3.1 Specimens for Isolation of bacterium

Stool, Urine, PUS, CSF Throat swab and sputum were collected from patient both out patient and impatient attending the general Hospital. Exhibiting the clinical symptoms of infection at, Government General Hospital.

Isolation of Bacteria from clinical specimens

Urine were cultured on McConkey Agar and blood Agar incubated at $37^\circ$C aerobically for overnight.

Stool sample were cultured on McConkey Agar and blood Agar incubated at $37^\circ$C aerobically for overnight.

PUS Were cultured on McConkey Agar blood Agar and Chocolate Agar incubated at $37^\circ$C aerobically for overnight.

Cerebro spinal fluid (CSF) cultured on McConkey Agar blood Agar and Chocolate Agar incubated at $37^\circ$C aerobically for overnight.

Throat Swab cultured on McConkey Agar blood Agar and Chocolate Agar incubated at $37^\circ$C aerobically for overnight.
Sputum sample cultured on McConkey Agar, blood Agar and Chocolate Agar incubated at 37°C aerobically for overnight.

Organisms were included in our study when they occurred as a pure culture and at a concentration of ≥ 10⁴ CFU/ml.

**Antimicrobial Susceptibility Testing**

Bacteria were tested for susceptibility to various Antibiotics using the disc diffusion method according to guideline set by the National Committee for Clinical Laboratory Standards (NCCLS).

**Briefly**

Organisms were grown overnight at 37°C in 3 ml Mueller Hinton (MH) broth.

Ten microliters of the overnight culture were used to inoculate a fresh 3 ml (MH) broth followed by incubations at 37°C with shaking until a 0.5 McFarland turbidity standard was obtained. A Sterile Swab was dipped into this culture and used to inoculate the surface of a fresh MH agar plate.

Antibiotic disc impregnated with Ampicillin (10 µg), chloramphenicol (30 µg), Streptomycin (300 µg), Tetracycline (30 µg) and Trimethoprim / sulfamethoxazole (1.25 µg / 23.75 µg) were placed on the surface of the inoculated agar plate. After incubation at 37°C for 24 hrs., Zones of Inhibitions around each antibiotic disc were measured. Using NCCLS guidelines, each organism was classified as either resistant or Susceptible to the antibiotics. Specifically, Organisms were considered resistant if the diameter of the Zone of Inhibitions was equal to or less than 13 mm for ampicillin, 12 mm for
chloramphenicol, 6 mm for streptomycin 14 mm for tetracycline, and 10 mm for Trimetha Prim / Sulfamethaxazole. Antibiotics disks were Purchased from (High Media India).

3.2 Collection, Transport and Examinations of Specimens

1. Specimen Urine:

**Collection and Transport of Urine**

Mid Stream Urine (MSU) for microbiological examination is collected as follows.

- Give the patient a sterile, dry, wide necked leak proof container, and explain the importance of collecting a specimen with as little contaminations as possible (Clean – catch specimen)
- Female patients should be instructed to clean the area around the urethral opening with clean water, dry the area, and collect the urine with the labia held apart.
- About 20 ml of urine should be collected if the patient is in renal failure or a young child. It may met be possible to obtain more than a few milliliters.
- Label the container with the date, the name and number of the patient, and the time of collection as soon as possible, deliver the specimen with a regent form to the Laboratory.
If immediate delivery to the laboratory is not possible, the urine should be refrigerated at 4°C if a delay in delivery of more than 1 hour is anticipated, boric acid should be added to the urine.

Specimens containing boric acid need not be refrigerated.

**Boric acid Preservations**

At a concentration of 10 g/l (1% W/V), bacteria remain viable without multiplying, white cells, red cells, and casts are also well preserved and there is no interference in the measurement of urine protein and glucose.

**Precaution**

Urine for culture must not be preserved with a bactericidal chemical such as thymol, bleach, hydrochloric acid, Acetic acid, or chloroform.

**Culture the Specimen**

It is necessary to culture urine which is microscopically and biochemically normal, except when screening for asymptomatic bacteruria in pregnancy. Culture is required if the urine contains bacteria, cells, casts, proteins, nitrite, or has a markedly alkaline or acid reaction.

