3. METHODS AND MATERIAL

MEDIA PREPARATION

Following of the media were used for culture and sensitivity testing for the study:

i. Nutrient agar

ii. Blood agar

iii. Mac Conkey agar

iv. Muller Hinton agar

v. Deoxycholate citrate agar

vi. Thioglycolate citrate bile salt sucrose agar

vii. Transport medium: Alkaline peptone water

viii. Transport medium: Carry Blair medium

ix. Brain Heart infusion broth

3.a. NUTRIENT AGAR

INTRODUCTION

Nutrient Agar media is a general purpose nutritional media, which is used for cultivation of microorganisms by supporting the growth of a broad range of non-fastidious organisms. Nutrient agar is commonly used media in microbiology laboratory because of its capability of growing a large variety of bacteria and fungi as it is constituted of many basic nutrients needed for the growth of bacteria. (MacFaddin et al., 2000)

PRINCIPLE

Nutrient gar has many constituents that are meat for a variety of functions. Peptone an enzymatic digest of protein is the basic unit of the composition of media. It is the sole source for
the organic nitrogen for the growth of bacteria. Beef or yeast extracts help in bacterial growth by providing vitamins, salts, organic nitrogen compounds and carbohydrates. Agar is used as solidifying material for the media. The role of sodium chloride in nutrient agar media is to maintain the salt concentration in the medium as of the cytoplasm of the microorganisms to balance osmosis. The solvent i.e., distilled water is the essential constituent for the media as it is required for growth of and reproduction of microbes. Water also acts as the medium through which various nutrients are transported. Though this media is used to grow and isolate non-fastidious bacteria, but with addition of some growth supplements like serum or blood, it is used for the cultivation of fastidious bacteria. (Downes and Ito, 2001)

**COMPOSITION** (Downes and Ito, 2001)

1. 0.5% Peptone
2. 0.3% beef extract/yeast extract
3. 1.5% agar
4. 0.5% NaCl
5. Distilled water
6. pH neutral (7.4) at 25 °C.

**PREPARATION** (Downes and Ito, 2001)

1. 28 g of nutrient agar powder was Suspend in 1000 ml of distilled water in a 2 litre borosil flask.
2. The mixture was heated and stirred to dissolve fully.
3. This dissolved mixture was autoclaved at 121^0^c for 15 minutes.
4. Once autoclaved, the flask was allowed to cool but not to solidify.
5. The media was poured into each plate (filling 25 ml in each plate) and the plates were left at room temperature on the sterile, uniform surface until the agar was solidified.
6. After the plates were solidified completely, these were stored in a refrigerator.
USES OF NUTRIENT AGAR (Downes and Ito., 2001)

1. It is commonly used for isolation and purification of microbial culture colonies.
2. It is also used for producing the bacterial lawns/ carpets for antibiotic susceptibility tests.
3. Also used for purity checking before susceptibility testing and biochemical reactions testing. (1, 2)
4. Used to solve teaching or demonstrative purposes, where prolonged growth and ambient temperature with nutrients is required.

EXPECTED COLONY CHARACTERISTICS (Downes and Ito., 2001)

Both GNB’s and GPC’s grew as Cream/pale to yellow homogeneous, free flowing powdery or Jelly like/ Firm colonies depending upon specific characteristics.

3.b. BLOOD AGAR

Introduction
Blood agar is an enriched, agar medium for bacterial growth. Some Fastidious bacteria, like streptococcus that do not grow well on ordinary growth media like nutrient media, blood is added for their fastidious growth requirements. (Murray et al., 2003)

Principle:
Since blood contains inhibitory proteins or enzymes for some bacteria like Neisseria and Haemophilus spp, it has to be heated to inactivate these inhibitors in cases if growth of these bacteria. Heating of blood coagulates he proteins present in blood so it become chocolate coloured and is known as chocolate agar, which supports the growth of these bacteria. (Downes and Ito., 2001)

Composition: (Downes and Ito., 2001)
1. Tryptones as protein source
2. Soybean protein digest,
3. Sodium chloride (Nacl),
4. agar
5. 5% sheep blood
6. casein produced by Pancreatic digest
7. of soy meal produced by Papaic digest
8. NaCl
9. Distilled water
10. pH 7.3.

**PREPARATION** (Murray et al., 2003)
1. The Blood Agar base was prepared as per the directions of the manufacturer.
2. This was sterilized by autoclaving at 121°C for 15 minutes.
3. The base as others was cooled to 50°C in Water bath or under running tap water.
4. When the agar base was cooled to 50°C, sterile blood was added to agar aseptically and was mixed well. Formation of air bubbles was avoided.
5. 15ml -25 ml of the prepared media was dispensed to sterile Petri plates aseptically.
6. The plates were Stored at 2-8°C in sealed plastic bags to avoid loss of moisture.

**USES OF BLOOD AGAR** (Murray et al., 2003)
Blood agar has two major uses:

1. To grow streptococcus spp.

2. To determine the type of haemolysis.

**EXPECTED GROWTH CHARACTERISTICS OF BLOOD AGAR** (Murray et al., 2003)
*S. pyogenes*: Beta-hemolysis
*S. pneumoniae*: Alpha-hemolysis
Satellitism in case of *H. influenzae*

**BLOOD AGAR AND HAEMOLYSIS** (Downes and Ito, 2001)
There are some bacterial species that produce extracellular enzymes, which lyse red blood cells present in the Blood agar, this is called as haemolysis. These haemolysins that are exotoxins radially diffuse outwards from the colony/colonies. This causes complete or partial destruction
of the red cells (RBC’s) in the medium and this also completely denatures the haemoglobin within the cells to form colourless products.

A streptococcus produces four types of haemolysis in Sheep Blood RBC’s; Alpha haemolysis, Beta haemolysis, gamma haemolysis and alpha wide zone alpha haemolysis.

Observation of Haemolysis is best done by incubating the plates in anaerobic conditions or examining sub-surface colonies under the source of light coming from behind.

In case of any haemolysis present, the zone of haemolysis will be available around the growing colony.

**ALPHA HAEMOLYSIS**

*The haemolysis that shows* Partial lysis of the RBC’s, which as result, produces a green-gray or brown discoloration around the colony. α haemolysis occurs due to the production of methaemoglobin by reducing haemoglobin of RBC’s, which finally is diffused in the medium surrounding the colony. There are many strains of streptococcus that produce alpha haemolysis but are normal body flora. But *Streptococcus pneumoniae* is one of the alpha haemolytic bacteria that cause serious pneumonia and other deadly infectious diseases in humans.

**BETA HAEMOLYSIS**

It is examined by complete lysis of Red Blood Cells in the agar plate, which causes a complete clearing of blood from the medium under and around the colonies e.g. Group A beta haemolytic streptococcus includes *Streptococcus pyogenes* and Group B beta haemolytic Streptococcus include *Streptococcus agalactiae*. In case of group A streptococci maximal activity of both types of haemolysins act maximally; Oxygen labile SLO and oxygen stable SLS. This occurs in absence of oxygen mainly, but some aerobic or partial anaerobic conditions also favour the haemolysis.

**GAMMA HAEMOLYSIS**

it is also known as non haemolysis. When there is no haemolysis in the medium, which is examined by absence of colour change under and surrounding the colonies in the medium.

**Alpha prime or wide zone alpha haemolysis**: when there is a small zone of intact RBC’s lying immediately adjacent to the colony, with a zone of complete haemolysis around the zone of
intact RBC’s, this known as alpha prime haemolysis. This type of haemolysis is usually confused with beta haemolysis.

TARGET HAEMOLYSIS
This is a characteristic type of haemolysis, occurs in Clostridium perfringens, which is readily, identified its characteristic “double zone” haemolysis of alpha and beta haemolysis. (Downes and Ito., 2001; Murray et al., 2003)

3.c. MAC CONKEY AGAR
INTRODUCTION
Mac Conkey agar is named after Mr. Alfred Theodore MacConkey, who was a bacteriologist for the Royal Commission on Sewage Disposal in 20th century. It was discovered as the first solid differential media that was formulated. Since Mac Conkey Agar is one of the good media for selective and differential growth and isolation of enteric (found in intestine) Gram-negative bacteria. This is the commonly used media for culture test; MacConkey agar media plates are one of the most common agar plates that are found in a clinical microbiology laboratory. The functions of selection and differentiation depend principally on its bile salts, indicator dye like neutral red- and carbohydrate composition (Wehr and Frank, 2004). It is a selective media to select the Gram negative bacteria, by differentiating the bacilli depending upon their carbohydrate (lactose) utilisation. The primary use of this media is to detect and isolate the members of family Enterobacteriaceae and Pseudomonas spp. The crystal violet and bile salts present in the media perform their roles by inhibiting the growth of GPC’s, so only GNB’s are allowed to grow. Since some enteric bacteria have ability to ferment lactose and other cant not, the principle of lactose fermentation is used to differentiate them under suitable pH with neutral red. (Eaton et al., 2005)

PRINCIPLE
Gram-negative enteric bacteria are only grown on this media because of the bile salts and Crystal violet present in it. As GNB’s have outer bile-resistant membranes and then that grow on MacConkey agar; they are differentiated by their ability of carbohydrate fermentation i.e., lactose. If any gram negative ferments lactose, as an end product acid is produced, that acid drops the pH of the
media. This drop in pH is shown by the change of Neutral red indicator to pink (at or below pH 6.8 i.e., acidic, Neutral red appears pink in colour) like other indicators. This is also called indicator medium or low selective medium. (Downes and Ito, 2001)

Strong lactose fermenters produce enough amount of acid, which is precipitated by bile salts around the growth. This precipitation reaction is seen as a pink halo around each colony and where isolated colonies are not seen, the areas of confluent growth are surrounded by the halo. But the halo is not seen in weaker lactose fermenters colonies. The bile salts and crystal violet dye play role of inhibiting the growth GPC’s and other hard to grow bacteria with their fastidious requirements like Neisseria species. Gram-negative bacteria that do not use or ferment lactose remain colourless on the media s no lactose fermented by which no drop in pH and the colour of the neutral red remains unchanged to pink. Hence the lactose fermenters show pink colour of the colonies, whereas, non lactose fermenters remain colourless on the plates. (Downes and Ito., 2001)

Composition of Mac Conkey Agar has specific roles. Casein, pancreatic digest of gelatin, and Animal tissue that provide required elements of nitrogen, minerals, vitamins, and essential amino acids for growth.

**Lactose**: that is a fermentable carbohydrate, which acts as carbon source and provides energy.

**Bile Salts and Crystal Violet**: these are the selective agents that cease the growth of GPC.

**Sodium Chloride**: it is necessary for transportation and maintaining osmotic balance in the cells of bacteria. It also helps in providing essential electrolytes.

**Neutral Red**: it is a pH indicator, of which the colour is red at or below pH 6.8. When lactose is fermented by GNB’s, the pH of the medium is decreased, which changes the colour of neutral red to pink.

**Agar**: it is a solidifying agent. (Eaton et al., 2005)

**COMPOSITION** (Eaton et al., 2005)

1. **Peptone** - 17 g
2. **Proteose peptone** - 3 g
3. **Lactose** - 10 g
4. **Neutral red** - 0.03 g
5. **Sodium chloride** - 5 g
6. **Bile salts** - 1.5 g
7. **Crystal Violet** - 0.001 g
8. **Agar** - 13.5 g
9. **Water** – 1000 ml
10. **pH** was adjusted to 7.1 +/- 0.2

To inhibit the swarming of Proteus species from the media, this media composition was edited by omitting sodium chloride. There are many variations of MacConkey agar depending on the need. If gram positive bacteria are also needed to be grown this media, **Crystal violet** was omitted. Sorbitol was added to MacConkey agar medium when it was suspected **E. coli O157**, which is an enteric pathogen.

**PREPARATION** *(Eaton et al., 2005)*
1. The measured amounts of above said powders were added to a 2 litre flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
4. The flask was Autoclaved at 121°C for 20 minutes.
5. Once autoclaved, the flask was allowed to cool but not to solidify.
6. The media was poured into each plate (filling 25 ml in each plate) and the plates were left at room temperature on the sterile, uniform surface until the agar was solidified.
7. After the plates were solidified completely, these were stored in a refrigerator.

**INTENDED USE OF MACCONKEY AGAR** *(Eaton et al., 2005)*
1. For Enterobacteriaceae selective isolation and identification from faeces, urine, wastewater and foods.

**EXPECTED COLONY CHARACTERISTICS** *(Downes and Ito, 2001)*
Lactose-fermenters: pink to red colonies with or without bile precipitation.
Non-lactose fermenters: colorless or pale or transluscent colonies.

3.d. MULLER HINTON AGAR

INTRODUCTION

Mueller-Hinton Agar is the media routine used in the laboratory for the purposes of disk diffusion method\(^1\) and for the cultivation of Neisseria spp. and *Moraxella* spp. this media is rich in nutrients that were originally being used for the cultivation of gonococcus and Meningococcus. But it is primarily used for antimicrobial susceptibility testing of microbes (Murray et al., 2003).

