post parcel. Refrigeration can be accomplished by placing the water tight specimen containers in ice and saw dust in a larger tin or container which itself should be water proof to avoid any leakage when ice melts. Each specimen contained in the same package should be properly labeled. From postmortem cases information mentioning the name of the owner of the dead animal, identification of the animal, date and time of death, time of postmortem, disease suspected, methods of preservation of specimen, type of examination desired, history, clinical symptoms and treatment, if any before death should be accompanied along with the material. A brief note on postmortem findings should also be send along with the specimens.

The type of specimen for bacteriological examination may be collected as follows:

Organs and tissues:

Specimens from postmortem animals should be collected as early as possible after the death and opening the body of the animal. Collections should always be made with the help of sterile instruments and equipment. With aseptic precautions and immediately send to the laboratory in leak proof sterile containers under refrigerated conditions.

Blood:

For the collection of whole bloods it should be citrated to prevent clotting. During life time blood from large animals is usually collected from the jugular vein. From post mortem cases blood is drawn from the right auricle or right ventricle of the heart with the help of syringe or a sterile Pasteur pipette after searing the surface with hot spatula. Blood can be collected in a leak proof screw capped vial and dispatched in ice and saw dust. If blood is drawn by Pasteur pipette, the same may be sealed over flame and dispatched as it is.

Serum is frequently required for serological reactions. Blood is collected in sterile test tubes, which is allowed to clot in a cold place. Serum is separated with the help of Pasteur pipette and transferred into another sterile screw capped tubes. Merthiolate in 1:10000 strength may be added as a preservative.

Pus, sputum, nasal discharge, throat and uterine discharges:

These specimens are usually collected directly from the sterile cotton swabs, sealed in screw capped test tubes and dispatched under refrigeration.

Exudates, transudates & other body fluids/clinical specimens should be collected with the help of sterile pipettes or syringes immediately after the body is opened and dispatched in leak proof screw capped sterilized container on ice or under refrigeration conditions.

Blood Exudates and tissue smears

Thin smears are prepared from blood; body exudates and external cut surfaces of various organs and properly marked for identification. Smears are dried in air, fixed in methyl alcohol and packed in non- absorbent papers with matchsticks between two adjacent slides to keep the smears apart from each other.

Pieces of intestines and their contents

The two ends of the representative portions of intestine should be tied with threads and separated from the rest of the organ. The pieces are packed in a wide mouth screw capped bottles and dispatched under refrigeration condition. Few drops of chloroform should be added as a preservative.

Milk

The udder should be washed with some antiseptic lotion. Discard first strippings before collecting15-20 ml milk from infected quarter and other quarters. Separate samples should be collected in separate screw capped test tubes and dispatched under refrigeration.

Urine

Urine samples should be collected with the help of a sterile catheter and dispatched in a screw caped tubes. Boric acid may be added as a preservative.

Hairs and scrapings from the skin

From dermatomycotic cases material may be collected after sterilizing the part with 70% alcohol. Scrapings should be collected from the margins of the lesions with the help of a blunt scalpel. Few hairs should also be pulled out and included in the specimen, which is packed in nonabsorbent paper and dispatched to the laboratory.

Collection of Material in Bacterial Diseases

Actinomycosis & Actinobacillosis: Smears from pus lesions, pus in vials on ice from affected materials.

Anthrax: Flame fixed blood smears of cattle and sheep. From subcutaneous swelling in horses, swine and dogs. Swab of blood from ear vein for cultural examination from dead animals. A small piece from tip of ear or muzzle (0.5 cm approx) in saline or without any preservatives in sterile glass test tubes or bottle on ice duly sealed. It is not advisable to open the carcass suspected for Anthrax in field. If opened, it should be properly disposed off by burning. All natural orifices of dead animal as well as bleeding surfaces may be sealed with cotton soaked in carbolic acid.

Bacillary White Diarrhea /Pullorum Disease (Poultry): Heart blood, faecal samples and tissues from liver, spleen an kidneys under refrigeration.

Black quarter: Impression smears from affected muscle tissues; exudates from lesion; pieces of affected muscles on ice.

Botulism: Intestinal contents and suspected food for demonstration of toxins under refrigeration.

Bovine genital campylobacteriosis:

In males: preputial mucus / smegma / secretions/washing, smear.
In females: vaginal mucus / lavage, aborted fetus, placenta, stomach contents, lungs, liver etc.

CCPP/ CBPP/Mycoplasmosis / Coryza : Swab from lesions nose and vagina in PBS on ice, paired serum.

Brucellosis: Paired serum, heart blood and abomasal contents of aborted fetus, placenta with 2-3 cotyledons, vaginal swabs in PBS, in separate bottle on ice, whole fetus, if small, on ice . Neat semen in sterile vial or semen straw on ice.

Chalmydosis / Psittacosis: Nasal swab, lung pieces in sterile container on ice, fixed impression smear from liver, lung and fetus. Sterile paired sera.

Contagious equine metritis: Urogenital swabs from mare & stallion, paired serum samples.

Cholerae/Shigellosis in human beings: Faecal swabs under refrigeration.

Chronic respiratory disease (CRD) in poultry: Swab from lesions nose and vagina in PBS on ice, paired serum.

Colisepticaemia in chick due to *E.coli*: Heart blood from live chicks or freshly dead or sick chicks, under refrigeration.

Coryza (Haemophilus paragallinarum): Swab from lesions nose & vagina in PBS on ice, paired serum.

Enterotoxaemia: Contents of small intestine with or without chloroform separately on ice, kidney, and urine.

Erysipelas in swine: Skin discharges or swabs and scrapings from 'Diamond skin disease' under refrigeration.

Fowl typhoid/ Paratyphoid / Salmonellosis: Intestinal swab, heart blood, bile, liver, Spleen etc. In sterile container on ice.

Glanders: Exudate from skin and lung lesions in vials on ice Impression smears from exudates duly fixed.

Johne`s disease: Rectal pinch smears, bowl washings (at least 10gm preserved in 10%neutral formol saline solution-For rectal pinch smear examination and not for the bacterial isolation).

Lamb dysentery: Contents of small intestine with or without chloroform separately on ice, kidney, urine.