**Estimating bacterial numbers**

It is necessary to estimate the approximate number of bacteria in urine. Normal specimens may contain a small number of contaminating organisms. Usually less than 10,000 (10⁴) per ml of urine. Urine from a person with an untreated urinary infection usually 1,000,000 (10⁵) or more bacteria per ml. The approximate number of bacteria per ml of urine can be estimated by using a calibrated loop or a measured piece of filter paper. Both methods are based on...
accepting that a single colony represent one organism. Ex. If an inoculum of 1/500 ml produce 20 colonies, the number of organism represented in 1/500 ml of urine in 20, or 10,000 in 1 ml (500x20).

The calibrated loop method in recommended for laboratories becomes it is inexpensive, simple to perform, and provides individuals calories that are easier to identify and remove for antimicrobial sensitive by testing.

**Routine Culture**

**Blood Agar and McConkey agar**

Mix the urine well by inverting the continue several times. Using a sterile calibrated wire loop using a loopful of urine an blood Agar and Maccaskey agar (a third or Quarter of each plate is adequate) the loop must be held vertical and only the loop must be dipped in the urine.

If the stem is also immersed, more than the required volume of urine will be inoculated the use of blood agar is recommended in addition to Mcconkey agar because it assist in the rapid Identification of pathogens, and enables haemolytic organism and other Gram positive organisms to be isolated which gram poorly or not at all on McConkey agar Incubate the Inoculated plates aerobically at 35° – 37° C. Overnight.
3.3 Collections, Transport, and Examination of PUS from wounds, abscesses, and burns

Collection of PUS from Wounds abscesses, and burns

Specimens of PUS should be collected by a Medical Officer as an experienced PUS from an abscess is best collected at the time the abscess is incised and drained, or after it has ruptured naturally. When collecting PUS from abscess, wounds on other sites, special care should be taken to avoid contaminating the specimen with commensals organisms from the skin. As far as possible a specimen from a wound should be collected before an antiseptic dressing is applied.

1. Using a sterile technique, aspirate and collect from a drainage tube up to 5 ml of PUS. Transfer to a leak-proof sterile container.

2. If PUS is not being discharged, use a sterile cotton wool swab to collect a sample from the infected site. Immerse the swab in a container.

3. Label the specimen and as soon as possible deliver it with a request form to the laboratory.

Culture the specimens

Routine

Blood agar and McConkey agar

Inoculate the specimen on blood agar plate and McConkey agar plate. Incubate the blood agar and McConkey agar plate aerobically at 35-37°C overnight.
Summary of the Laboratory Examination of Pus Wounds, and Abscesses and Burns

Routine Investigations

Report

Colour of pus
Whether it contains granules

Inoculate:

Blood agar (two plates)
Incubate aerobically and anaerobically

Neomycin blood agar Incubate anaerobically up to 48 h

MacConkey agar
Incubate aerobically

Cooked meat medium
Subculture at 24 h, 48 h, and 72 h as indicated

Examine

Gram smear
Look for pus cells and bacteria

Additional Investigations

Day 1

1. Describe the Appearance of Specimen

2. Culture the Specimen

Inoculate

L J slope,
Infection is suspected

3. Examine Specimen Microscopically

Examine

Ziehl Neelsen
Tuberculosis is suspected

KOH
Day 2 and Onwards

Examine the blood agar, neomycin blood agar, and MacConkey agar cultures especially for:

- *Staphylococcus aureus*
- *Streptococcus pyogenes* (Group A)
- *Clostridium perfringens*
- *Pseudomonas aeruginosa*
- *Proteus species*
- *Escherichia coli*
- *Enterococci*
- *Bacteroides species*
- *Anaerobic cocci*
3.4 Collection Transport and Examination of throat and Mouth Specimens

Collection & Transport of Throat and Mouth Specimens

1. Whenever possible throat and mouth swabs should be collected by a medical officer or an experienced nurse.

2. In a good light and using the handle of a spoon to depress the tongue, examine the inside of the mouth.

3. Look for inflamations, and the presence of any membrane, exudates or PUS.

4. Swab the affected area using a sterile cotton or Alginate wool swab taking care not to contaminate the swab with saliva, return it to its sterile Container.

Important

For 8 hours before swabbing the patient must not be treated with antibiotics or Antiseptic mouth washer (gargles).

Cautioun

It can be dangerous to swab the throat of a child with acute hemophilus epiglottitis. Because this may cause a spasm that can obstruct the child’s airway, blood for culture should be collected instead.