PRINCIPLE

Mueller Hinton Agar has some of the properties that help the media to be used as appropriate media for susceptibility testing. First is that it is a general, non-selective, non-differential medium, which allows all types of bacteria to grow. Another reason for its selection for susceptibility testing is that it contains starch, which is considered to be good for absorbing any toxins released by bacteria; hence the effect of toxins on the antibiotics is reduced. Also it is a loose agar that allows the antibacterials to diffuse better than most other media. A better diffusion helps in formation of a better and true zone of inhibition (Murray et al., 2003).

COMPOSITION (Murray et al., 2003)

1. Casein peptone H 17.5 gms
2. starch 1.5 gms
3. pH 7.4 + 0.2
4. agar 17.0 gms
5. beef infusion 3.0 gms
6. distilled water 1000 ml

Five percent and nicotinamide adenine dinucleotide or 5 % of sheep blood can be added in case of susceptibility testing for *Streptococcus* species and *Campylobacter* spp.
**PREPARATION** (Murray et al., 2003)

1. The measured amounts of above said powders were added to a 2 litre flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
4. The flask was Autoclaved at 121°C for 20 minutes
5. Once autoclaved, the flask was allowed to cool but not to solidify.
6. The media was poured into each plate (filling 25 ml in each plate) and the plates were left at room temperature on the sterile, uniform surface until the agar was solidified.
7. After the plates were solidified completely, these were stored.

**USES**

1. For cultivation of Neisseria and Morexella spp.
2. For antimicrobial susceptibility testing of bacteria and yeast.

**EXPECTED COLONY CHARACTERISTICS** pale yellow colonies. (Murray et al., 2003)

3.e. **DEOXYCHOLATE CITRATE AGAR** (Speck., 1984)

**INTRODUCTION**

Deoxycholate Citrate Agar is used to grow the pathogenic bacteria selectively e.g., Salmonella and Shigella from stool.

**PRINCIPLE**

This media is modification of Leifson (Leifson., 1935). This media was formulated to maximise the recovery of almost enteric pathogens especially to Salmonella and Shigella spp. from foods (Speck., 1984). Since to isolate Shigella on the culture media, the medium should have less inhibitory properties; this media provides all the requirements for Shigella. Sodium deoxycholate from pH 7.3 to 7.5 acts as inhibitory agent for gram positive bacteria. Citrate salts also play role
by inhibiting growth of gram-positive bacteria as well as most other normal intestinal flora. Heart infusion provides source of carbon and Proteose peptone provides carbon, nitrogen, vitamins and minerals. To inhibit or suppress Coliform bacteria and gram-positive bacteria sodium deoxycholate, sodium citrate and ferric ammonium citrate salts are used. The medium is buffered by Dipotassium phosphate buffers. Lactose provides carbohydrate source and also helps in differentiating the pathogens on the basis of colour. As in the MacConkey agar lactose degradation causes acidification of the medium around the relevant colonies and the colour of the pH indicator neutral red is changed to red to pink. Some Surface colonies of non-lactose fermenters generally take a little pinkish colour from the medium and these organisms are often mistaken for coliforms. (Leifson., 1935) Precipitation around the colonies is seen as the precipitation reaction of salts and acid. The salt Sodium deoxycholate combines with pH indicator neutral red in an acidic environment, which causes the dye to go out of the solution to form precipitation of salt. (Leifson., 1935) The black colour is formed at the time of reduction of ferric ammonium citrate to iron sulphide where black iron sulphide is formed. Since both Salmonella and Shigella species do not utilise lactose but Salmonella spp. sometimes produce H₂S, so colourless colonies with or without black centres are seen. The agar is strongly hydrolysed by Citrate and iron combination when heated; the agar becomes soft and unelastic. Autoclaving makes the agar soft which is almost impossible to streak. (Leifson., 1935) Salmonella Gallinarum is inhibited by increases concentration of sodium Deoxycholate (>0.1%) (Leifson., 1935)

**COMPOSITION** (speck, 1984)

1. Heart infusion solids  10 g
2. Protease peptone  10 g
3. Lactose  10 g
4. Sodium Deoxycholate  5 g
5. Neutral red  0.02 g
6. Sodium citrate  20 g
7. Ferric ammonium citrate 2.0 g
8. Agar 13.5 g
9. pH 7.5 ± 0.2
10. distilled water 1000 ml

PREPARATION (speck, 1984)

1. The measured amounts of above said powders were added to a 2 litre flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
4. This media should not be autoclaved.
5. Once boiled, the flask was allowed to cool but not to solidify.
6. The media was poured into each plate (filling 25 ml in each plate) and the plates were left at room temperature on the sterile, uniform surface until the agar was solidified.
7. When the plates were solidified completely these were stored in refrigerator.

USES

It is used for the cultivation and isolation of organisms that cause bacillary dysentery, food poisoning or typhoid fever.

EXPECTED COLONY CHARACTERISTICS (speck., 1984)

I. Coliform bacteria: pink colonies.

II. Salmonella: transparent colonies, with or without black centres.

III. Shigella: transparent colonies.
3.f. THIOGLYCOLATE CITRATE BILE SALT SUCROSE AGAR (Clesceri et al., 1998)

INTRODUCTION
Thiosulphate citrate bile salt sucrose agar is used for the cultivation and isolation of Vibrio cholerae and other enteropathogenic Vibrio's responsible for food poisonings selectively. This was discovered by Nakanishi (Nakanishi., 1963) and modified by Kobayashi et al (Kobayashi.,1963). The media was initially discovered for two types of Vibrio i.e., V.cholerae and V. Parahaemolyticus, but all types of vibrio are grown in this media that are identified by various colony characteristics (Forbes et al., 1998).

PRINCIPLE
TCBS media that is enriched with Alkaline Peptone Water that helps V.cholerae to grow on the medium, lavishly. (Murray et al., 2003) yeast extracts and Proteose peptone act as nitrogenous sources ad also provide other nutrients like vitamin B complex. Bile salts are taken from ox gall that with the help of sodium citrate inhibit gram-positive bacteria and coliforms. source of sulphur is taken from Sodium thiosulphate that when combined to ferric citrate is is reduced to hydrogen sulphide. As source of fermentable carbohydrate sucrose is utilised for growth of Vibrios. the vibrio that consumes sucrose are seen as yellow colonies in the alkaline pH with the help of Bromothymol blue and thymol blue pH indicators. According to the studies due to its selectivity and inhibitory properties this medium is not appropriate for oxidase testing of Vibrio species. (Murray et al., 2003)

COMPOSITION (Murray et al., 2003)

1. Yeast Extract 5 gm
2. Proteose peptone 10 gm
3. Sodium Citrate 10 gm
4. sodium thiosulphate 10 gm
5. Ox Bile or Beef extract 8 gm
6. Sodium Chloride 10 gm
7. Ferric Citrate 1 gm
8. Sucrose 20 gm
9. Bromothymol Blue 0.02 gm
10. Agar 15 gm
11. Distilled water 1000 ml

**PREPARATION** (Murray et al., 2003)

1. The measured amounts of above mentioned powders were added to a two litre flask under sterile conditions.

2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.

3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.

4. This media should not be autoclaved.

5. Once boiled, the flask was allowed to cool but not to solidify.

6. The media was poured into each plate (filling 25 ml in each plate) and the plates were left at room temperature on the sterile, uniform surface until the agar was solidified.

7. After the plates were solidified completely, these were stored.

**USES:** used to grow Vibrio spp. responsible for choleras and other diarrhoeal diseases.

**EXPECTED COLONIES CHARACTERISTICS** (Murray et al., 2003)

I. V.cholerae : Strains of V. cholerae - yellow colonies
II. V.alginolyticus - yellow colonies
III. V.parahaemolyticus - blue-green colonies
IV. V.vulnificus - blue-green colonies
V. Proteus species though less grown are sucrose fermenters and may form yellow colonies (9).

3.g. ALKALINE PEPTONE WATER

INTRODUCTION
Alkaline Peptone Water is used for enrichment of Vibrio species and for transportation of stool samples suspected for Vibrio infections.

PRINCIPLE
This is the enrichment medium that helps to cultivate the small number of isolates in suspected clinical samples. (Forbes et al., 1998) The alkaline pH provides favorable environment for the growth of Vibrio. Amino acids as the source of energy are extracted by performing the digesting the animal tissue enzymatically. The nitrogenous sources for energy are also derived from this digestion. Sodium chloride maintains osmotic equilibrium. Sodium chloride like another medium helps in maintaining osmotic balance. The growth is seen in the form of turbidity in the tube, prolonged incubation of samples in APW helps in eliminating contaminants or other normal flora due to high pH (Downes and Ito, 2001)

COMPOSITION (Forbes et al., 1998)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Gms / 1000 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>animal tissue by Peptic digest of</td>
<td>10 gm</td>
</tr>
<tr>
<td>Nacl</td>
<td>10gm</td>
</tr>
<tr>
<td>pH (@25°C)</td>
<td>8.4±0.2</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

PREPARATION (Forbes et al., 1998; Downes and Ito, 2001)

1. The measured amounts of above said powders were added to a 2 liter flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was heated with frequent agitation and was boiled for 1 minute to completely dissolve the powder in distilled water.
4. The media was dispensed in borosil tubes.
5. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
6. The flask was Autoclaved at 121°C for 20 minutes
7. Once autoclaved, the flask was allowed to cool.
8. These tubes were stored at 2°C-4°C.

3.h. CARY- BLAIR MEDIUM

INTRODUCTION
This is used for collection and transportation of stool samples. This Transport Medium is a chemically defined, non-nutritive, semisolid, buffered medium. Carry and Blair devised this medium with lesser nutrients, low redox potential with high pH (Cary and Blair., 1964). Because of high pH the Vibrio samples can also be transported. Along with the recovery of Salmonella and Shigella species. (Murray et al., 2003)

PRINCIPLE
The sole purpose of Cary- Blair Medium is to maintain the viability of bacteria at the time of collection since examination of the specimen. The reason of being non nutritive is to limit the number of bacteria during Transportation. Cary-Blair Medium is made with composition of minimal nutrients to enhance the survival of bacteria without multiplication. Sodium Thioglycolate helps in maintaining low oxidation-reduction potential. Alkaline pH of the medium helps in minimizing bacterial destruction due to acid formation. Sodium chloride acts by maintaining the osmotic equilibrium whereas the medium is buffered by Disodium phosphate. (Murray et al., 2003)

COMPOSITION (Murray et al., 2003)
1. Disodium phosphate                  1.1gm
2. Sodium thioglycollateb              1.5gm
3. Sodium chloride                       5.0gm
4. Agar                                         5.0gm
5. pH @ 25°C                                  8.4±0.2
PREPARATION (Murray et al., 2003)

1. The measured amounts of above said powders were added to a 2 litre flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
4. The flask was Autoclaved at 121°C for 20 minutes
5. After heating the medium the medium was added with 1% aqueous calcium chloride solution.
6. The media was dispensed in borosil tubes.
7. Once autoclaved, the flask was allowed to cool.
8. These tubes were stored at 20°C-40°C.

3.i. SELENITE BROTH (SELENITE F BROTH)

INTRODUCTION

It is enrichment medium for selective growth of Salmonellae from stool sample, urine sample or another clinical samples. Inhibitory effects of selenite on some organisms selectively were discovered by Klett (Klett, 1900). It was first demonstrated by selective and Guth (Guth, 1926) to isolate Salmonella Typhi. The product was fully investigated and constituted by Leifson (Kelly et al., 2003)

PRINCIPLE

This Enrichment media is employed for detection of Salmonella in the chronic stages of sickness in Faecal specimens or in convalescent state or when there may be very less number of organisms may be present. The hydrolysate of Casein by enzymatic digestion provides the source of nitrogenous substances. The carbohydrate source, the pH of medium by providing lactose to the medium. Depending upon the type of bacteria, acid or alkali is produced in the media by consumption of Selenite. If alkali produced, the pH of the medium is raised by which toxicity of the selenite is lowered, leading to overgrowth of bacteria. In cases when acid is produced, that helps in selective growth of desired bacteria. Thus lactose plays an important role in growth of
bacteria to maintain the pH of the medium. Selenite is reduced by bacterial growth, alkali is produced. This increase in pH reduces the toxicity of the selenite and leads to overgrowth of other bacteria. The acid that is produced by bacteria due to lactose fermentation helps in maintaining the pH of required range. Sodium phosphate acts as buffering agent and also reduces the toxicity produced by selenite (Kelly et al., 2003).

**COMPOSITION** (Kelly et al., 2003)

**Part I**
- Enzymatic Casein hydrolysate 5.0gms
- Lactose 4.0gms
- Sodium phosphate 10.0gms

**Part II**
- Sodium hydrogen Selenite 4.0gms
- Final pH (at 25°C) 7.0±0.
- Distilled water 1000 ml

**PREPARATION** (Kelly et al., 2003)

1. The measured amounts of above said powders were added to a 2 litre flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
4. The flask was Autoclaved at 121°C for 20 minutes
5. After heating the medium the medium was added with 1% aqueous calcium chloride solution.
6. The media was dispensed in borosil tubes.
7. Once autoclaved, the flask was allowed to cool.
8. These tubes were stored at 2°C-4°C.