Leptospirosis: Blood, serum, milk, 20ml urine in sterile vials by adding 1drop of formalin (for microscopic examination).

Listeriosis: Aborted fetus, brain, placenta and all internal organs in sterile vials on ice.

Necrobacillosis /Foot rot disease in sheep: Affected tissues under refrigeration.

Pasteurellosis/Haemorrhagic septicemia/Fowl cholera: From sick animals fixed smears from blood and throat swelling and from dead animals, smears from heart blood and liver, heart blood in a sterile pipette / bottle, lymph nodes and spleen on ice.

Tetanus: Deep swab from the local tissues of affected part under refrigeration

Tuberculosis: Cough material in sterile container from live animal, sample of milk in sterile container,smear from lesions fixed by heat, lymph gland, lung lesions in sterile container for bacterial isolation in 50% buffered glycerin.

Ulcerative lymphangitis/Caseous lymphadenitis: Smears and swabs from lesions on ice.

White scours in calves: Faecal swabs from live animal and mesenteric lymph gland, intestinal tissues and its contents under refrigeration from the postmortem.

Collection of Material From Mycotic infections

Aspergillosis: Affected tissue, nodular lesions from the lungs in case of Brooder's Pneumonia, under refrigeration.

Blastomycosis/Candidiasis/Coccidiodomycosis: Affected tissue under refrigeration.

Cryptococcosis: Affected tissue under refrigeration and brain tissues should be sent in Zenker's fluid.

Histoplasmosis: Smears and swabs from lesions and affected tissues under refrigeration.

Rhionosporiodosis: Affected tissues (Polyps) fresh.

Ringworm: Skin scabs or dead tissue (keratinised tissue), hairs, nails etc to be dispatched in an envelope.

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Practical 13

CHARACTERIZATION OF STAPHYLOCOCCUS

Morphology

Gram positive, Spherical cocci, measuring 0.8 to 1.0 um in diameter. Arranged in 'Grape like' clusters. Cluster formation is due to cell division occurring in more than one plane with daughter cells remaining closer together. In the presence of some antibiotics or ageing of culture, cocci stains Gram Negative.

Non-spore forming, Non-motile, Non-capsulated (Exceptions rare strains)

Cultural characteristics

1. Nutrient Agar

Staphylococcus aureus	Golden – yellow, 1-2 mm diameter, glistening colonies giving 'oil paint' appearance.
Staphylococcus albus	Ivory white / Cream colored 1-2 mm diameter, glistening colonies
Staphylococcus citreus	Lemon yellow colored, 1-2 mm diameter, glistening colonies giving 'oil paint' appearance.

Pigment production can be enhanced by incubating at 22^0C / addition of 1% Glycerol acetate/incorporation of milk into the medium. Pigmentation is due to carotenoids.

2. Blood agar

Colony characteristic similar to those on Nutrient agar, But are surrounded by the zone of *alpha, beta* - hemolysis. Hemolysis is marked on bovine, sheep & rabbit blood agar; weak on horse agar.

3. MacConkeys Agar

Colonies are smaller in size and impart pink colour due to lactose fermentation.

4. Mannitol Salt Agar

1% Mannitol,7.5% sodium chloride & 0.0025% Phenol red is added to the Nutrient agar. This is both selective and Indicator medium. Most strains of *Staphylococcus aureus* ferments mannitol therefore produce acid, due to which yellow zones surround colonies. Colony characters are similar to that of Nutrient agar.

Addition of Potassium tellurite imparts black colour to the colonies.

Bio-chemical Properties

Staphylococcus aureus is catalase positive. (Catalase production may function to inactivate toxic hydrogen peroxide and free radicals formed by myeloperoxidase system within phagocytic cells after ingestion of microorganisms.)

Oxidase negative.
Ferments glucose. maltose, lactose, mannitol and sucrose with the production of acid but no gas. Most strains of *Staphylococcus aureus* ferment mannitol, whereas most strains of *Staphylococcus epidermidis, Staphylococcus saprophyticus* are Mannitol negative.

Indole Negative, MR Positive, VP Positive
Urease Positive, Hydrolyzes gelatin
Reduces nitrates to nitrites (NO_3---NO_2)
Lipolytic- when grown on media containing egg yolk, produce a dense opacity.

Tests for Identification

1. Catalase Test

This test is used to differentiate those bacteria that produce the enzyme catalase. Such as *Staphylococci*, from non-catalse producing bacteria such as *Streptococci*.

Principle

Catalase acts as catalyst in the breakdown of hydrogen peroxide to oxygen and water.

Requirement

Hydrogen peroxide - 3% H_2O_2 (10 volume solution)

Method

Pour 2-3 ml of the hydrogen peroxide solution into a test tube.
Using sterile wooden stick or glass rod, remove a good growth of the test organisms and immerse it in the hydrogen peroxide solution. Look for immediate bubbling.

Results

Active bubbling - Positive test - Catalase produced.
No release of bubble - Negative test - No Catalase produced.

2. Slide Coagulase Test

Place a drop of saline (0.85% NaCl) solution or water on a clean microscope slide. With the minimum of spreading, emulsify a small amount of solid culture, e.g. one or two colonies, in the drop of saline to form a smooth milky suspension. If the strain is auto-agglutinable and a smooth suspension cannot be obtained, do not proceed with the slide test.
Dip an inoculating loop or straight wire into undiluted plasma warmed to room temperature and stir the adhering traces (not a loopful) of plasma into the drop of bacterial suspension on the slide.
Coarse clumping becoming visible to the naked eye within 5-10 sec is a positive result. A slower reaction is a negative result but the strain giving a slow reaction should always be retested by the tube method or by alternative identifying test such as the deoxyribonuclease test.

3. Tube Coagulase Test

Prepare 1 in 10 dilution of the plasma in saline (0.85 % NaCl) solution and place 1ml of the diluted plasma in the small tube Inoculate the strain test into the tube preferably by adding 0.1ml of an 18-24 in broth culture (about 10^8 cocci). Incubate the tube at 37^0c and examine for coagulation at 1,3 and 6th hour leave the negative tubes at room temp. Overnight and re-examine.