5. Within two hours of collection, deliver the swab with a request form to the laboratory.
Culture the Specimens

Routine

Nutrient agar, Blood agar & Chocolate Agar

Inoculate the swab on a plate of (NA, B.A. & C.A) If the swab is received in silica gel mol stem it first with sterile nutrient broth and then inoculate the plate.

Note: (Human blood must not be used in preparing plates for the Isolation of Streptococcus pyogens or other beta-hemolytic streptococcus. Sheep, goat, or horse blood should be used). Add a bacitracin disc to the plate, this will help in the identification of streptococcus pyogens Incubate the plate preferably in aerobically or in a carbon dioxide enriched atmosphere overnight at 35-37°C.

Examine the Specimen Microscopically

Routine

Grams smear: Make an evenly spared of the specimen an a slide allow the smear to air dry in a safe plate. Fix the smear an stain by the grams technique.
Summary of the Laboratory Examination of Throat and Mouth Swab

Routine Investigations

Inoculate:

**Blood agar**
- Add a bacitracin disc
- Inoculate preferably

Examine:

**Gram smear**
- Look for pus cells and Vincent's organisms (Spirochetes and fusiform rods)
- Look for yeast-like cells if thrush is suspected
- Look for pleomorphic rods if diphtheria is suspected

Additional Investigations

Day 1

1 Culture the Specimen

Inoculate:

**MTM and TBA**, if diphtheria is suspected

**Sabourand agar**, if thrush is suspected

Examine:

**Albert stained smear**, if diphtheria is suspected

Day 2 and Onwards

3 Examine and Report Cultures

Examine the MTM and TBA cultures for

*Corynebacterium diphtheriae*

Examine the Sabouraud culture for

*Candida albicans*
3.5 Collection, Transport and Examination of Sputum

Collection & Transport of Sputum

Sputum for Microbiological investigation is collected and transported as follows.

1. Give the patient a clean, dry, wide necked leak proof container, and request him or her to cough deeply to produce a sputum specimen.

Caution:

(When a sputum specimen is being collected adequate safety precautions should be taken to prevent the spread of infectious organisms)

Important

The specimen must be sputum, not saliva, sputum is best collected in the morning soon after the patient wakes and before any mouth wash in used. If the patient is a young child and it is not possible to obtain sputum, gastric washing can be used for the Isolation of Mycobacterium tuberculosis other respiratory Pathogens.

2. Label the container and fill in a request form

3. If Pneumonic or bronchopuenamia is suspected deliver the sputum to the laboratory with delay as possible because organism such as S.pumnoniae and H. Influenzae required culturing as soon as possible.

Note: Specimen for the isolation of S.pumnoniae and H.Influenzae must never be refrigerated. If Pneumonic plague is suspected deliver the sputum to the laboratory as soon as possible. Make sure the specimen in marked “HIGH RISK”.
Examine the Specimen Microscopically

Routines:

Gram smear: Using a piece of stick, transfer a purulent part of the sputum to a glass slide, and make a thin smear allow the smear to air dry in a safe place. Fix and stain by Grams technique.

Examine the smear for PUS cells and bacteria

Culture the Specimens

Routine

Blood agar & chocolate agar

Wash a purulent part of the sputum in about 5 ml of sterile physiological saline. Inoculate the washed sputum on plates of blood agar and chocolate (heated blood) agar. Add an optochin disc to the chocolate agar plate; this will help to identify S. Pneumoniae. Incubate the blood agar plate aerobically and the chocolate agar plate in a carbon dioxide enriched atmosphere at $35^\circ - 37^\circ$ for up to 48 hours examining for growth after overnight incubation.
Summary of the Laboratory Examination of Faecal Specimens

Day 1

Routine Investigations

Additional Investigations

1. Describe the Appearance of Specimen

2. Examine the Specimen Microscopically

Report

Colour
Whether formed, semi formed, unformed, or fluid

Contents, i.e. blood, mucus, pus, worms, tapeworm segments

Examine:
Saline and eosin preparations look for

Examine
Methylene blue preparation if specimen is unformed
Look for faecal leucocytes

Basic fuchsin smear for campylobacters, if specimen is unformed and, or, contains blood, pus, or mucus