3.j. **BRAIN HEART INFUSION BROTH (BHIB)**

**INTRODUCTION**
Brain heart infusion Broth (BHIB) is the enrichment medium that helps in isolating and cultivating all blood pathogens. (Murray et al., 2003)

**PRINCIPLE**

Calf brain and beef heart provide nitrogenous sources to the media for growth of all fastidious organisms. Proteose peptone provides amino acids to the bacteria for growth. NaCl helps in maintaining osmosis whereas Disodium phosphate keeps the pH adjust by buffering the medium; the fermentable carbohydrate requirement is satisfied by addition of dextrose in the medium (Murray et al., 2003).

**COMPOSITION (Downes and Ito, 2001)**

1. Calf brain infusion - 200 Gms
2. Beef Heart infusion - 250 Gms
3. Proteose Peptone - 10 Gms
4. Sodium Chloride - 5 Gms
5. Di sodium phosphate - 2.5 Gms
6. Dextrose - 2 Gms
7. Distilled water - 1 litre
8. pH - 7.4-7.6

**PREPARATION (Downes and Ito., 2001)**

1. The measured amounts of above said powders were added to a 2 litre flask under sterile conditions.
2. These powders were suspended and mixed slowly in the position of eight in sterile distilled water. The flask was covered with cotton plug.
3. The material was required to heat and shake well, which further was boiled so that the powder in water can be dissolved completely.
4. The flask was Autoclaved at 121°C for 20 minutes
5. After heating the medium the medium was added with 1% aqueous calcium chloride solution.
6. The media was dispensed in borosil tubes.
7. Once autoclaved, the flask was allowed to cool.
8. These tubes were stored at 2°C-4°C.

3.k. AUTOCLAVING

PRINCIPLE

The principle of functioning of autoclave is based on moist heat. Pressurized steam is used in the autoclaves to destroy microorganisms. Since they are very effective, they are considered to be the most important systems to be depended upon for the decontamination and sterilization of biological, clinical waste and glassware, media, or reagents. The steam flushes the air out of the autoclave chamber for effective heat transfer that reaches to all parts of autoclaves and sterile the material available in it. At the first step of starting the autoclave, the waste pipe for evaporation that is located downwards at the machine must be checked and cleaned in case of blockage. The sieve of the equipment must be checked for cleaning as if it is congested with debris the results may not be satisfactory. (Laboratory Biosafety Guidelines., 2001)

CONTAINER SELECTION (Laboratory Biosafety Guidelines., 2001)

- **Polypropylene bags:** these autoclave bags are also known as biohazard bags or, though the autoclave bags cannot be torn, but care must be taken as these bags have tendency to be burst or punctured during autoclaving. So care must be taken in terms of placing these bags during autoclaving. Therefore, the bags should be placed in rigid container first. The size and colors of these bags vary and some kind of bags is available in printed versions with an indicator that changes colour during processing.

- Since these bags do not allow steam to penetrate, so these bags are loosely tied to allow steam to move into the articles present in these bags.

- For this reason the bags must not be twisted tightly or taped.

- **Polypropylene pans, baskets or containers:** these are basically kind of plastic, which can withstand high pressure of autoclaving and they are also capable of retaining heat without its transfer. So these containers take longer time to penetrate heat and the whole process of
autoclaving is prolonged. Hence if the time of autoclaving is required to be reduced the lid of the container should be open and diameter of the container must be broad with shorted sides to increase the surface.

- **Stainless steel containers and pans**: These are considered to be best containers for autoclaving, as they are good conductors of heat so the chances of prolonged time remain low. Stainless steel material is costlier to the plastic ones.

**PREPARATION AND LOADING OF MATERIAL** (Laboratory Biosafety Guidelines., 2001)

- The liquid containers were filled only half.
- The screws were loosened.
- The bags of biological waste were always put into pans to hold spills in case.
- Plastic bags were positioned on the sides of baskets with loosely taped ends.
- To allow the proper circulation of steam, the placement of items was done loosely.
- The lids after keeping the items were closed.
- The pressure valve was released to check the formation of steam.
- The autoclave was turned on.
- When the steam started being formed the valves were tightly closed.
- The pressure was allowed to rise for 15 lbs for 15 minutes.
- After exactly 15 minutes, the autoclave was switched off and the steam was released.

**REMOVING THE LOAD** (Laboratory Biosafety Guidelines., 2001)

- Before removing the lid the chamber pressure was checked for zero setting.
- Overall dress code like apron, eye shields, gloves for biological and heat protection, feet covered completely.
- After the opening the door, the liquids were allowed to cool for 20 minutes before removing.

**3.1. SAMPLE COLLECTION** (Ganguli *et al.*, 1984)

**INTRODUCTION**

It is obviously hard to collect all the guidelines for sample collection and transportation in the laboratory to ensure quality patient care. The results of the microbiology tests depend upon the quality of samples received. As a result of poor collection or poor transportation of specimen, the
causative organism is missed to be isolated, for which, as a consequence, failure to treat the patient occurs. As a protocol, the direct sample smears help in determining the quality of the sample and this also provides rapid information for diagnosis of infection and treatment. Direct smears also allow the clinician to judge if additional, better quality samples are required (Baron et al., 2005).

1. Meticulously clean working habits were adopted during all the procedures. In case of Spillages 0.5-1% Hypochlorite was used for coverage.

2. Proper methodsof discarding the wastewere adoptedas per the guidelines of Punjab Pollution Control Board.

3. Hand washing with soap before leaving the working area was strictly followed.

4. All samples were treated as biohazards.

5. The concerned laboratory workers used appropriate barrier protection e.g., gloves, apron etc.

6. In case of splashing of the specimen in laboratory, eyewear, face masks, and aprons were considered necessary to wear, before dealing with the samples.

7. It was instructed that the external surfaces of the collection container and the related paperwork should not be contaminated with any of the sample at any stage of processing, starting from sample collection till discarding the samples.

8. To avoid contamination, direct dealing with patient regarding sample collection and processing was minimised.

9. It was ensured to clinicians that the samples that were collected in syringes i.e., aspirates etc. Should be sent to the laboratory in transportation tubes.

10. If there was a little material in the syringe, the syringe was rinsed with normal saline and then the material was transferred to the sterile tube.

11. The clinicians were advised to use protective equipment before transferring specimen from syringe to tube e.g., capping of needle before removing from the syringe.

12. All the specimens were collected before the antibiotic treatment was started.
13. For collection of all types of samples for culture and susceptibility, proper sterile equipment was used to prevent the interference of normal environmental flora during procedures.

14. Labelling of specimens should be clearly done with name, age, sex, address and contact number with sample details and date of collection.

15. During collection, it was kept in mind that sufficient amount of sample can yield appropriate and accurate results.

16. Geographical and epidemiological conditions were also considered while proceeding with the samples.

17. It was ensured that there must not be any leakage of samples- all the samples must be properly covered or stoppered.

18. Samples were preferably collected prior to the start of antibiotics.

19. Samples were handled in way that they must not get contaminated with external organisms or commensals present in environment.

20. Missed identification of samples was excluded.

Basically the samples for microbiological tests were collected in the following way to avoid above mentioned errors.

i) **Blood** – for blood sample collection, properly sterile brain hear infusion broth was used.

ii) **Urine** - for collection of urine samples, properly sterile, wide mouth container with lid was used.

iii) **Sputum** - for collection of sputum samples, properly sterile, wide mouth container with lid was used.

iv) **CSF, pleural, Peritoneum fluid or other body fluids** were collected in sterile penicillin vials.
v) **Stool** - for collection of stool samples, properly sterile, wide mouth container with lid was used.

vi) Any fluids /discharges that were in very less amounts were collected with the help sterile swabs, ensuring that they were properly plugged. The ends of the sticks were not broken off and were allowed to project out.

### 3.m. COLLECTION AND TRANSPORT OF BLOOD SAMPLES

Blood culture samples were required in people those are at higher risk of all infections like immune suppression or patients those had a recent infection, prosthetic heart valve replacement or any surgical procedure. New borne and old age people are more prone to sepsis who may had an infection but may not show the typical signs and symptoms of sepsis (Claudio *et al.*, 2004).

Therefore, blood collection skills may help in holistic and timely treatment. For blood sample collection, the sample of blood was commonly collected vein from cubital fossa that is anterior to the elbow. Since, this vein lies close to the skin surface, with not a large nerve supply, this vein was considered to be most appropriate vein to prick (Calfee and Farr, 2002).

**PREPARATION OF BLOOD COLLECTION SITE**

1. The blood samples were drawn under aseptic conditions.

2. To maintain aseptic conditions, the skin was cleaned with savlon, then 2% Iodine or 10% povidone.

3. Iodine was cleaned with alcohol or spirit to prevent its irritation caused by iodine on skin.

4. After cleaning the skin the phlebotomist waited for a minute to draw the blood.

5. For blood culture sample collection, 5 ml of blood is appropriate in adults, which was immediately put in 50 ml of BHI broth. For paediatric age group the ratio was maintained 1:10 by reducing the volume of sample and broth to 1ml and 10 ml respectively.

6. These blood culture bottles should never be refrigerated after addition of blood sample (Baron *et al.*, 2005).
3.n. BLOOD SAMPLE COLLECTION

As we know that study of blood in various aspects is most important diagnostic tool for clinicians. The reports collected from blood studies usually help the clinical setting in treating mass of symptoms or diseases. If the patient presents the signs of septicemia blood culture is considered to be the gold standard method to verify the infections. It helps in verifying infections if present; it also helps in identification of microorganism, which is responsible for the infection e.g., pelvic inflammatory disease, pneumonia, puerperal fever, neonatal epiglottitis, sepsis, and pyrexia of unknown origin (PUO) or any other bacterial infection. Overall, the purpose of blood cultures is find any bacteria present in blood responsible for causing disease and to guide the clinician for treatment. This Testing is done to identify the reason of septicaemia, which may lead to sepsis (a serious and life-threatening complication) in less immune patients.

Blood cultures are required when the patient has symptoms of sepsis, which means that any bacteria or fungi or their toxic metabolites are harming the body of the patient, this involves following initial symptoms:

1. Chills or fever or both
2. Nausea, vomiting
3. Tachypnoea, tachycardia
4. Confusion
5. Oliguria

Later on if untreated the blood infection may lead into severe symptoms:

1. Inflammation in the body
2. The formation of blood clots in blood vessels
3. Hypotension
4. organ failure

Since these infections are serious, they are required to be treated immediately. The patients suspected for sepsis are treated with intravenous antibacterial agents that act on wide strand of bacteria meanwhile the blood culture and its susceptibility testing reports are done. According to the results the antibiotics may be changed (Claudio et al., 2004).
METHODS KNOWN

Basically there are two methods to collect the blood samples for cultures and microbiological studies: vacutainer system method and syringe method. These methods are chosen depending upon the availability of infrastructure. Age is the most important factor to select the method for venipuncture.

VACUTAINER SYSTEM

It is the most appropriate method to collect blood from vein. This system is comprised of closed evacuated tube system, where chances of contamination are very low. This system contains a Vacutainer and a vacuette. The vacuette with plastic hub and hypodermic needle is used where blood is required with any interference of environmental factors. The blood with this technique is directly collected into Vacutainer closed tube under vacuum (Calfee and Farr, 2002).

Under some circumstances, a syringe or a butterfly needle with which plastic catheter is attached may be used.

In developing countries, syringe method is commonest method for blood sample withdrawal due to cost issues with vacutainer system (Calfee and Farr, 2002).

Apart from vacutainer set required in vacutainer system, the blood samples are collected with the help of following:

1. SYRINGE METHOD

For collection of blood for blood cultures, there are many types of needles, depending upon their sizes required by various age groups and conditions. The most commonly used needles in various sizes are: 21gauge (green coloured), 22gauge (black coloured), and 21gauge (green coloured) butterfly needle, 23gauge (blue coloured) butterfly needle and 25gauge (orange coloured label) butterfly needle. The butterfly needles are used in paediatrics cases or in special circumstances. The problem with this needle is that it is so small that it may haemolyse RBC’s during blood collection (Calfee and Farr, 2002).

2. BUTTERFLY NEEDLE METHOD
These needles are fine needles that are used in cases of kids as finer needles are less painful. But the blood collected with needle has to flow through the rubber tubing, which may cool down the sample, increasing chances of clotting. The blood clotting in the butterfly needle is as fast as it may happen in the tube during the collection of blood. Thereby, the phlebotomist has to prick the patient for collection sometimes.

By using common needles for collection of blood samples, the blood is not clotted as rapidly as in butterfly needle. Since here there is no tubing is this method used, blood flows straight to the test tube. So the clotting doesn’t occur rapidly with less chances of haemolysis.