The conversion of plasma into a smooth or stiff gel, best seen on tilting the tube the horizontal position is a positive result. Since the coagulam may be liquefied sometimes after it has been formed, it is necessary to examine the tubes at each of the times prescribed above.

Control test of known coagulase positive and coagulase negative cultures and a tube un-inoculated plasma should be set up with each batch of tests.

DNAase Test

The test is used to differentiate *Staphylococcus aureus* which produces the enzyme DNA from other Staphylococci which do not produce DNAase. It is particularly useful if plasma is not available to perform a coagulase test or when the results of a coagulase tests are difficult to interpret.

Principle

Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA).The test organism is cultured on medium, which contains DNA. After overnight incubation, the colonies are tested for DNAase production by flooding the plate with a weak HCl solution. The acid precipitates unhydrolyzed DNA. DNAase producing colonies surrounded by clear areas indicating DNA hydrolysis.

Requirement

DNAase Agar Plate - upto 6 organisms may be tested on the same plate. Hydrochloric acid 1 Mol/litre.

Method

Divide a DNAase Agar plate into the required number of strips by marking the underside of the plates. Using a sterile loop or swab spot-inoculate the test and control organisms. Make sure each time area is clearly labelled.

Incubate the plate at 35-37^0C overnight.

Cover the surface of the plate with 1 mol/l hydrochloric acid solution. Tip off the excess acid. Look for clearing around the colonies within 5 minutes of adding the acid.

Results

Clearing around the colonies - DNAase Positive strain. (*Staphylococcus aureus*)

No clearing around the colonies - DNAase Negative strain. (*Staphylococcus epidermidis*)

Exercise

1. Explain : a. Hot-Cold Lysis; b. Halophillic
2. Enlist the animal disease conditions caused by *Staphylococcus* species.

*****

Practical 14

CHARACTERIZATION OF STREPTOCOCCUS

Morphology

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.
The majority of the strains when grown under ordinary conditions do not produce capsules.
Capsule producing strain - *Streptococcus epidemicus.*

Cultural characteristics

Nutrient Agar: Colonies are small smooth, glistening, dewdrop, finely granular with age become opaque with raised central position. No pigment is produced.

Blood Agar: Beta-type of hemolysis is seen. After 24 hours - 48 hours incubation .
Bouillon is uniformly clouded in early stages of growth, finely granular sediment as the culture ages, which settles on the sides and bottom of the culture. No pellicle is formed.

Crystal violet Blood Agar
The addition of low concentrations (1:500000, i.e., 0.0002%) of crystal violet to blood agar inhibits the growth of some bacteria, notably Staphylococci. Crystal violet blood agar is therefore a selective medium for the isolation of streptococci.

Sterile Nutrient agar	90ml
Sterile horse blood	10ml
Crystal violet in 1:1000 aqueous solution	0.2ml

Melt the agar to, cool to 50^{O}C, add the blood and crystal violet and pour plates.

Edwards Agar Medium (Blood Agar, Crystal violet & Aesculin)

Str. agalactiae	Small Transparent, Bluish Grey in colour, Hemolytic/ non-hemolytic.
Str. dysgalactiae	Green discolouration, non-hemolytic.
Str. uberis	Dark colonies surrounded by black or brown zones of discolouration.

Biochemical properties

Streptococcus pyogenes

Produces acid but no gas from glucose, lactose, salicin, sucrose, trehalose, does not ferment sorbitol, inulin, arabinose or raffinose. It produces a final pH of 6.0-4.8 in glucose broth.
Indole negative, Catalase negative

Nitrates are not reduced
Sodium hippurate /starch is not hydrolyzed. Not soluble in bile, but does not grow on 40% bile blood agar.

Mastitic Streptococcus

Streptococcus agalactiae produces acid but no gas from lactose, salicin, trehalose , does not ferment sorbitol , inulin , arabinose or raffinose.

Name	Hemolysis / Lancifield group	Salicin	Mannite	Inulin	Lactose	Raffinose	Tre-halose	sorbitol	Sodium hippurate
Streptococci agalactiae	B / B (Double zone of hemolysis)	+	-	-	+	-	+	-	+
Streptococci dysgalactiae	NH / C	-	-	-	+	-	+	-	-
Streptococci uberis	NH /…	+	+	+	+	-	+	+	+

Sodium hippurate is hydrolyzed by *Streptococcus agalactiae* and *Streptococcus uberis*.

Test for Identification

CAMP test : Christie, **A**tkins and **M**unch - **P**eterson first described CAMP test in 1944. Reported that hemolytic activity of Staphylococcal beta lysine on erythrocytes is enhanced by an extracelluar facto or produced by group B Streptococci, known as CAMP factor, wherever two reactants overlap in a sheep or bovine blood agar plate.

Make a single streak of the streptococcal test strain perpendicular to the Staphylococcal streak. Leave about 1 cm space between the two inoculation lines. Two streak lines must not touch each other. Incubate the inoculated plate at 37oC for 24 hours in air or in 10 % CO_2.

Streptococci produce a positive reaction in the absence of oygen. CAMP factor produced by group B *Streptococci* enhances the *B*- lysin produced by *Staphylococcus* and an increased area of lysis appears at the junction of two organisms assume the shape of an arrow head.

Test for diagnosis of mastitis caused by Streptococcus

Bromocresol purple test

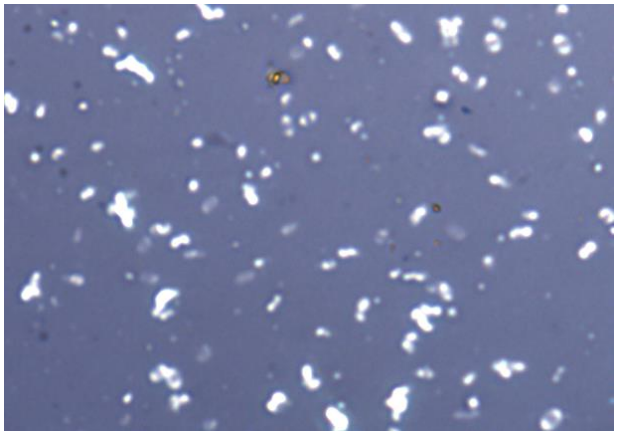
Add 0.5 ml of sterile 0.5 % aqueous solution of bromocresol purple to 9.5 ml of milk. Incubate 24 hours at 37oC & Read the test Appearance of canary yellow colonies of bacterial growth along the walls and in the bottom of the tubes is diagnostic of *Streptococci agalactiae* with clumps. (Fermentation of lactose to acid changes the bromocresol purple to yellow range of indicator.) *Streptococci dysgalactiae* & *Streptococci uberis* though ferments lactose, but do not form clumps.