Alkaline peptone water preparation for vibrios, if cholera is suspected

Inoculate:
Campylobacter selective medium.
If the patient is under 2 years or Campylobacter enteritis suspected Incubate in CO₂

Alkaline peptone water for subculture to TCBS, if cholera is suspected

MacConkey or SS agar (with additional bile salt), if Yersinia enterocolitica infection is suspected. Incubate at 20-28°C
Day 2 and Onwards

Examine XLD agar culture for:
- *Salmonella species*
- *Shigella species*

Differentiate using MIU and KIA media or the APTZ test identify serologically

Examine the C

Campylobacter culture for:
- *Campylobacter species*

Examine the TCBS culture for:
- *Vibrio cholerae*
- *Vibrio parahaemolyticus*

Occasionally other pathogenic Vibrio species may be isolated

Examine the MacConkey or SS agar (room temperature) culture for:
- *Yersinia enterocolitica*
3.6 Collection, Transport and Examination of faeces (Stool) Specimens

Collection & Transport of Faeces

Faeces for Microbiological examination should be collected during the acute stage of diarrhea

1. Give the patient a clean, dry disinfectant free bedpan or suitable wide necked container in which to pass a specimen. Ask the patient to avoid contaminating the faeces with urine.

2. Transfer a portion (about a spoonful) of the specimen specially that which contain mucus, PUS, or blood into a clean, dry, leak proof container.

Rectal Swabs: If it is not possible to obtain faeces, collect a specimen by inserting a cotton wool swab into the rectum for about 10 seconds, care should be taken to avoid unnecessary contamination of the specimen with bacteria from the anal skin.

3. Label the specimen and send it with a request form to such the laboratory within 2 hours.

Examine the Specimen Microscopically

Routine:

Saline and eosine Preparation for Microscopically

Place a drop of freshly physiological saline on one end of a slide and a drop of eosin stain on the other. Using a piece of stick or wireloop, mix a small a mount of specimen (especially mucus and blood) with each drop cover each preparation
with a cover glass. Examine the preparation using the 10x and 40 x objectives with the condenser Iris closed sufficiently to give good contrast

**Culture the Specimen**

If the specimen is formed or semi formed, make a thick suspensions of it in about 1 ml of sterile peptone water.

**McConkey agar and Salmonella, and Shigella agar** (S S agar)

Inoculate a loopful of specimen an McConkey agar or modified Salmonella, and Shigella agar (S.S) which has been added an auditioned amount of sodium dioxycholate (0.2 g/10 ml) of medium Inoculate acerbically at room tem(20-28° 0) for up to 48 hours examining for growth after overnight incubation.
Summary of the Laboratory Examination Sputum

Routine Investigations

Report whether:
- Purulent, mucopurulent
- Mucoid or mucosal
- Contains blood

Examine:
- Gram stain
  Look for pus cells and bacteria
- Z Neelsen or Zinorochrome

Inoculate:
- Blood agar
  Incubate aerobically
- Chocolate agar
  And an optchin dish
  incubate in CO₂

Additional Investigations

Day 1

1 Describe the Appearance of Specimen

2 Examine Specimen Microscopically

Examine:
- Saline preparation, if Paragonimiasis suspected
- Eosin preparation, if asthma or other organic condition suspected
- KOH preparation, if a fungal infection suspected
- Gram stain, if Histoplasmosis or pneumonic plague is suspected

3 Culture the Specimen

Inoculate:
- LJ slope, if tuberculosis suspected. Decontaminate the specimen first with sodium hydroxide.
Day 2 and Onwards

Examine the blood agar and chocolate agar cultures for:

- *Streptococcus pneumoniae*
- *Staphylococcus aureus*
- *Haemophilus in
- *Klebsiella pneumoniae*
- *Pseudomonas aeruginosa*

4. Examine and Report Cultures

Examine the LJ culture for *Mycobacterium tuberculosis*
3.7 Collection, Transport and Examination of Cerebrospinal Fluid (CSF)

Collection of (CSF)

CSF must be collected by experienced medical officers. Health workers must collect CSF as aseptically as possible to prevent organisms from being introduced into the central nervous system. The fluid is usually collected from the arachnoid space using a sterile wide-bore needle inserted between the fourth and fifth lumbar vertebrae, allowing the CSF to drip into a dry sterile container. A vertical puncture is sometimes performed to collect CSF from infants. Laboratory staff should be advised before a lumbar puncture is performed so they can be prepared to receive and examine the specimen immediately. A delay in examining CSF reduces the chances of isolating a Pathogen.