Other material required at the time of blood collection for blood culture is discussed below:

1. 70% isopropyl alcohol pad
2. Povidine iodine solution
3. Spirit
4. Tourniquet
5. Gauze pieces
6. Adhesive bandages

PROCEDURE OF VENIPUNCTURE

1. VENIPUNCTURE WITH EVACUATED OR VACUUM TUBES

PRINCIPLE

It works in a system that includes a cannula with the vacuum pressurized sleeve, which is attached to a holder that is used to hold the needle-sleeve combination. When the needle once is inserted into patient, the holder leads the negatively pressured collection vial to attach to holder. When the collection tube is inserted; until the vein is pierced properly the blood doesn’t move to come in the collection tube that is already attached to holder. These systems are commonly used in developed countries like US, UK, and Australia. In developing countries some hospitals and private doctors’ offices and community labs use this system (Calfee and Farr, 2002).

PROCEDURE

U.S. Company BD marketed the Vacuum tubes for the first time under the market name Vacutainer tubes. The vacutainer tubes (glass or plastic made) are like test tubes with vacuum,
which automatically aspirates blood into itself from vein when with help of hub, the needle is inserted into the vein. The benefit of using these tubes is that multiple tubes meant for different samples with different additives can be attached one by one for collection of blood in a single procedure (Calfee and Farr, 2002).

2. VENIPUNCTURE WITH SYRINGE AND NEEDLE

PRINCIPLE

This method was adopted in this study being most appropriate method by cost, effectiveness (less haemolysis chances that may participate in false positive or negative blood culture results). There are many health care workers that opt for a syringe-needle technique for Venipuncture. There are many manufacturing companies that make syringes and needles for this purpose. The venipuncture method is based on the principle that blood sample withdrawal by syringes has to done manually; thus the pressure on succession is controllable by the operator. This adds a feature to the operator when the patients is presented with very thin vein for blood sample collection, as by this the chances of collapse under pressure are minimized.

So this method is preferred on burnt patients/ severely ill patients/ elderly patients or oncology patients. Over weight patients with very thin, small, deep or fragile veins are helped by this method. In case of children, where small amounts of blood are required, this method may be helpful (Calfee and Farr, 2002).

PROCEDURE

The principles of both methods may vary, but the procedure and purpose of collection of blood from vein is same as discussed below:

Through venipuncture, 1-5 ml of blood was required for blood culture, depending upon the age of the patient.

1. Brain heart infusion broth should be kept on the table before starting venipuncture to directly transfer blood from syringe to bottle. The blood samples were collected using aseptic techniques. To maintain the aseptic techniques, it was required to clean both mouth of the blood collection bottle and the median cubital fossa. sample collection in the patient prior to collection by swabbing with 70% isopropyl alcohol, povidone and again spirit in three consecutive steps in
circular motion. The site of collection of blood was left for one minute to dry to prevent any kind of chemical interference with the blood cultures (Calfee and Farr, 2002).

Blood Culture Media were always considered according to the age of the patient i.e., pediatric or adult: for pediatric patients: 10 ml of BHIB was used and for adults 50 ml of BHIB was used.

The sample was collected in the following way for blood cultures:

2. The vein for collection was first prepared for collection of blood sample as discussed above.

3. The site was allowed to dry for 30 seconds to one minute.

4. It was kept in mind that the venipuncture site after cleaning must not be retouched for palpitation. If re palpitation is required, the site has to be cleaned again.

5. With the help of syringe and needle the required volume of sample was collected.

6. After withdrawal of blood and syringe from vein, the vein was pressed gently with dry gauge to cease bleeding.

7. The venipuncture site was then taped to prevent any infection through skin.

8. The caps of blood culture bottles were cleaned with 70% isopropyl alcohol and removed after allowing the tops to dry.

9. The blood samples that were collected were injected into bottles.

10. The bottles were labelled simultaneously with name, age, and sex and laboratory number of the patient.

11. Needle and syringe were discarded into sharps container according to the guidelines of Punjab Pollution Control Board.

12. The culture bottles were immediately incubated at desired temperature. Sterile supplies and aseptic techniques for collection of specimens were followed to prevent specimen contamination by microorganisms. The blood samples were collected before the use of antibiotics.

13. The usual risks of venipuncture include hematoma, air embolism or collection that lead to false positive results (3-4%), which thereby may guide the clinician wrong for inappropriate treatment (Claudio et al., 2004; Calfee and Farr, 2002).
3.0. COLLECTION AND TRANSPORTATION OF URINE SAMPLES FOR CULTURE AND SENSITIVITY TESTING

URINE SAMPLE COLLECTION

Urine culture test was demanded to investigate many kinds of bacterial and fungal infections. For urine culture test, Mid-stream urine sample is the most appropriate sample, because of the reduced chances of cellular or microbial contamination. This was collected after instructing the patients properly. The first part of urine was allowed to escape as it significantly reduces the chances for contaminants to enter into the urine stream. The genitalia were cleaned properly to for collection of mid-stream urine in wide mouthed clean container. The inner side of the cap or bottle was advised not to be touched. The transportation of samples should be immediately done to the lab. The samples for the cultures should be processed within 3-4 hours of collection. Catheterised samples can also be collected under aseptic conditions. The midstream urine sample can be collected anytime of the day or night. Suprapubic aspiration under aseptic techniques and proper guidance may help in getting appropriate results in case of infants where mid-stream results may be doubtful (Vernon et al., 1997). Urine culture is helpful in diagnosis of all cases of complicated urinary tract infections. In case of upper UTI i.e., pyelonephritis and in lower UTI i.e., cystitis and in children with urinary infections the test is recommended. In cases of Asymptomatic bacteriuria (ASB) the test is very much important when the patient does not show any symptoms of infection but general symptoms persist, the culture shows positive results, which can guide clinician to treat the infection that showed no symptoms but could have led to serious illness (Lewis, 1998).

Inappropriate antibiotic treatment of urinary tract infections may cause unnecessary side effects to the patient and it may increase antibiotic resistance. Another risk factors cover increased costs of healthcare and recurrent UTI in young women (Lewis, 1998).

The samples that were collected for culture and sensitivity were properly labelled and the samples were delivered to the processing area immediately. Care must be taken during collection, transportation and processing of samples:

1. Complete instructions of collection of urine samples should be given to the patient or responsible person for the collection.
2. In case of delay in transportation to processing area, the samples should be kept in ice or refrigerator i.e., 2°C - 8°C.

3. The urine container that was meant for collection should have following information labelled on it: patient’s name, age, sex, and lab. No. and date and time of collection (Giddens and Robinson, 1998).

METHODS KNOWN

1. The vacutainer system container

2. Sample collection in simple sterile and wide mouthed container with lid

These are the two methods by which the urine samples are collected for culture. In developing countries, the simple container is used due to the high price of vacutainer system (Cohen et al., 1997).

MATERIALS REQUIRED

1. Sterile, clean and dry, disposable, leak-proof containers with lid

2. Disposable gloves

3. Betadine swabs or towelettes

4. Sterile and dry gauze pieces

5. Patient’s bedpan or urinal, if patient is bed ridden

6. No calibration is needed for this procedure of urine collection.

7. Preservative in case of delay or transportation: boric acid in tablet or powder form.

Apart from basic requirements, following materials are required for collection of urine samples by vacutainer system:

1. Plastic collection container

2. Yellow top tube
3. Grey top tube with preservative
4. Towelettes

**PRINCIPLE**

The mid-stream urine sample is collected for blood culture because in the first part of urine the urethra is washed away and the debris is also removed along with this. It generally becomes impossible for contaminants to enter the mid-stream urine. So the chances of contamination are reduced. In case of delay in processing the preservative boric acid helps in maintaining the refrigerated state of the sample, which reduces the chances of contamination by deterring the shedding of bacteria or other microbes which may have resulted in false positive culture or overgrowth of pathogens. Preserved samples are easy to store at room temperatures. The non-buffered boric acid may give some adverse effects on bacteria so it should not be used (Cohen *et al.*, 1997).

**PROCEDURE**

1. The midstream urine sample was advised to the patient.
2. The procedure was explained carefully to increase the chances of proper sample collection.
3. Towelettes were given to the patients for cleansing.

**URINE SAMPLE COLLECTION IN FEMALES**

1. Females were advised to sit on bedpan or toilet seat.
2. They instructed to clean the genitalia by first cleansing the labia with one downwards stroke towards rectum. The towelettes were discarded.
3. With the help of other towelettes, the vulvar part was also cleaned. The towelettes were discarded.
4. The females were advised to allow the first part of urine void, and the midstream urine was advised to collect.

**URINE SAMPLE COLLECTION IN MALES**
1. Male patients were given clean urinals.

2. They were asked to clean the genitalia properly before voiding urine.

3. As first portion of urine washes out urethra and contains debris, the first part of urine was allowed to escape.

4. The mid-stream urine was advised to catch in clean sterile container.

5. The patients were advised not to touch inside the container.

6. The patients were allowed to finish voiding.

7. The urine samples in the containers were sent for processing.

8. In case of vacutainer system, after 15-20 minutes of collection of urine, the protective safety label on the container was peeled back, and the grey coloured tube was pushed into recessed channel in cup lid, with the help of vacuum, the urine came in the tube until the tube was filled and the vacuum was used up. The grey top tube was removed from the container. The tube was inverted many times to dissolve the preservative in it (plate 1).

9. The urine samples in the tubes were sent for processing.

10. **Urine sample collections in catheterised patients** - The samples were not collected from collection bag or by opening the closed drainage system. For this the area of collecting tubes as cleaned and it was punctured with the help of sterile needle and syringe. By puncturing with syringe the samples were collected in desired volumes (Cohen et al., 1997).
3.p. STOOL SAMPLE COLLECTION (Tam et al., 2012)

A stool culture test is useful in identification of bacteria or viruses which are causing an infection. Though more than fifty different types of commensals normally live in the intestines, a big number of pathogenic bacteria, viruses, fungi, or parasites can start growing in the intestines and cause infections and diseases. Stool culture test is done to find out the reasons of some gastrointestinal diseases like severe or bloody diarrhea, flatulence, vomiting, nausea, loss of appetite in any infection and other associated symptoms like bloating, abdominal pain/cramping and fever, which may arise from infection from food or consumption of non-potable water.

Stool culture test helps in identifying the pathogen responsible for the diseases like food poisoning, colitis (inflammation of the colon), Salmonella and Vibrio infections etc. Another important role of stool culture is to identify the carriers that harbour the pathogens without having infection themselves and are able to transmit the pathogens to healthy people. The test also helps in judging the effectiveness of antibiotics to infection (Tam et al., 2003).
To do a stool culture test, stool sample is collected in a clean container and is incubated under conditions that allow the pathogenic bacteria or other organisms to grow. The cause of infection is identified by judging the colony characteristics, by biochemical tests on the colonies isolated, and by observing the sample and colonies microscopically.

Depending upon the type of infection to be detected from the stool, one may only need to test only one stool sample, or more than one stool samples with a definitive period of days (Tam et al., 2012).

1. It was advised that laxative should not be used by the patient before sample collection.
2. The bladder was completely empty before collection of stool so that the urine sample may not be mixed with stool.
3. The samples were collected in clean, disposable, dry container.
4. Water contact of stool was avoided.
5. Samples on diapers should be avoided.
6. In case of children with diarrhoea, a diaper line was fastened with the diaper and the sample was collected from that line with the help of the plastic spoon and was put in container.
7. The parts of stool that were slimy or mucoid or bloody were collected preferably while choosing for culture test.
8. The patients were advised to fill the container 1/3rd full.
9. The patients were also advised not to overfill the container and they were advised to wash their hands after collecting the stool samples.
10. The personal details of the patient with laboratory number and date of collection were mentioned on the label on the container.
11. In case of delay in processing the samples were refrigerated to prevent deterioration of the bacteria in the stool.
12. The samples were processed within 8 hours of collection.

**MATERIALS REQUIRED** (Kubota et al., 2011)
There are two methods of sample collection for stool cultures:

1. Sample collection by patient in the sterile containers and
2. Anal or rectal swabs in case of no stool samples available.
3. Material required
4. Sterile, clean and dry, disposable, leak-proof containers with lid
5. Disposable gloves
6. Towelettes
7. Patient’s bedpan, if patient is bed ridden
8. Dry sterile cotton Swabs in case of rectal swab collection
9. Sterile swab sticks in case of rectal or anal swab collection

**PRINCIPLE**

Stool should be passed into a clean dry container as already present environmental flora may interfere in the results. If wooden applicator stick is used to mix the sample the stick must be removed from the stool sample immediately. As wooden stick has an absorbing property that will absorb the moisture of the stool, rendering stool sample dry and unacceptable for the culture. In terms of weight the stool sample should be 50 grams if solid, 20 ml if fluid (Scallan et al., 2006).

**PROCEDURE**

1. The patient should be enquired by the clinician about history of antibiotic consumption.
2. History of travelling should be noted.
3. The recent contrast material test like barium meal tests or barium enema was also noted, if any.
4. The patient was advised to give stool sample 1/3rd full of the container.
5. The sample was collected in dry, clean, leak proof container. It was made sure that the sample was not mixed with urine, water, soil or other material.
6. Ideally the sample should be processed immediately after collection.