Exercise

1. Explain CMT
2. Enlist mastitis causing bacteria and fungi.
3. Write chloride content, pH, Sp.gravity of normal & mastitic milk.

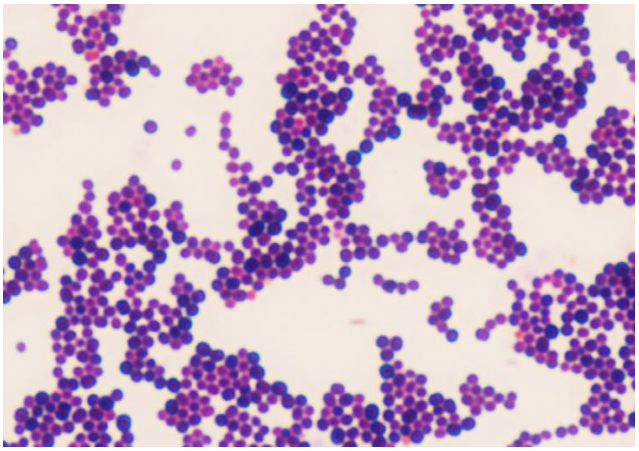
*****

PLATE 1

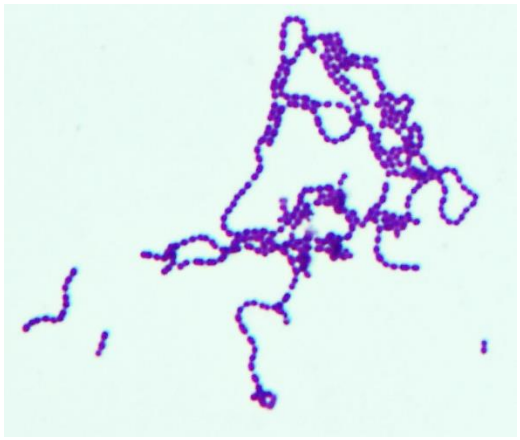


Bacterial Negative Staining

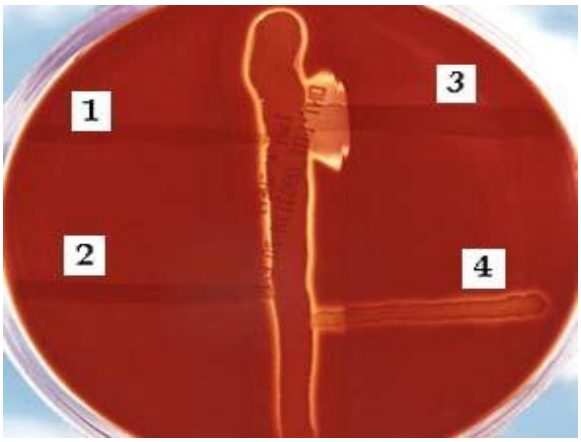
Culture Sensitivity Test



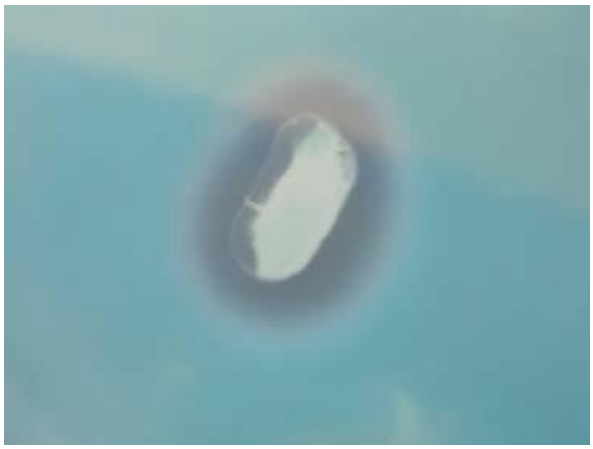
Microscopic picture of *Staphylococcus*



Microscopic picture of *Streptococcus agalactiae*



CAMP Test: The vertical streak is a beta -hemolysin producing strain of *Staph aureus*, & at right angles to it is (3) *S. agalactiae* *Note the large area of complete lysis where the extracellular compound of *S. agalactiae* encounters the beta-lysin of *S. aureus*



DNAase activity of Staphylococcus aureus

Practical 15

CHARACTERIZATION OF ESCHERICHIA

Escherichia coli

Morphology

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.

Cultural characteristics

Nutrient agar: White to yellowish white turning to brown or golden brown with age, are moist, glistening, opaque and circular with entire edge measuring 2-3 mm in diameter, low convex, colorless, opaque or partially translucent colonies.

MacConkey's agar: Lactose fermenting pink red colonies

Hektoen enteric (HE) Agar: Yellow orange or yellow green Brilliant green agar: Yellow colour colonies

Blood Agar: Complete zone of hemolysis is shown by some strains.

Eosin Methylene Blue Agar : Colonies are very dark, almost black, when observed directly against the light. By reflected light a green sheen 'Metallic sheen' can be seen which is due to the precipitation of methylene blue in the medium from the very high amount of acid produced from fermentation.

Triple Sugar Iron Agar Slant:

Organism	Slant	Butt	Gas	H2S
Escherichia,	Acid (A)	Acid (A)	Pos (+)	Neg (-)

IMViC for *Escherichia coli*: **++--**

Exercise

1. Explain Rabbit Ligated Ileal Loop Assay (RLIL Assay).
2. Explain Congo red Binding Test.

*****

Practical 16

CHARACTERIZATION OF SALMONELLA

Salmonella Pullorum & Salmonella Gallinarum

Morphology

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.

Cultural characteristics

Nutrient Agar: Greyish white, smooth, glistening, opalescent, entire colonies. Variants with rough, dry & irregular edges, mucoid colonies are encountered.

MacConkey's Agar: Colourless or pale yellow, non-lactose fermenting colonies.