- Take two sterile, dry screw-cap containers and label one No. 1 (used for culture) and the other No. 2 (used for other investigations).
- Collect about 1 ml of CSF in container No. 1 and about 2-3 ml in container No. 2.
- Deliver immediately the sample with a request to the laboratory.

Microscopic Examinations

Routine

Gram strain: CSF must be examined without delay, and the result of the examination reported to the medical officer as soon as they become available, especially the gram
smear report. The fluid should be handled with special care because it is collected by lumbar Puncture and only a small amount can be withdrawn.

**Culture the Specimen**

Culture of the CSF is necessary if the fluid contain cells and or the protein concentration is abnormal

**Important**

CSF should be cultured as soon as possible after collection if a delay is unavoidable the fluid should be kept at 35-37°C (never refrigerated) if the CSF appear only slightly cloudy, centrifuge it in a sterile tube for 15-20 mitrs and use the sediment for inoculating the plates.

**Routine**

**Chocolate (heated blood) agar**

Inoculate the specimen an chocolate agar and inoculate in a carbondioxide enriched atmosphere at 35-37°C for up to 48 hour checking for growth after overnight incubation.

**McConkey agar and blood agar**

Inoculate the specimen an McCoanly ager and blood agar Incubate soft plates of 35-37°C overnight.

**3.8 BIOCHEMICAL REACTIONS**

**3.8.1 IMVIC TESTS**

The IMVIC test consist of four deferent test (i) Indole production (ii) Methyl - red (iii) voger – Proskamer and (iv) citrate utilizations the name IMVIC stands for the first letter of the name of the test in the series
with the lower case I included for ease of pronunciation the IMVIC test were designated to differentiate gram negative bacilli (Family Enterobacteriaceae) on the basics of their biochemical properties and Enzymatic reaction in the presence of specific substrates.

**Indole Production Test**

Tryptone broth 1%, (Dissolve 10 g of peptone in 1 liter of distilled water) Sterilize in the autoclave at 15 psi (121%C) for 15 minutes. Inoculate one tryptone both with organism any another in uninoculated corporative control. Incubate inoculated and uninoculated tubes at 35°C for 48 hours after 48 hours of incubation add 1 ml of Kovac’s reagent (Para dimethyl amino benzaldehyd 2 g, Isoamyl alcohol 30 ml, hydro chloric acid concentrated 10 ml ) to each tube the including control. Shaken gently after intervals for 10 – 15 minutes, allow the tubes to stands permit the reagent to come to the top and Examine the tubes as to the color in the reagent “Layer”

**Positive:** Development of Cherry (deep) red colour in the top layers of the tube

**Negative:** test for Iodole production Absence of red coloration Iodole negative

<table>
<thead>
<tr>
<th>Tryptophanase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan → Indole + Pyruv acid + NH₃</td>
</tr>
<tr>
<td>HCL</td>
</tr>
<tr>
<td>Indole + Kovac’s reagent → Rosindole + H₂O</td>
</tr>
<tr>
<td>butanol(cherry red compound)</td>
</tr>
</tbody>
</table>

39
Methyl – red and Voges – Proskaur test (MRVP)

MRVP broth (Peptone 7.0 g, dextrose/glucose 5.0 g, Potassium phosphate 5.0 g D/W 1000 ml, PH. 6.9) pour the 5 ml of broth in each tube and sterilize by autoclaving at 15 lb pressure for 15 minutes. Inoculate HR VP tubes both organism and tube as an inoculated corporate control. Inoculate all five tubes at 35°C for 48 hours. 5 drop of methyl red to the one set of the MR tube and add 12 drops (VP-I & VP II) of VP reagent to other set of V-P tube as well as to an inoculated tubes shake the tubes gently for 30 seconds. Allow the reaction to complete for 15-30 minutes. Absence the tubes for change in color of MR-VT tubes.

In the MR Test the methyl red indicator in the PH range of 4 will remain red (thought tube) Which is indication of a positive test while turning of methyl red to yellow is a negative test. In the VP test the development of crimson – to ruby pink (red) colour, may be most intense of the surface, is indicative of positive VP test while no change in coloration is a negative test.