7. It was ensured that the stool containers after collection of the sample were sealed well.

8. The containers after proper labelling and sealing must be put in the plastic bags (Warwick, 2004).

**RECTAL SWAB**

The method of collecting stool sample from rectum or anus is used when the stool sample is not available. The tip of the swab is passed beyond the anal sphincter 1 cm in kids and 4 cm in adults. The swab is rotated at the angle of $360^\circ$ from anal crypts for 30 seconds.

The stick of the swab is broken and put in the transport media (Selenite F broth or Salmonella Shigella medium or alkaline peptone water) for transportation to the laboratory.

The dealing person must wear gloves during the procedure; otherwise risk of getting infected is high in dealing these samples without gloves (Tam et al., 2003).

**3.q. CSF OR OTHER NORMALLY STERILE BODY FLUIDS FOR CULTURE AND SENSITIVITY** (Ogunsola et al., 1998)

1. The collected body fluids samples were transported to the processing section of the laboratory immediately.

2. Cell lysis in fluids may begin within one hour of collection which may interfere in the results.

3. The source of fluid must be stated.

CSF and other body fluids were collected by physicians under aseptic conditions and transported immediately to the laboratory. These samples were never refrigerated. In case of delay these were kept at room temperature (Ogunsola et al., 1998).

**3.r. PUS SWABS AND OTHER DISCHARGES**

1. Before collecting the swabs of any wounds or discharges, the site was not cleaned with antiseptics. The lesions were cleaned with normal saline.
2. 1-2 ml discharge samples were collected in sterile penicillin vials in case of fluids, and sample in case of pus, it was preferable to collect the samples with swab.

3. In case of swabs, two sterile swabs were taken; one for direct microscopy and another for culture.

4. In cases if vaginal discharges, high vaginal swabs were collected after actual Visualisation by clinician by keeping in mind that it should not touch the sides of vaginal walls (Mulu et al., 2006).

3.s. SPUTUM (Mulu et al., 2006)

1. For collection of sputum samples, the morning “Coughed up” samples after rinsing the mouth were collected in a screw capped container.

2. These samples were transported to the working area within 3-4 hours of collection.

PROCESSING OF ISOLATES
3.t. PROCESSING OF SAMPLES

After the collection of appropriate samples, they were processed differently, according to the type of samples.

3.t.I. BLOOD CULTURE

TIMING OF COLLECTION

The timing of collection of samples for culture is not as important as other factors in patients with intravascular infections because organisms present in blood samples are present throughout the time. A single sample was taken prior to antibiotics were started.

Blood culture bottles were transported immediately to laboratory and placed into the incubator at 37°C for 24 hours (Li et al., 1994).

3.t.II. CHOICE AND RANGE OF MEDIA FOR BLOOD CULTURE

The use of a single good all-purpose medium can give nearly as many positive results as the use of a range of different media, with the advantages of economy in costs and labour and avoidance of confusion. In some cases, however, a range of special media must be used e.g., bile salt broth for isolation of Salmonella species from blood.

If a single medium is used as a routine, it must be richly nutritive and suitable for aerobes and anaerobes when incubated under suitable conditions. A good choice is a Robertson type cooked meat medium based on brain heart infusion broth with a layer of pieces of lean meat.
If two bottles have been seeded from the samples, their caps may be loosed and one is incubated in air plus 5%-10% CO₂ while the other is incubated anaerobically with added CO₂. Modern commercial systems exploit the advantages of shaking broths during incubation to encourage growth. In the present study blood was collected in BHI broth and incubated at 37°C (plate 2) (Lapage et al., 1970).

![Blood culture collection bottle](image)

**Plate 2.**

3.t.III. METHOD

The sample of blood after collection in required amount was added into blood culture bottle (Ganguli et al., 1984).

**INCUBATION OF BLOOD CULTURE**

After collecting blood sample was incubated at 37°C for 24 hour.
3.t.IV. EXAMINATION OF BLOOD CULTURE

1) Blood culture bottles were opened with care to avoid contamination and observed for turbidity.

2) From liquid media, subcultures were done on MacConkey and blood agar to observe colony characteristics for final diagnosis.

3) Blood culture was examined once on the first day and thereafter at 24 hours intervals.

4) Subcultures were done after 48, 72 hours and 7 days. It is usual to continue incubation and inspection for up to 5-7 days with a final subculture then. Prolonged incubation was recommended, before the bottles were discarded, in the investigation of some infections such as bacterial endocarditis, or if the patient was on antibiotics (Wolach, 1997).

3.t.V. PLATING OF BLOOD CULTURE

With aseptic technique described above the samples were plated on suitable media including MacConkey agar and blood agar and were incubated at 37°C overnight. Isolated pure colonies were further characterized by physical characteristics, gram staining and biochemical reactions.

3.t.VI. OBSERVATION

The isolated bacteria were tentatively identified on the basis of Colony characteristics and biochemical reactions.

3.t.VII. URINE CULTURE

the urine samples were collected in sterile containers under prescription described above.

TIMING OF COLLECTION

The timing of collection of urine samples is not as important as other factors if bacteria present in urine, it will persist in the urine samples of all day. A single sample was taken prior to antibiotics were started (Cohen et al., 1997).

3.t.VIII. CHOICE AND RANGE OF MEDIA FOR BLOOD CULTURE
The use of a single good all-purpose medium can give nearly as many positive results as the use of a range of different media, with the advantages of economy in costs and labour and avoidance of confusion. In some cases, however, a range of special media must be used.

If a single medium is used as a routine, it must be richly nutritive and suitable for aerobes and anaerobes when incubated sealed and unexposed to an external atmosphere. A good choice is a cysteine lactose electrolyte deficient medium or routine medium like MacConkey agar and nutrient agar along with blood agar can also be used.

Here in this study, MacConkey agar and nutrient agar along with blood agar were used to observe the various characteristics of pathogens on the basis of haemolysis, lactose fermentation etc. (Cohen et al., 1997).

3.t.IX. URINE CULTURE

METHOD

the sample was processed for identification and isolation of respective bacteria in two methods discussed below:

- Wet mount preparation
- Streaking on media

WET MOUNT PREPARATION

following materials were required for preparation of wet mount:

1. Microscopic glass slides
2. Cover slips
3. Inoculating nichrome wire loop
4. Micropipette
5. Bunsen burner
6. Urine sample
**PROCEDURE**

1. A clean sterile and scratch free glass slide was taken.
2. The slide was labelled with the name of the patient.
3. 15 - 20 µl of urine sample to be tested was kept on the centre of the slide.
4. The slide was covered with cover slip avoiding formation of bubbles.
5. This was focussed and examined under 10X and 40X for motile organisms. First of all, the edge of the cover slip was focussed under 4x objective of the microscope.
6. A bubble in the liquid suspension was looked for and the fine focus was adjusted on the edge of the bubble.
7. The objective was then switched to the 10x objective and the focusing was repeated carefully.
8. Once focussed the objectives were switched to the 40x objective and again the focusing was repeated carefully.
9. The slide was observed for any kind of microorganism on it in urine sample.
10. The slide after examining was cleaned up with alcohol as it could have had live bacteria on it. Then it was discarded (Lulich *et al.*, 2004).

**STREAKING ON MEDIA**

The sample of urine after collection in required amount was streaked on three different media discussed above. The required amount of urine becomes a loopful of urine sample that holds 0.001ml of sample in 4.8 mm diameter (Calfee and Farr, 2002).

**INCUBATION OF URINE CULTURE**

After collecting blood sample was incubated at 37°C for 24 hour.

**3.t.X. EXAMINATION OF URINE CULTURE**

1) The plates were observed next day of culture after incubation for colony morphology.

2) If mixed growth was found the colonies were purified on different MacConkey and blood agar plates.
3) These isolated colonies were incubated for next 24 hours and looked for different colony characteristics.

4) In case of sterile plates the plates were reincubated for next 24 hours and then were reported as sterile (Tam et al., 2003).

3.t.XI. OBSERVATION

Various morphological characteristics were observed to interpret the pathogen.

When a single type of colony was found with > 1 lac colony forming units/mililitre in the sample collected properly, Bacteriuria was reported. If the sample was collected from the catheter, the rate was considered to be as 100 CFU/ml. same CFU rate was considree in women presenting symptoms of urine infection (Colgan et al., 2006).

In 0.001 ml loop one colony was equalant to 1000 CFU per ml of urine. $10^5$ CFU were considered as positive for urine culture and CFU $10^3$ or $10^3$ were taken as contaminants. But with significant history this concentration was also considered as positive urine culture (Lin and Fajardo, 2008).

3.t.XII. STOOL CULTURE

The stool samples were collected in sterile containers under prescription described above.

TIMING OF COLLECTION

The timing of collection is not as important as other factors in patients with gastro intestinal infections because organisms present in stool are found at a constant rate. A single suitable sample was collected before the start of antibiotics therapy (Warwick, 2004).

3.t.XIII. CHOICE AND RANGE OF MEDIA FOR STOOL CULTURE

The use of a single good all-purpose medium can give nearly as many positive results as the use of a range of different media, with the advantages of economy in costs and labour and avoidance
of confusion. In some cases, however, a range of special media must be used. In stool culture initially stool sample requires a good quality enrichment or transport medium for the pathogens of stool to survive (Tam et al., 2003).

For plating, single medium cannot be used as a routine; the medium must be different according to the nutritional requirement of different bacteria of stool. Here in the study the enrichment medium used was alkaline peptone water and Selenite F broth. The solid medium for plating was done on selective mediums. Selective medium used here was MacConkey agar, blood agar, Deoxycholate citrate agar and TCBS.

METHOD

In order to isolate and identify the causative bacteria, the stool samples were processed by two methods: Wet mount preparation as discussed above and Streaking on media, which was done on various enrichment media mentioned earlier (Lulich et al., 2004).

INCUBATION OF STOOL CULTURE After putting the stool sample in enrichment medium, this was incubated at 37°C for 6-8 hours (Tam et al., 2003).

3.t.XIV. EXAMINATION OF STOOL CULTURE

1) After six hours of incubation the subcultures were done from both enrichment medium onto the plates of solid mediums i.e., MacConkey agar, blood agar, Deoxycholate citrate agar and TCBS medium.

2) Both solid medium and enrichment medium were incubated for next 24 hrs.

3) The plates were observed for any kind of growth.

4) If mixed growth was found the colonies were isolated on different selective medium plates.

5) Other subcultures were made from enrichment medium onto selective mediums and the plates were again incubated for 24 hrs.
6) These isolated colonies were incubated for net 24 hours and looked for different colony characteristics.

7) In case of sterile plates the plates were reincubated for next 24 hours and then were reported as sterile (Lewis, 1998).

**OBSERVATION**

Colony Morphology was observed for tentative identification of causative pathogen or bacteria.

### 3.t.XV. BODY FLUID CULTURE AND PUS CULTURES

**FLUIDS AND PUS**

The samples were collected in sterile containers or on sterile swab sticks by physicians under prescription described above and were sent to the laboratory for study (plate 3).

**TIMING OF COLLECTION**

The timing of collection is not as important as other factors in patients with infections because if the patient is suffering from infection even a single organism may be isolated from the sample. A single suitable sample was taken prior to antibiotics were started (Lewis, 1998).

### 3.t.XVI. CHOICE AND RANGE OF MEDIA FOR BODY FLUIDS OR PUS CULTURE

The use of a single good all-purpose medium can give nearly as many positive results as the use of a range of different media, with the advantages of economy in costs and labour and avoidance of confusion.

If a single medium is used as a routine, it must be richly nutritive and suitable for aerobes and anaerobes when incubated sealed and unexposed to an external atmosphere. Here in this study, MacConkey agar and nutrient agar along with blood agar were used (Lewis, 1998).
Sample collection by Swab stick

3.t.XVII. METHOD In order to isolate and identify the causative bacteria, the samples were processed by two methods: Wet mount preparation as discussed above and Streaking on media, which was done on various media, mentioned earlier (Lulich et al., 2004).

INCUBATION OF CULTURES

After putting the stool sample in enrichment medium, this was incubated at 37°C for 6-8 hours (Tam et al., 2003).

3.t.XVIII. GRAM’S STAIN (Reynolds et al., 2009)

REPARATION OF SMEARS FOR GRAM STAIN

a) SWAB SAMPLES

IF two swabs were provided for testing one was directly put for cultures while the other was used for making smear. The swab was rolled lightly on the clean, grease free glass slide. If only one swab was taken, first it was cultured and then was used for smear examination.
b) **PUS AND DISCHARGES**

These samples were spread over a clean slide. It was kept in mind that the smear should not be very thick.

c) **SPUTUM SAMPLES**

The smear was made as pus but if mucous was present it was taken as representative sample.

d) **CLEAR FLUIDS AND CSF**

A loopful of fluid was taken on a clean glass slide and it was allowed to air dry as such without spreading.

e) **CULTURES**

With the help of a sterile loop, a loopful of broth culture were taken and spread on clean glass slide and were allowed to air dry. In case of colony, a loopful of sterile normal saline was taken on a slide. The colony to be examined was touched with the loop and a suspension was formed gently with the normal saline while spreading it outwards. The size of the smear should be 1x2 cm.