Hektoen Enteric (HE) Agar: Blue green with black centres. Brilliant green agar: Whitish pink colour colonies.

XLD Agar: Colourless colonies with black centres.

Salmonella – Shigella Agar: Colourless colonies with black centres.

Eosin Methylene Blue Agar: Non-lactose-fermenting colonies produce no acid from fermentation, so the lighter-colored alkaline reaction is seen.

Triple Sugar Iron Agar Slant:

Organism	Slant	Butt	Gas	H2S
Salmonella	Alkaline (K)	Acid (A)	Pos (+)	Pos (+)

IMViC for *Salmonella*: **-+-+**

Tests for diagnosis of Salmonellosis

1. Plate agglutination test

Plate agglutination test can be employed for the diagnosis of Fowl Typhoid or Pullorum disease, by the detection of antibodies in suspected sera / blood. The whole blood test provides a rapid test for Fowl Typhoid & Pullorum disease that can be used on the farm. The sensitivity of whole blood test is low and in inexperienced hands false positive and false negative results may be recorded. The test is useful when large no of samples / birds to be tested, i.e., field level.

Material Required

Salmonella polyvalent colored antigen, Suspected sera / blood, Glass plate.

Method

Serum 0.02 ml is mixed with polyvalent crystal violet stained antigen (0.02ml).

The plate/slide is rocked gently for 2 minutes, after which the test is read.

Interpretation

Positive Test: Agglutination / Clump formation within 2 minutes.
Negative Test: No agglutination (No clump formation)

The test components are stored at 4^{O}C and must have reached room temperature before being used. Due to transient, non-specific reactions, positive/suspicious sera should be retested after heat inactivation at 56^{O}C for 30 min.

Exercise

1. Write the pre-enrichment growth requirement for *Salmonella* species to be isolated from faecal samples.

*****

Practical 17

CHARACTERIZATION OF KLEBSIELLA AND PROTEUS

Klebsiella pneumoniae

Morphology

Gram-negative rods, Non-motile,
Possess well-defined polysaccharide capsule,
Measuring 0.9um-1.7 um by1.8um-3.7um in length with parallel or bulging sides and slightly pointed or rounded ends.
Arranged either in pairs or singly.
Coccid form most prevalent in older cultures Non-spore forming.

Cultural characteristics

Nutrient agar: Large, raised and round colonies. Yellowish in color, moist glistening and viscid colonies and mucoid appearance because of loose slime accumulation.
Gelatin stab: 'Nail-head' type of growth.
MacConkey's agar: Lactose fermenting, large, mucoid, red colonies with entire edge.
Christensen's medium (Urease test): Urease positive
Eosin Methylene Blue Agar: colonies are less dark. Often a dark centre is seen surrounded by a wide, light-colored, mucoid rim – resulting in a 'Fish-eye' type of colony (Black centre colonies).

Triple Sugar Iron Agar Slant:

Organism	Slant	Butt	Gas	H2S
Klebsiella	Acid (A)	Acid (A)	Pos (+)	Neg (-)

IMViC for *Klebsiella pneumoniae*: **--++**

Proteus vulgaris /Proteus mirabilis

Morphology

Gram-negative plump rods measuring 0.5um by 1-3um in size,
In young cultures most of them are long, filamentous and curved.
Motile by means of peritrichous flagella.
Non-spore forming and Non-capsulated.

Cultural characteristics

Nutrient agar: Transparent, thin film like swarming growth on nutrient agar is seen. Emits characteristic "fishy / seminal smell"
MacConkey's agar: Non-lactose fermenting colorless / pale yellow colonies

Christensen's medium (Urease test): Urease positive

Eosin Methylene Blue Agar: Non-lactose-fermenting colonies produce no acid from fermentation, so the lighter-colored alkaline reaction is seen.

Triple Sugar Iron Agar Slant:

Organism	Slant	Butt	Gas	H2S
Proteus	Alkaline (K)	Acid (A)	Pos (+)	Pos (+)

IMViC for *Proteus vulgaris*: **++-v**, *Proteus mirabilis*: **-+vv**

Exercise

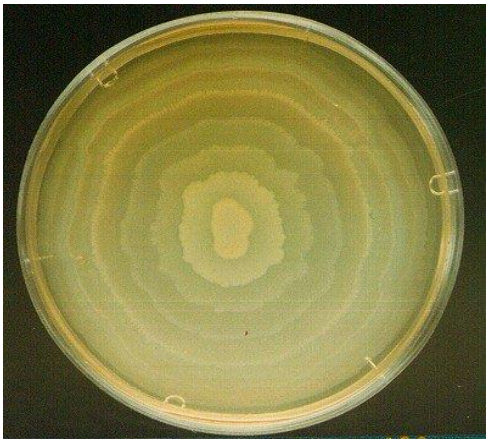
1. Name the Friedlander's bacillus.
2. Explain: Diene's Phenomenon?

*****

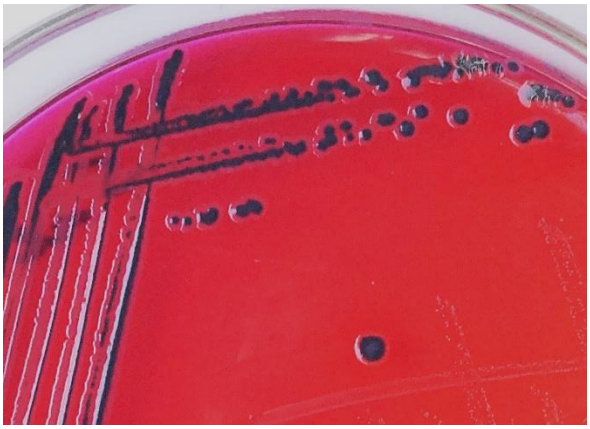
PLATE 2

7.*Escherichia coli* on MacConkeys Agar Lactose Fermenting Pink Red Colonies

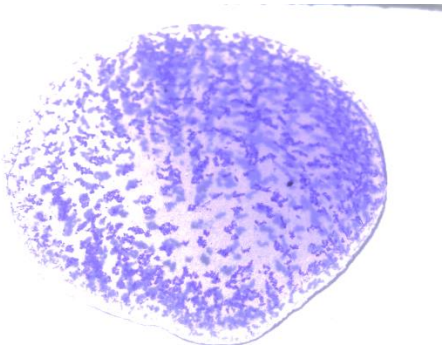
8. *Escherichia coli* on EMB: Characteristic "Metallic sheen''