**PRINCIPLE OF GRAM'S STAIN**

The basic principle of the stain is the power of any bacteria to conserve the colour to which it is exposed or not under the presence of decolorizer. Peptidoglycan, the main constituent of the cell of bacteria is found in high content in some bacteria. These bacteria when treated with two different dyes step by step, due to the thickness of cell wall, they retain the colour of the first dye and with the treatment of decolorizer even, they don’t lose the first dye i.e., crystal violet. Whereas, some bacteria don’t possess the peptidoglycan in higher amounts. In spite of peptidoglycan in the cell wall they contain few lipids. Thus when they are treated with many dyes step by step and in presence of decolorization, they lose the colour of first dye due to lipid dissolution in presence of decolorizer and they take the colour of second dye i.e., Safranin.

The bacteria that retain the color of first dye are stained purple and called as Gram Positive bacteria. Those bacteria that take the colour of second dye are seen red or pink under microscope.
are called as Gram Negative bacteria. However the time of decolorization play an important role as prolonged time may affect the cell wall of gram positives also and they may lose the colour, thus resulting to false reports. There are some bacteria that retain the colour of both dyes. They are called as Gram variable bacteria (El-Garnal et al., 2009).

**REAGENTS FOR THE GRAM STAIN**

1. Crystal violet

2. Gram's iodine.

3. Ethyl alcohol (95%).

4. Counterstain solution

**PROCEDURE**

1. The smear was air dried.

2. The smear was heat fixed by passing it over a flame 3-4 times, smear side upwards.

3. Crystal violet stain was flooded over the slide for 1min. This was washed under running tap water.

4. After washing the slide was flooded with Gram's iodine 1 min. It was again washed under running tap water.

5. The slide was rinsed with acetone for few seconds for decolourisation.

6. The excessive acetone was washed off with water.

7. Safranin stain was added over the smear for 1min, it was again washed under running tap water.

8. The slide was blotted dry gently.

9. Exam the smear 1st under 10x and then add 1 drop of oil (cedar wood oil) and

   The smear was examined under oil immersion lens.
10. On the basis of their dye retaining properties, the bacteria were categorised as GPC or GNB. The bacteria that retained the colour of primary dye considered as gram positive bacteria and the bacteria that took the colour of secondary dye were considered as gram negative bacteria.

In case of throat swabs Albert stain was done along with gram stain (Nester and Eugene, 2004).

3.t.XVIX. PRINCIPLE

Albert's staining technique is a special staining technique as a special structure in bacteria is demonstrated by this. It is principally used to demonstrate metachromatic granules of bacteria *Corynebacterium diphtheria*, which causes the disease named diphtheria. The name *Corynebacterium* has been is taken from a Greek word "Coryne", meaning the club shape of the bacteria formed during prolongation of cultures. The energy is stored in storage granules of bacteria. The granules consist of polymetaphosphates and have many other names like volutin bodies, Babe-Ernst granules or polar bodies. These granules have a property of metachromasia that is why they are known as metachromatic granules. The granules take the colour of the stain other the colour taken up by bacillary body.

When the smear is stained with polychrome methylene blue, the granules take the colour violet, whereas the rest of the bacillary body appears to be blue. The bacteria in appropriate medium produce the abundant granules. When the bacteria are stained with Albert's stain, the bacillus stains green and the granules stain bluish black.

REQUIREMENTS

Apart from other basic requirements of smear making, there were two reagents that were used in the staining process: Albert's A solution and Albert's B solution.

2. Albert's A solution: it is consisted of Toluidine blue, malachite green, glacial acetic acid, and ethyl alcohol.

3. Albert's B solution: it is consisted of Iodine and Potassium iodide in water.

4. Smear on glass slide

5. Staining rack
6. blotting paper
7. immersion oil, microscope

PROCEDURE
1. The slide was placed on the staining rack with smear facing upwards.
2. The smear was covered with Albert's A solution and was allowed to act for 7 minutes.
3. The stain was poured off and was washed the slide in Albert's B solution (NOT tap water).
4. The slide was placed back on the rack and the smear was covered with Albert's B solution for two minutes.
5. The stain was poured off and the slide was wash in tap water.
6. The slide was dried using blotting paper,
7. A drop of immersion oil was placed on the smear and was observed under oil immersion objective.
8. On the basis of the bacilli arrangement in smear and the presence of metachromatic granules the bacteria were identified.

STREAKING ON MEDIA
The sample of body fluid or pus after collection in required amount was streaked onto three of the medium: MacConkey agar, Blood agar and Nutrient agar.

INCUBATION OF PUS OR BODY FLUID CULTURE
Once the sample was collected, it was plated on suitable media and incubated at 37˚C overnight.

EXAMINATION OF CULTURE (Tam et al., 2003)
1) The plates were observed next day of sample collection after incubation for colony morphology.

2) If mixed growth was found the colonies were isolated on different MacConkey and blood agar plates.

3) These isolated colonies were incubated for net 24 hours and looked for different colony characteristics.

4) In case of sterile plates the plates were reincubated for next 24 hours and then were reported as sterile.

3.u. OBSERVATION

Even a single colony found on any medium was considered as significant and the colony morphology was observed.

After 24 hours of incubation at 37°C, various types of colonies that used to grow on various media were presumptively identified as following:

**Staphylococcus aureus**

**MacConkey agar:** *S. aureus* formed small pink color colony due to lactose fermentation.

**Blood agar:** *S. aureus* formed white, golden yellow with beta-hemolytic (Cole et al., 2001).

**Enterococcus species**

**MacConkey agar:** Pin point magenta colored colony.

**Blood agar:** showed beta hemolysis / alpha hemolysis or non-hemolytic colonies (Matthews et al., 1997, Fisher and Phillips, 2009).

**E.coli**

**MacConkey agar:** *E.coli* shows non mucoid, bright pink colony due to lactose fermentation.
**Blood agar:** grayish smooth large colonies (Vogt and Dippold, 2005).

**Klebsiella species**

**MacConkey agar:** Klebsiella formed faint pink colony due to mucoid nature of capsule.

**Blood agar:** grayish smooth large, mucoid and shiny colonies (Zheng, 2013).

**Pseudomonas species**

**MacConkey agar:** Pseudomonas aeruginosa formed non-lactose fermenting colonies of pale yellow color or with green pigment. The colonies were flat with sharp edges.

**Blood agar:** the colonies were greyish and spreaded sometimes showed green ting of pigment (Poole, 2004).

**Acinatobacter species**

**MacConkey agar:** Acinatobacter showed dome shaped pink white color colonies suggestive of late lactose fermentation.

**Blood agar:** the colonies were grayish and spreaded (Yeom et al., 2013).

**Salmonella species**

**MacConkey agar:** the colonies were non lactose fermenters with pale yellow color.

**Blood agar:** the colonies were grayish and spreaded (Nye, 2002).

**Citrobacter species**

**MacConkey agar:** the colonies were non lactose fermenters with pale yellow color.

**Blood agar:** the colonies were grayish and spreaded (Ong et al., 2010).

**Proteus species**
MacConkey agar: the colonies were flat, non-lactose fermenters with pale yellow color with swarming.

Blood agar: the colonies were grayish and spreaded and flattened (Matsuyama et al., 2000).

For identification and confirmation of the isolated organisms, gram staining was done.

3.v. IDENTIFICATION OF ISOLATES

1) By Staining methods: gram stain and albert stain (in case of throat swabs) once the causative bacteria were purified and isolated from the culture plates, these were preliminary identified as GPC or GNB. Their morphology was also observed by smear.

2) Depending upon their gram characteristics and morphology, appropriate biochemical tests were applied to identify the isolates, responsible for infections.

GRAM POSITIVE COCCI: 1. Catalase test

1. Coagulase test

GRAM NEGATIVE BACILLI: 1. Indole test

2. Vogus proskauer test

3. Methyl red test

Citrate test

Urease test

Oxidase test

Motility test

Triple Sugar Iron agar test
Identification of GPC: The isolates that were gram positive cocci according to the gram stain; they were further identified on the basis of the two tests discussed below:

3.v.I. CATALASE TEST
this is the test that is used to differentiate between gram positive cocci. The enzyme catalase is used by bacteria that live in oxygenated environment where toxic metabolites of oxygen like hydrogen peroxide are produced. This enzyme neutralizes the bactericidal effects of H2O2 and protects the bacteria. Anaerobic bacteria generally do not contain the catalase enzyme (Chelikani et al., 2004).

PRINCIPLE
The H2O2 that is produced by bacteria in presence of oxygen is lysed by catalyse enzyme present in bacteria into hydrogen and water. The same is implemented in vitro, when the hydrogen peroxide (3%) is added to bacterial colony the bacteria if having catalase enzyme will neutralise the HO2 added by splitting into water and hydrogen, which can be seen as water bubble or effervescence in the colony. lack of catalase enzyme in bacteria is evident, when there is no or weak bubble production (Chelikani et al., 2004).
Strict aerobes as well as facultative anaerobes contain the enzyme Catalase, hence these are considered as catalase-positive bacteria. As they all are considered to have the capability to respire using oxygen as a terminal electron acceptor. Generally, anaerobes don’t contain this enzyme and are considered to be Catalase-negative bacteria and there are some facultative anaerobes that only ferment and do not use oxygen for respiration as a terminal electron acceptor e.g., Streptococci.

USES OF CATALASE TEST (Chelikani et al., 2004)
1. The catalase test is basically used to distinguish among Gram-positive cocci: genus Staphylococcus and genus Streptococcus and Enterococcus.
2. To differentiate between aerotolerant strains of Clostridium and Bacillus species. Catalase test can be used as an identification test for of Enterobacteriaceae.

REQUIREMENTS
1. Culture: 24-48 hour on nutrient agar was preferred to perform the test.
2. Hydrogen peroxide (H₂O₂) 3%

**EQUIPMENT**

1. Bunsen burner.
2. Inoculating loop.
3. Cover slips

**PERCENTAGE OF H₂O₂ USED ON CATALASE TEST**

(Chelikaniet al., 2004)

1. For routine testing of aerobes for catalase test, 3% hydrogen peroxide was used.
2. For routine testing of anaerobes for catalase test, 15% hydrogen peroxide was used.

**PROCEDURE**

A small amount of bacterial colony was transferred to a surface of clean, dry glass slide with the help of a loop or sterile wooden stick.

1. Drop of 3% H₂O₂ was placed on the slide and mixed.
2. The results were noted.
3. The slide was disposed in the biohazard glass disposal container (Chelikaniet al., 2004).
4. For this test, a metal loop or needle was not used as it may cause false positive results or may degrade the metal.
5. The colonies were picked from nutrient agar and the blood agar plate was avoided. In case of picking colonies from blood agar plate, the care was taken in scraping the colony only as the blood in the blood agar plate also may give false positive results.
6. The results were noted within 20 to 30 seconds because some other bacteria may possess enzymes other than catalase that can degrade hydrogen peroxide may give false results (Chelikaniet al., 2004).

3.v.II. COAGULASE TEST (Ryan and Ray, 2004)

**INTRODUCTION**
PRINCIPLE

The enzyme Coagulase is present in staphylococcus aureus only. No other strains of staphylococcus possess the enzyme. This enzyme is capable of converting soluble fibrinogen in plasma to insoluble fibrin. That in the form of clot or clumping is seen in the test tube or slide.

The bacteria, Staphylococcus aureus, produces two forms of Coagulase i.e., bound and free. Two types of test are done to detect the presence of enzyme Coagulase (Ryan and Ray, 2004).

Types of test:

1. Slide Coagulase test for bound Coagulase or clumping factor.

2. Tube Coagulase test for free Coagulase.

Slide Coagulase Test: this is the commonly used test in the microbiology because the time consumption of this test is very less(Ryan and Ray, 2004).

REQUIREMENTS

1. Culture: 24-48 hour on nutrient agar was preferred to perform the test.

2. Horse or rabbit plasma

EQUIPMENTS

1. Bunsen burner.

2. Inoculating loop.

3. Cover slips

4. Glass slides

5. Test tubes

6. Water bath or incubator

Procedure

1. A clean grease free and dry glass slide was taken.
2. A drop of rabbit plasma was placed over the surface of the slide at one side.

3. At another side of the slide, next to plasma drop, a drop of normal saline was put as negative control.

4. A portion of the isolated colony to be tested was emulsified in each drop, and a smooth milky suspension was made.

5. The slide was rocked gently in to and fro motion for 5 to 10 seconds to look for clumping (coagulation)(Ryan and Ray, 2004).

**Expected results:**

1. **Coagulase Positive:** clumping visible by in 10 seconds or less in coagulated plasma drop with no clumping in saline or water drop.