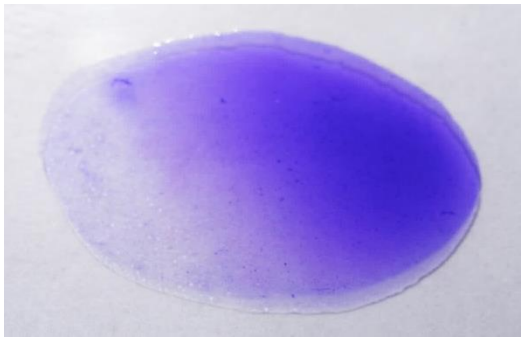
9. Swarming of *Proteus mirabilis* on an agar plate showing - typical growth rings.



10. Salmonella on XLD agar : Colourless colonies with black Centres



11. Salmonella Plate Test- Positive test



12. Salmonella Plate Test- Negative test

Practical 18

CHARACTERIZATION OF PSEUDOMONAS

Pseudomonas aeruginosa

Morphology

Gram-negative rods, Motile bacteria (Polar flagella), Non spore forming , Non-capsulated.

Cultural characteristics

Nutrient Agar: Diffuse spreading colonies with typical 'Pungent' odour. Characteristically produce water soluble pigments which diffuse through the medium i.e., Pyocyanin (Blue-green), pyoverdine (yellow-green, fluorescent), and pyorubin (red-brown).

Biochemical & Sugar fermentation tests:
Catalase Positive
Oxidase Postive
Cetrimide Positive
Nitrate Reduction Positive
Gelatin Hydrolysis Positive
Arginine dehydrolase & Lipase Positive
H2s & Urease Negative

IMViC for Pseudomonas : ---+

Ferments Mannitol;
Does not ferment Glucose, Inulin, Lactose, Maltose, Sorbitol & Sucrose.

Triple Sugar Iron Agar Slant:

Organism	Slant	Butt	Gas	H2S
Pseudomonas	Alkaline (K)	Alkaline (K)	Neg (-)	Neg (-)

Exercise

1. Explain nosocomial infection.
2. Name the animal disease conditions caused by *Pseudomonas* species.

*****

Practical 19

CHARACTERIZATION OF PASTEURELLA

Pasteurella multocida

Morphology

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.

Growth Requirements and cultural characteristics

Aerobe & facultative anaerobe. Optimum temperature for growth is 370C.
PH range is 6-8.5, optimum being 7.2-7.4.

Brain heart infusion agar
Fluorescent and iridescent colonies are moderate in size, white, opaque(pathogenic).
Intermediate colonies vary in appearance in between Fluorescent and blue colonies.
Blue forms/R forms: Small dew drop like colonies, bluish, rough colonies (with low virulence).
Better growth is obtained when blood or serum is added to the medium.

MacConkeys Agar: No growth.

Blood Agar: White opaque or dew drop like colonies as that of on Brain Heart Infusion agar medium, No Hemolysis.

Biochemical Properties

Oxidase positive, Indole positive,
Ferments glucose, saccharose, mannose and mannitol.
Do not ferment salicin, dextrin, starch, trehalose, inositol.
Urease negative. Do not liquefy gelatin. H2S not detectable.

Differentiation between *Pasteurella* and *Manhaemia*

Particulars	*Pasteurella multocida*	*Manhaemia hemolytica*
Motility	Non-motile	Non-motile
Growth on MacConkeys Agar	-	-
Hemolysis on Blood Agar	-	+
Indole formation	+	-
Litmus milk	Neutral	Acid
Raffinose	-	+

Blood Smear Examination: Detection of Bipolar Organism

Leishman's Stain

Leishman Stain powder 0.15 gm (Grind in the mortar)
Methyl alcohol 100 ml. (Pure, pH 6.5)
Mix it well. Filter and use. As far as possible always use freshly prepared Leishman's stain.

Procedure

1. Pour the undiluted stain on the unfixed smear and allow it to act for 1 minute.
2. Add double the volume of the distilled water and mix the fluid by pipette or rubber teat alternately sucking and expelling. Allow the diluted stain to act for 12 minutes.
3. Flood the slide gently with distilled water, allowing the preparation to differentiate in the distilled water until the film appears bright pink in colour (usually about 30 sec)
4. Remove the excess of water with blotting paper and dry in the air.
5. Observe under oil immersion.

Interpretation

Pasteurella multocida appears to be 'bipolar' rods with hallow space surrounding the bacilli indicating the presence of capsule.

Exercise

1. Justify - *Pasteurella multocida* appears to be 'Bipolar' .
2. Differentiate between *Pasteurella multocida* and *Escherichia coli*.

*****

Practical 20

CHARACTERIZATION OF CLOSTRIDIUM

Clostridium tetani

Morphology

Gram-positive rods, long slender rods. Measuring 0.5u in diameter by 2u to 4 um in length.
Filamentous forms occurs which lateron breaks into shorter rods.
Ends are rounded. Occur singly or in short chains.
Sluggishly motile by means of peritrichous flagella
Spores found 2 or 3 times the diameter of the cell and are situated terminally, giving a "drumstick" appearance.

Growth requirements and cultural characteristics:

Obligate anaerobe. Optimum temperature 37^{O}C. Optimum pH 7.0 to 7.6.

Colonies on agar surfaces are irregular, often spreading from a denser center (effuse), have a filamentous edge, are glistening and grayish – yellow, becoming brownish with age.
Gelatin stabs: Fir tree growth occurs.
Blood agar: Alpha type of hemolysis first appears, followed by complete hemolysis.

Reinforced cooked meat medium: Blackening occurs after a few weeks.

Biochemical properties:

- Carbohydrates are not fermented by Clostridium tetani
- Although small amount of glucose is utilized.
- Litmus milk remains unchanged.
- Coagulation on prolong incubation..
- Robertson cooked meat medium /: Blackening occurs after a few weeks.
- Slight NH3 is produced, Reduce nitrates to nitrites slightly,
- Slight H2S is produced,
- Indole negative, Methylene blue reduction, MR and VP negative.
- Phenol positive.