2. **Coagulase Negative:** No clumping in any drop.

The test negative by slide method were confirmed by tube Coagulase test (plate 4) (Ryan and Ray, 2004).
Coagulase test

**TUDE COAGULASE**

the test was done in the case of negative slide test to confirm the negativity of the test (Ryan and Ray, 2004).

**REQUIREMENTS**

1. Culture: 24-48 hour on nutrient agar was preferred to perform the test.
2. Horse or rabbit plasma

**EQUIPMENTS**

1. Bunsen burner.
2. Inoculating loop.
3. Cover slips
4. Test tubes
5. Water bath or incubator
TEST PROCEDURE

1. 0.5 ml of rabbit plasma was taken in a clean, sterile and dry glass tube.

2. Several isolated colonies to be tested were emulsified in the plasma to form a milky suspension.

3. The tube was incubated at 35°C for 4 hours.

4. The tubes after four hours were checked for clot formation, if negative at 4 hours, the tubes were reincubated at room temperature overnight and checked again for clot formation (Ryan and Ray, 2004). Identification of GNB’s: the bacteria that were characterized as gram negative rods in gram stain; they were further identified by following biochemical methods.

3.v.III. INDOLE TEST

INTRODUCTION

Indole test is a biochemical test that is performed on gram negative bacteria to determine their ability to convert tryptophan into the indole, this reaction in the test tube is performed by a series of different intracellular enzymes, which are referred to as "tryptphanase"(washington, 1996).

PRINCIPLE

This is based on the enzymatic reactions where in some bacteria the enzyme tryptophanases is present that converts tryptophan to intermediate molecule indolepyruvic acid, which finally is converted into indole. this is basically a reductive deamination process. this deamination process is catalysed by tryptphanases with the help of coenzyme pyridoxal phosphate in which the amine (-nh₂) group of the tryptophan molecule is removed. final products of the reaction that are formed are indole, ammonium (nh₄⁺), pyruvic acid and energy. The test is indicative of positive reaction when pink to red ring is formed on the surface. This red ring is the result of series of reactions. Tryptophan the amino acid present in the peptone broth is lysed by some bacterial enzyme tryptphanases. This finally turns into indole. This indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indole. 4 (p)-dimethylaminobenzaldehyde, present in kovac’s reagent reacts with indole to from rosinolde. The rose indole in the presence of alcohol and acid id precipitated and accumulated at surface in the form of red ring (forbes et al., 2002).
**REQUIREMENTS**

1. **Culture:** 24-48 hour peptone water broth culture was preferred to perform the test.
2. **Kovac’s reagent**
3. **Bunsen burner**
4. **Inoculating wire loop.**

**PROCEDURE**

1. Indole test is also indicated by the change in colour like after addition of reagent, many other biochemical tests in microbiology.
2. A loopful of pure culture bacterial colonies was incubated in peptone water.
3. These peptone water tubes were incubated at 37°C for 24–48 hours.
4. After 24–48 hours the tubes were taken out and, 5 drops of Kovac’s reagent (isoamyl alcohol, para-Dimethylaminobenzaldehyde, concentrated hydrochloric acid) were added to the culture broth.
5. The colour changes were noted with presence of pink coloured ring in some bacteria.

**3.v.IV. VOGES-PROSKAUER TEST**

**INTRODUCTION**

The liquid media that is used to test Methyl Red and Voges-Proskauer for identification of bacteria is routinely used Glucose Phosphate Broth. This broth is ideal for the testing of the procedures to differentiate the coli-aerogenes group of bacteria (Voges., 1898). To identify the isolated bacteria that are gram negative bacilli, this is one of the common test that is conducted on GNB’s. These tests were initially discovered by Voges and Proskauer and modification was done by Clark and Lubs.

**PRINCIPLE**

When the bacteria grow in the media, they utilize carbohydrates. The catabolism of the sugars is detected by this test. It is clear that glucose is fermented by all members of Enterobacteriaceae.
When glucose is consumed by bacteria, acid are produced. The bacteria that are MR positive continue to produce acid for 2-5 days. The acid reduces the pH of the test media, thus indicated by addition of methyl red reagent, that gives very red colour and it becomes positive for MR.

The bacteria that don’t consume glucose for longer time, decarboxylation process is initiated there. As the mid product of this process, neutral acetyl methyl carbinol (acetoin) is formed. By this the pH of the media is raised which with the atmospheric oxygen and alkaline environment, gives rise to acetoin and 2, 3- butanediol. These end products are neutral in pH and they are oxidized to diacetyl that reacts with creatine to produce a red colour, giving a positive reaction.

The Methyl Red (MR) test takes the time of 5 days after incubation at 30°C. Whereas, The Voges-Proskauer test (VP) takes 1-2 days’ time. Voges-Proskauer is based on the principle of detecting end product of the series of reactions i.e., acetoin. For this test the VP broth is incubated with bacteria overnight. This broth is then treated with alpha-naphthol and potassium hydroxide. For the positive results a red colour is formed in the broth. The utilization of glucose by bacteria present in glucose phosphate broth is detected. The glucose is utilized by bacteria to form acetyl methyl carbinol. This reacts with Alpha-naphthol and potassium hydroxide to change the colour to red (MacFaddin, 2000).

**REQUIREMENTS**

1. **Culture:** 24-48 hour tryptic soy broth culture was preferred to perform the test.

2. **Media:** MR-VP medium

**PREPARATION OF MEDIA**

1. 5 grams glucose, 5 grams peptone and 5 grams dipotassium hydrogen phosphate were weighed separately.

2. All the ingredients were suspended in distilled water.

3. The volume was made up to 1000 ml.

4. pH was set to 6.9.

5. 3 ml of the media was dispensed into each test tube and was plugged; the tubes were sterilised at 121°C.

**REAGENTS**
Barritt's reagents: Barritt's A and Barritt's B.

Preparation of Barritt's reagent: **Barritt's reagent is composed of two solutions;**

1. Solution A: 6 grams of $\alpha$-naphtholin in 100 ml of 95% ethyl alcohol.
2. Solution B: 16 grams of potassium hydroxide in 100 ml of water.

**EQUIPMENTS**

1. Bunsen burner.
2. Inoculating loop.

**PROCEDURE**

1. Using sterile technique, the test organism was inoculated to the properly labelled tube of MR VP medium with the help of inoculation loop. These cultures were incubated overnight at optimum temperature.
2. The loop was sterilized in vertical position in the blue flame of the Bunsen burner until it gets till red hot. Heating was started from the base of the wire to tip of the wire slowly.
3. From the rack, the test tubes containing the peptone broth cultures were taken that had been kept for 24 - 48 hours at 37°C.
4. The neck of the test tubes were sterilised by the flame.
5. Using aseptic techniques, a loop full of organisms was taken off from the broth.
6. The neck of the tubes was gain flamed and plugged immediately.
7. Again flame the neck of the tube and replace the cap and place the tube in the test tube rack.
8. The inoculum was put in the MR-VP broth tube under aseptic results.
9. The tube was incubated for 24 to 48 hours at 37°C.
10. After 24-48 hrs. The broths were removed from the incubator.
11. The cotton plugs from the tubes were removed and 10 drops of Barritt's A reagent and 10 drops of Barritt's B reagent were added to broth.
12. The tubes were shaken gently for several minutes.
13. The colour formation was observed (MacFaddin, 2000).
3.v.V. METHYL RED TEST

INTRODUCTION

Methyl Red (MR) test determines production of mixed acids in presence and utilization of glucose. Types and quantities of end products of fermentation that are produced by anaerobic fermentation of glucose is one of the key help lines that which help to differentiate various bacteria of Enterobacteriaceae. The reaction was developed by German bacteriologists Daniel Wilhelm Otto Vogues and Bernhard Proskauer in 1898 at the Institute for Infectious Diseases (MacFaddin, 2000).

PRINCIPLE

When mixed acid fermentation occurs in MR VP tests, one of the two broad patterns, 2-3-butanol fermentation occurs. In mixed acid fermentation, basically, three acids (acetic acid, lactic acid and succinic acid) are formed. The mixed acid pathways give 4 mol of acidic products in different proportions (mainly lactic and acetic acid). By these acids produced, the pH levels fall below 4.4, which are visualised by pH indicator methyl red (p-dimethyl amino benzene-O-carboxylic acid) that gives yellow colour above pH 5.1 and red colour at pH 4.4 (MacFaddin, 2000).

REQUIREMENTS

- Culture: 24-48 hour tryptic soy broth culture was preferred to perform the test.
- Media: MR-VP medium
- Bunsen burner
- Inoculating loop.

PROCEDURE

As same broth is used for both of the tests, the same test tube broth was used and the tests MR VP were done simultaneously.
1. The tube for MR VP were inoculated with test bacteria under sterile conditions.
2. The tubes were incubated at 35° C for 4 days.
3. Five drops of the methyl red indicator was added to the tube.
4. The colour change was observed.
3.v.VI. CITRATE TEST

INTRODUCTION
This test was used to detect the ability of an organism to use citrate as the only source energy in from of carbon (Forbes et al. 10 edi.).

PRINCIPLE
As other biochemical test this test is also based on the colour change after various reactions. When the Bacteria are inoculated on a sodium citrate medium containing a pH indicator such as bromothymol blue, this citrate is used. In presence of inorganic ammonium salts, the reaction is executed, which are used by bacteria as sole source of nitrogen. The enzyme citritase, if present in bacteria, breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down by series of reactions to pyruvate and carbon dioxide (CO₂). As end products of the reactions, sodium bicarbonate (NaHCO₃) and ammonia (NH₃) are formed, which result in turning the environment alkaline by alkaline pH. In alkaline pH colour of the medium is changed from green to blue.

REQUIREMENTS
• Culture: 24-48 hour tryptic soy broth culture was preferred to perform the test.
• Media: Simmons’s citrate medium
• Bunsen burner
• Inoculating loop

PROCEDURE
1. The citrate medium was taken.
2. The growth of bacteria was taken with sterile straight wire and was inoculated on the Simmons citrate agar (citrate medium).
3. It was incubated overnight at desired temperature.
4. Colour change was observed. (MacFaddin, 2000)

3.v.VII. OXIDASE TEST
INTRODUCTION

The enzyme oxidase that if present in bacteria is detected by this test (Isenberg, 2004).

PRINCIPLE

A type of hemoprotein enzyme known as indophenol oxidase that is a protein, which contains iron or cytochrome oxidase are the two enzymes that are present in few bacteria. They help in Electron Transportation Chain or ETC and assist the reaction by moving the electrons from donor compounds (NADH) to electron receptors (O₂). Generally, aerobic bacteria possess this enzyme. With help of this enzyme bacteria can use oxygen for energy purposes. H₂O or H₂O₂ are produced as end products of the reaction. These end products are detected by addition of the test reagent, TMPD. The role of Dihydrochloride in TMPD is to act as electron donor in thereaction artificially. The bacterial enzyme Oxidase catalyses this reaction and the resultant is fromed. If present the enzyme Oxidase, the results are seen as purple colour. Some bacteria produce cytochrome c oxidases for their metabolic activities. Reagents like N, N, N’, N’-tetramethyl-p-phenylenediamine (TMPD) or N,N-dimethyl-p-phenylenediamine (DMPD) the redox indicators are used to check their activity. This enzyme is nly found in aerobic bacteria.

OX+ result means the bacteria contains cytochrome c oxidase, which can have an electron transfer chain to use oxygen for energy production.

OX- result indicates that the bacteria don’t possess cytochrome c oxidase so oxygen is not used for electron transfer so that energy is produced (plate 5) (Isenberg, 2004).

REQUIREMENTS

• Culture: 24-48 hour on nutrient agar was preferred to perform the test.

• Oxidase discs

• Bunsen burner

• Inoculating loop

• Cover slips

PROCEDURE

1. The Himedia disc of oxidase was taken on a clean glass slide.
2. Two-four colonies were taken with the help of sterile nichrome wire loop or with the edge of cover slip and streaked on the disc.

3. The results were observed within $n$ 30 seconds (Isenberg, 2004).

Plate 5. Oxidase test disc

3.v.VIII. TSI

INTRODUCTION

Triple sugar iron agar is a biochemical test that is used to differentiate the bacteria depending upon the utilization of sugar present in test media (plate 6) (Prober, 1997).

COMPOSITION

Lactose 10 parts
Sucrose 10 parts
Glucose 1 part
Ferrous sulphate
Peptone
Agar
Distilled water

MEDIA PREPARATION

1. Measured amounts of powders were added to a glass flask.

2. Those were mixed with the help of distilled water.
3. The water levels we made as per required.
4. The media flask was heated to mix the contents thoroughly.
5. The media was dispensed in pre sterile test tubes.
6. These tubes were plugged tightly with cotton and were set in racks to put in autoclave for sterilization.
7. These tubes were autoclaved at 121°C for 15 minutes under 15 lbs pressure.
8. After autoclaving, the tubes were lied down in slanting position to solidify, so that the slant of the media can be prepared.
9. These slants were stored in refrigerator.

**PRINCIPLE**

Three different sugars in the media are used by different bacteria, which are indicated by the colour change of the indicator phenol red. Acidification after sugar utilization occurs in the media by which, the pH of the media drops down, and the colour of the indicator is changed to yellow.

1. In case of iron consumption, the ferrous sulphate is utilized and H2S is formed which is indicated by blackening of the media. Different colors on slants or butt signify different sugars consumptions.

**PROCEDURE FOR TRIPLE SUGAR IRON AGAR (TSI) TEST**

1. The top of a well-isolated colony was touched with the help of sterilized straight inoculation needle.
2. Then the colony was stabbed into the TSI tube by this is done by a direct stab in the center of the tube, which is then streaked towards outwards on the surface of media.
3. Incubation of the test tubes were given at 37°C overnight.

**INTERPRETATION OF TRIPLE SUGAR IRON AGAR TEST**

1. The consumption of sugar like lactose or sucrose is indicated by red butt and yellow slant due to a large amount of acid was produced.
2. Some organisms generated gases that produced bubbles or pockets formation of bacteria.
3. In situation like when consumption of lactose was not observed only glucose was consumed in small amounts, the colour of the butt was seen as yellow due to non-availability of oxygen. The slant in this case was seen red or pink in colour.

4. In case of neither lactose/sucrose nor glucose was fermented, both the butt and the slant was seen as red.

5. In case when hydrogen sulphide was produced, black colour of ferrous sulphide was observed in the media.

Plate 6. Triple sugar iron agar

3.v.IX. MOTILITY TESTING

Motility testing is done for detection of mobility of any bacteria. This was done by following methods (Van and Peter, 2011):

1. Direct microscopic wet mount
2. Hanging drop method
3. Soft agar stabbing or tube method.

WET MOUNT SLIDE METHOD
The procedure was followed as mentioned earlier.

**Focussing technique:**
Unstained bacteria are transparent and difficult to see. Therefore, while focusing, the lighting was reduced sufficiently to make the organisms visible.

For proof of true motility, look for directional movement that is several times the long dimension of the bacterium. The movement will also occur in different directions in the same field.

Brownian movement was ignored by ruling out the vibrational movement caused by invisible molecules that bombard bacterial cells. This was differentiated by observing the directional movement of bacteria.

1. If only a few cells were seen exhibiting motility, characteristically, it was considered that the bacteria were motile.
2. Wet mount was always examined immediately to avoid water movement due to temperature change and capillary action of the fluid (Heintzmann and Rainer, 1999).
3. This helps in determining cellular shape and arrangement.
4. This method is very less time consuming cost effective
5. The wet mount slide dries out quickly, which renders the organisms immotile.
6. Handling of pathogenic organism on slide raise the chances to spread infections to the handlers.
7. There is the possibility of danger to the person in handling viable organisms on a slide.
8. The slide of Wet mounts cannot be stored.

**HANGING DROP SLIDE (Heintzmann and Rainer, 1999)**

**REQUIREMENT**

- Cavity slides and cover slips
- Petroleum jelly / Vaseline
- Inoculating loop
- Toothpick
- Cultures to be tested.

**PROCEDURE**
1. With the help of a toothpick, Vaseline or Petroleum jelly was spread on the four corners of a clean cover slip.

2. With the help of the inoculating wire loop, the thoroughly mixed culture was taken and a loopful was put on the centre of the cover slip.

3. The cavity slide was lowered with the concave side facing down, onto the coverslip. This made the drop protruding into the centre of the concavity of the slide.

4. The slide was pressed firmly to attach to cover slip.

5. This was turned up and placed on the stage of the microscope. The drop was examined by first locating its edge under 10X and focusing on the drop. The objective was then switched to the high-dry objective (40 X).

6. The diaphragm was closed to increase the contrast and to see the unstained bacteria clearly.

7. Again Differentiation between actual motility and Brownian movement was made.

8. After observing the results the slide was discarded. (Heintzmann and Rainer., 1999)

9. A hanging drop preparation was always examined immediately as the organisms become less motile with time.

10. Particular care was taken to avoid breaking the cover slip as it was more vulnerable because it was supported only around its edges.

11. It was always made sure that the specimen was on the top side of the slide (Heintzmann and Rainer., 1999).

12. The wet mount slide dries out quickly, which renders the organisms immotile.

13. Handling of pathogenic organism on slide raise the chances to spread infections to the handlers.

14. There is the possibility of risk to the person in handling viable organisms on a slide.

15. The slide of Wet mounts cannot be stored (Heintzmann and Rainer, 1999).

16. Method is less time consuming cost effective than soft agar method (Heintzmann and Rainer, 1999).

1. **SOFT AGAR STABBING (TUBE METHOD)** (FDA, 1998)(plate 7)

**REQUIREMENT:**

Two test tubes with motility medium
Inoculating needle

Required test Cultures

**PROCEDURE:**

The tubes of semisolid motility media were labelled properly.

1. The sterile inoculating straight wire was taken and was inserted into the culture to pick the isolated colonies (plate 6).
2. Simultaneously the neck of the soft agar media was flamed to make it sterile and then it was stabbed $2/3^{rd}$ of the way down to the bottom. The neck of the tube again was flamed before recapping the tube.
3. The tubes were incubated at optimum temperature overnight.
4. The motility cultures tubes were Examined for the motile lines or fanning around the stab line (FDA, 1998).

**ADVANTAGES**

It is an accurate method with precise results.

No need of microscope and with naked eye the results can be observed (FDA, 1998)

Plate 7. Motility test medium (soft agar stabbing)
3.v. X. UREASE TEST

INTRODUCTION
This is the biochemical test that is used to identify the urease producing bacteria that have ability of lysed urea in the media (Zimmer, 2000).

REQUIREMENTS
Urease test medium
Test organism
Inoculating wires
Bunsen burner

PRINCIPLE
There are many bacteria that have urease enzyme, which has the ability to split urea in the presence of water to form ammonia and carbon dioxide. The ammonia further combines with carbon dioxide and H2O to form (NH4)2 CO3 that turns the hydrogen ion concentration of medium alkaline that turns the colour of phenol red indicator from orange yellow colour to bright pink (plate 8) (Zimmer, 2000).

PROCEDURE
1. The urease agar slants were streaked with sterile straight wire having isolated colony of test organism.
2. The tube was incubated overnight at 37°C.
3. The results were observed (Zimmer, 2000).

USES
For identification of many genera and species of Enterobacteriaceae including Proteus, Klebsiella and Yersinia species (Zimmer, 2000).
GENERAL PRECAUTIONS WHILE PERFORMING THESE TESTS (Richmond and McKinney, 1999)

1. Gloves mask and lab coat were worn always.
2. The hairs were tied properly to prevent any contamination from the culture. When you enter the lab switch on the exhaust fans.
3. Oculars and objective lenses were cleaned with lens paper before and after each use. It was made sure that the microscope was working properly.
4. The illumination was adjusted according to the preparation before using the microscope.
5. The working area was cleaned with disinfectant before starting work to prevent the contamination.
6. The flame of the Bunsen burner was set properly to blue coloured flame.
7. Labelling at every step was done properly.
8. The inoculating wire loop was flamed to red hot.
9. After sterilising the loop it was made sure that the loop doesn’t lie down or doesn’t touch anything. If it touched the surface or anything the loop was made sterile again by heating.
10. It was made sure that the loop was cooled before touching the colony or inoculum to avoid killing of bacteria.

11. When removing the caps from tubes, the caps of the tubes were always kept in the hands; they were never set on the table to avoid contaminations.

3.v.XI. **ANTIMICROBIAL SUSCEPTIBILITY TEST BY KIRBY-BAUER METHOD:**

Once the type’s bacteria were confirmed by various biochemical tests they were taken to the next step of testing antimicrobial sensitivity.

1. Isolated colony taken from media was transferred to a test tube containing 1.5 ml sterile saline.
2. The density of the suspension is visually equivalent to the barium sulphate standard, 0.5 McFarland units.
3. Before use, the standard should be shaken vigorously.
4. A cotton swab is dispensed into the suspension and taken out by squeezing the extra broth on the side of the wall by rotating the swab stick.
5. The swab stick containing the bacterial inoculum was spreaded on the plate by lawn and carpet method.
6. After the inoculum has dried, discs are applied with the forceps, a dispenser and pressed gently to make a good contact with the plate containing medium to avoiding losing the grip from medium.
7. Note: Only six discs can be accommodated on a single circular plate.
8. The antibiotics pane tested for each organism are shown in Table I,II,III
9. The organisms were defined as sensitive, intermediately sensitive and resistant by breakpoints defined by CLSI. The summary of break point for E.coli, Klebsiella spp., Acinetobacter, Staphylococcus and Enterococci is given in table.
10. QC strains used were E.coli (ATCC 35218), Staph aureus (ATCC 29213), Enterococcus fecalis (ATCC 33186) and Pseudomonas aeruginosa (ATCC 27853)

<table>
<thead>
<tr>
<th>Organism →</th>
<th>Staphylococcus aureus</th>
<th>Enterococcus spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotics ↓</td>
<td>S I R</td>
<td>S I R</td>
</tr>
<tr>
<td>Antibiotic name</td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>-----</td>
<td>-------</td>
</tr>
<tr>
<td>Penicillin (10 µg)</td>
<td>≥29</td>
<td>-</td>
</tr>
<tr>
<td>Oxacillin (1 µg)</td>
<td>≥13</td>
<td>11-12</td>
</tr>
<tr>
<td>Gentamycin (10 µg/120 µg)</td>
<td>≥15</td>
<td>13-14</td>
</tr>
<tr>
<td>Teicoplanin (30 µg)</td>
<td>≥21</td>
<td>16-20</td>
</tr>
<tr>
<td>Cotrimoxazole (1.25 µg/23.75 µg)</td>
<td>≥18</td>
<td>13-17</td>
</tr>
<tr>
<td>Erythromycin (15 µg)</td>
<td>≥23</td>
<td>14-22</td>
</tr>
<tr>
<td>Clindamycin (2 µg)</td>
<td>≥21</td>
<td>15-20</td>
</tr>
<tr>
<td>Linezolid (30 µg)</td>
<td>≥21</td>
<td>20</td>
</tr>
<tr>
<td>Vancomycin (30 µg)</td>
<td>≥17</td>
<td>15-16</td>
</tr>
<tr>
<td>Tetracycline (30 µg)</td>
<td>≥19</td>
<td>15-18</td>
</tr>
<tr>
<td>Cefoxitin (30 µg)</td>
<td>≥22</td>
<td>21</td>
</tr>
<tr>
<td>Amoxicillin-Clavulinc Acid 20 µg /10 µg</td>
<td>≥18</td>
<td>14-17</td>
</tr>
</tbody>
</table>

Table 1. Breakpoints of Gram Positive Cocci
<table>
<thead>
<tr>
<th>Drug</th>
<th>Symbol</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
<th>Upper Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amikacin (30 µg)</td>
<td>≥17</td>
<td>15-16</td>
<td>≤14</td>
<td></td>
</tr>
<tr>
<td>Netilmicin (30 µg)</td>
<td>≥17</td>
<td>15-16</td>
<td>≤14</td>
<td></td>
</tr>
<tr>
<td>Tobramycin (10 µg)</td>
<td>15</td>
<td>13-14</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone (30 µg)</td>
<td>23</td>
<td>20-22</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime (30 µg)</td>
<td>≥23</td>
<td>15-22</td>
<td>≤14</td>
<td></td>
</tr>
<tr>
<td>Ceftazidime (30 µg)</td>
<td>≥18</td>
<td>15-17</td>
<td>≤14</td>
<td></td>
</tr>
<tr>
<td>Gatifloxacin (5 µg)</td>
<td>≥18</td>
<td>15-17</td>
<td>≤14</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin (5 µg)</td>
<td>≥21</td>
<td>16-20</td>
<td>≤15</td>
<td></td>
</tr>
<tr>
<td>Ampicillin (10 µg)</td>
<td>≥21</td>
<td>15-20</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Imipenem (10 µg)</td>
<td>≥16</td>
<td>14-15</td>
<td>≤13</td>
<td></td>
</tr>
<tr>
<td>Cotrimoxazole (1.25µg /23.75 µg)</td>
<td>≥18</td>
<td>13-17</td>
<td>≤12</td>
<td></td>
</tr>
<tr>
<td>Colistin (10 µg)</td>
<td>11</td>
<td></td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Polimixin B (300 unit)</td>
<td>12</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Aztreonam (30 µg)</td>
<td>22</td>
<td>16-21</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Piperacillin Tazobactem (100 µg /10 µg)</td>
<td>≥21</td>
<td>18-20</td>
<td>≤17</td>
<td></td>
</tr>
<tr>
<td>Cefaparazone Sulbactem (75 µg /10 µg)</td>
<td>23</td>
<td>20-22</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

**Table II. Breakpoints of gram negative bacilli**

**DATA ANALYSIS**

Different statistical test like t and z test was used to analyse the data.