Clostridium chauvoei

Morphology

Rods with rounded ends. 0.6u x 3-8u in size. Occurs singly or in chains.
Spores elongated and arranged subterminally or terminally giving 'Pear Shaped ' appearance.
Motile. Non-capsulated.

Growth Requirements & Cultural characteristics

Strict Anaerobe
Temp 37^{O} C
Slightly alkaline pH favourable

Agar Plate
Small irregular, effuse ,transparent colony,finely granular in the center becoming almost invisible towards the periphery-edges resembles 'Wisps of Hairs' . Bluish gray in transmitted light.
Liquid-Uniform, turbidity form white flaky deposits

Reinforced Cooked Meat Medium: Saccharolytic turns meat medium pink

Biochemical Properties
Produce Acid from glucose, fructose,maltose, lactose and sucrose.
Do not ferment mannitol, glycerol, dulcitol, salicin, inulin.
Indole, MR, VP, NH_3,,Methylene Blue, Catalase: Negative

Clostridium perfringens

Morphology
Gram-positive rods, measuring 0.8u to 1.5 u in diameter by 4u to 8 um in length,
Ends are truncated /square or slightly rounded.
Short, coccal forms and long filaments may be found. Occur singly, in pairs or in short chains.
Non-motile (Differs from other members of the genus)
Spores are large and oval with slightly flattened ends, located subterminally or centrally .
Sporulation is favored by an alkaline pH, do not occur in unusual media containing fermentable carbohydrates i.e., below pH 6.6.
Capsules may be demonstrated in body fluids and in media containing serum.

Growth Requirements & Culture Characteristics:
Grow well in ordinary media, Optimum growth temperature 37^{O} C.
Organism grows best in alkaline media.

From two main types of surface colony

A. One is round, 2-4mm in diameter, low convex, amorphous, grayish, yellow, opaque, with smooth surface and entire edge.

B. Umbonate, opaque brownish centers and light, more translucent radially striated periphery with crenate edge.

Horse Blood Agar:
Colonies are surrounded by a zone of B-hemolysis, also by an outer zone of partial lysis (Target Hemolysis) owing to the action of Theta () and alpha () toxins respectively.

Egg-yolk Agar:
Clostridium welchii produces a diffuse lecithinase – C opacity, which is inhibited by Clostridium welchii antitoxin; No lipolysis is produced. i.e., Nagler's reaction

Reinforced Cooked Meat Medium:
Early growth develops, gas is produced, meat particle turn pink.

Plain Milk Medium:
Characteristic stormy fermentation reaction is seen.

Biochemical properties:
Clostridium welchii forms acid and gas from glucose, fructose, galactose and sucrose,But not from mannitol, dulcitol or salicin.
Litmus milk is coagulated, acid and gas is formed giving a stormy fermentation within 24 hours.
Slight NH3 is produced,
Reduce nitrates to nitrites slightly,
H2S strongly positive, Indole negative,
Methylene blue reduction, Catalase, MR and VP negative.

Clostridium botulinum

Morphology

Clostridium botulinum is large bacilli measuring 0.9u to 1.2u by 4u to 6 u,
Occurs singly or in pairs, Spores are terminal. Sluggishly motile by means of 4-8 perithichate flagella. Non-capsulated, Gram positive in young cultures.

Growth requirement and culture characteristics
Strict anaerobe, optimum temperature 25^{O} to 30^{O}C poorly at 37^{O}C.
Requires slightly alkaline medium.
Growth enhanced by 0.5% glucose and 0.5% K_2HPO_4.

Nutrient agar : Irregular, small translucent, grayish, white, yellowish brown, fimbriate edge, i.e., "Woolly edge".
Liquid Medium: Turbid flaky deposits in tube.

Biochemical reactions
MR, VP, methylene blue reduction, nitrate, catalase and indole negative.
NH3 & H2S positive.
Different types produce different changes in Carbohydrate fermentation.
Do not ferment Raffinose, mannitol and dulcitol.

Test for assessment of *Clostridium welchii* toxin

Mouse Inoculation Test

Method

1. Mix contents of small intestine i.e., 2gm with 2 ml of antitoxin of Clostridium welchii type D.
2. Inject 1ml of above mixture intramuscularly in mice. (Group A).
3. For Group B, mix contents of small intestine i.e., 2gm with 2 ml of distilled water and inject 1ml. intramuscularly in mice.
4. After 24 –48 hours take the results.

Interpretation

If Group A survives and Group B dies, it indicates the presence of toxin of *Clostridium welchii* type D.

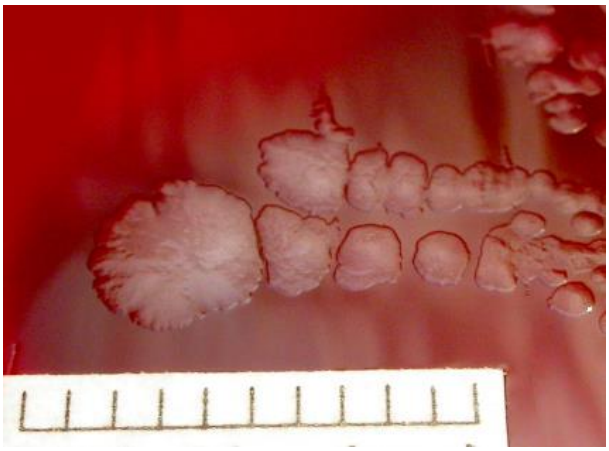
Exercise

1. Write the lethal dose of toxin produced by *Clostridium welchii* for Guinea pig.
2. Explain the principle of ‘ Gas-Pack ’ system.
3. Explain: Nagler’s reaction.
4. Write procedure of inoculating the suspected material for isolation of Clostridia using Reinforced cooked meat media.

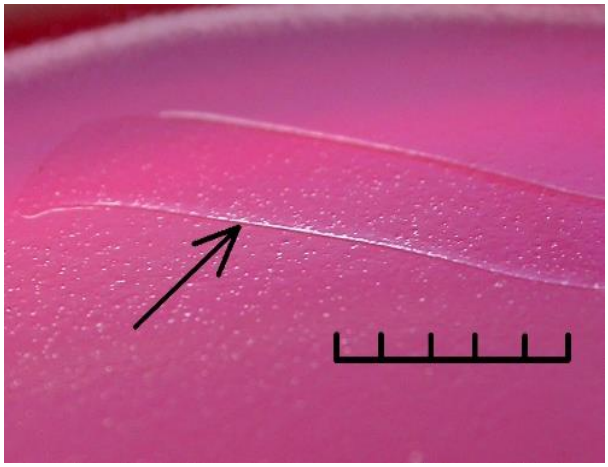
*****

PLATE 3

Klebsiella pneumonia on MacConkeys Agar



Clostridium chauvoei
Anaerobic cultivation on Blood Agar



Clostridium tetani on FFA
Fastidious Anaerobic Agar

Reinforced Cooked Meat Medium: Note the growth of Saccharolytic ***Clostridia***- Pink Coloration

Practical 21

ISOLATION AND IDENTIFICATION OF BACTERIA FROM CLINICAL CASES OF MASTITIS

Clinical Sample Collected:			
Case No.:	Date:		
Species:	Breed:	Age:	Sex:
Case History:			
Presumptive Diagnosis:			
Tests Employed:			
Results:			
Interpretation:			

Signature of Course Teacher

Practical 22

ISOLATION AND IDENTIFICATION OF BACTERIA FROM CLINICAL CASES OF ABORTION

Clinical Sample Collected:			
Case No.:	Date:		
Species:	Breed:	Age:	Sex:
Case History:			
Presumptive Diagnosis:			
Tests Employed:			
Results:			
Interpretation:			

Signature of Course Teacher

Practical 23

ISOLATION AND IDENTIFICATION OF BACTERIA FROM CLINICAL CASES OF ENTERIC INFECTION

Clinical Sample Collected:			
Case No.:	Date:		
Species:	Breed:	Age:	Sex:
Case History:			
Presumptive Diagnosis:			
Tests Employed:			
Results:			
Interpretation:			

Signature of Course Teacher

Practical 24

ISOLATION AND IDENTIFICATION OF BACTERIA FROM CLINICAL CASES OF RESPIRATORY INFECTIONS

Clinical Sample Collected:			
Case No.:	Date:		
Species:	Breed:	Age:	Sex:
Case History:			
Presumptive Diagnosis:			
Tests Employed:			
Results:			
Interpretation:			

Signature of Course Teacher

Practical 25

ISOLATION AND IDENTIFICATION OF BACTERIA FROM CLINICAL CASES OF PYOGENIC INFECTION

Clinical Sample Collected:			
Case No.:	Date:		
Species:	Breed:	Age:	Sex:
Case History:			
Presumptive Diagnosis:			
Tests Employed:			
Results:			
Interpretation:			

Signature of Course Teacher

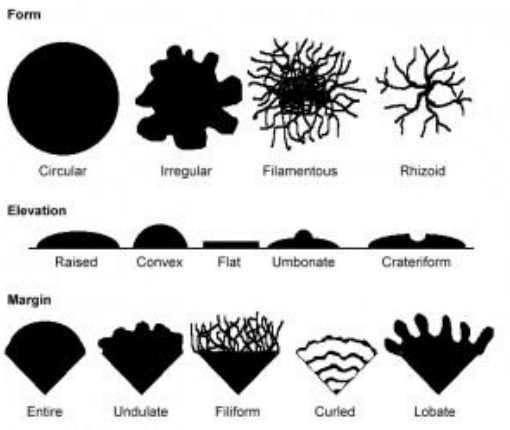
APPENDIX

1. BACTERIAL CULTURAL CHARACTERISTICS

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

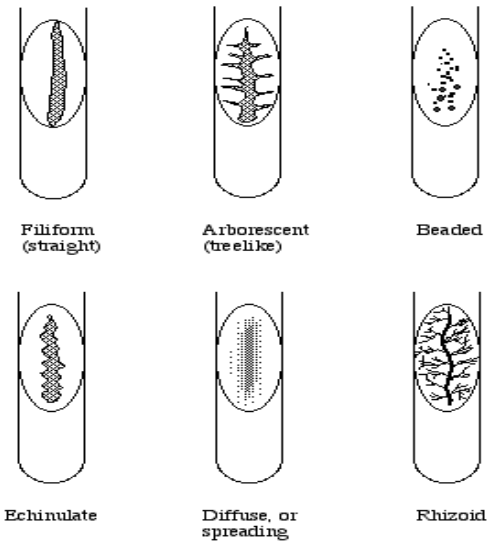
Growth on solid media

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
Consistency	- Butyrous, friable, viscid
Emulsifiability	- Easy, difficult, forms homogenous or granular suspension.



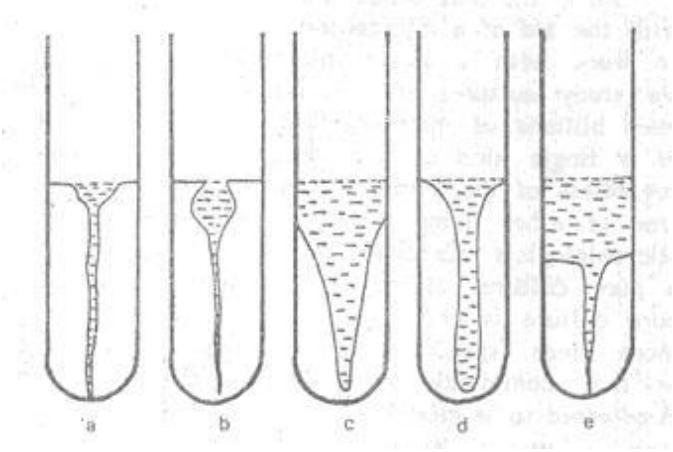
Growth on slant

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

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2. 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 phosphate	K_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 (KNO_3) 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 + 0.02 % Potassium nitrate & 0.55 peptone ---------- Nitrate Reductase ---------- Nitrite ---------- Sulphanilic acid Alpha Naphtylamine ---------- Diazo Red Dye

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 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 Durham's 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

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