Citrate Utilization test

Simmon’s citrate agar (Ammonium dihydrogen sulphate 1.0 g, Dipotassium phosphate 1.0 g, NaCl 5. g sodium citrate 2.0 g. Magnesium sulphate 0.5 g, agar 15.0 g, Bromothymol blue 0.8 g, D/W 1.00 ml, PH. 6.9) pour the medium in the culture tube and sterilize by autoclaving at 15 lb pressure for 15 minute and prepare the slants. Inoculate one set Simmon’s citrate agar slat with organs by means of a stab and streak inoculators and one set as an
inoculated comparative control. Inoculate all the slants at 37°C for 48 hours. Observe the slant culture for the growth any coloration of the medium.

Positive: The medium colour turn blue (eg. Citrate positive)
Negative: which no change in the colour of medium

3.8.2 Catalase test

Trypticase soy agar (Trypticare 15.0 g, phyton 5.0g Sodium chloride 5.0 g, agar 15.0 g D/W 100 ml PH-7.3) pour the medium in culture tubes as flasks an sterilize by autoclaving at 15 lb pressure for 15 minutes.

Inoculate trypticare soy agar slant and keep an Inoculated typticare soy agar slant as control incubate the cultue at 35° C for 24-48 hours while holding the inoculated tubes at an angle allow 3-4 drops of hydrogen peroxide to flow over the growth of each slant culture observe the each culture slant for the appearance or absence of gas bubbles.

Positive: A catalase positive culture will produce bubbles of oxygen within 1 minute Negative: After addition of H₂O₂, negative cultures shows no bubbles seen

3.8.3 Coagulase Test

Tube Coagulase test (Gillespie 1943)

1. Prepare a 1 in 10 dilution of the plasma in saline (0.85% Nacl) solution and place 0.5 ml of the diluted plasma in a small tube.

2. Inoculate into the diluted plasma 0.1 ml of the 18-24 years broth culture (about10⁸ cocci ) of the strain under test.
Alternatively, to avoid a day’s delay while the broth culture is gram, emulsify a few colonies to give a dense suspension in a small volume of broth and add 0.1 ml of this suspension about \((10^9 \text{ cocci})\) to the diluted pleasure.

3. Setup control text with known coagulase positive and coagulase negative culture and include a tube of unseeded diluted plasma to confirm that it does not coagulate Spontaneously.

4. Inoculate the tubes at \(37^\circ\text{C}\), preferably is a water both an examination them for coagulation at 1,3 and 6 hours and again if still negative, after standing overnight at room temperature.

**Positive:** Read as positive a test in which the plasma has been converted into a stiff gel.

**Negative:** Read as negative a test in which the plasma remain wholly liquid and free flowing read as doubtful a test showing a large & small organized clot or several small un organized clots surrounded by clear liquid.

### 3.8.4 Oxidase Test

**Filter Paper Method**

Strips of Whatman’s No.1 filter paper are soaked in a freshly prepared 1% solution of tetramethyl – P – Phenylene – diamine dihydrochloride. After draining for about 30 seconds the strips are freeze dried and stored in a dark bottle. For use a strip is removed, laid in a petridish and moistend with D/W the colony to be tested is picked up with a Platinum loop and smeared over the moist area.
Positive: A positive reaction is indicated by an intense deep purple blue, appearing within 5 to 10 seconds, a delayed positive reaction in 10 to 60 seconds. 

Negative: A negative reaction is by absence of coloration.

3.8.5 Urease test

Christensen’s medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>K$_2$H$_2$PO$_4$</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Phenol red (1 in 500 aqueous solution)</td>
<td>6.0 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0 gm</td>
</tr>
<tr>
<td>D/W</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Glucose 10% solution</td>
<td>10 ml</td>
</tr>
<tr>
<td>Urease 20% solution Sterile</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Sterilize the glucose and urea solution by filtration. Prepare the basal medium. Without glucose or urea, adjust PH 6.8 – 6.9 and sterilize by autoclaving in a flask at 121°C for 30 minutes cool to about 50°C add the glucose and urea and tube the medium as deep slopes. Inoculate heavily over the entire slope surface and incubate at 37°C Examine after 4 hours and after overnight incubations.

Positive: The urease positive culture changes the colour of the indicator to purple – pink

Negative: No change of the indicator colour.
3.8.6 Preliminary Identification of Enteric pathogens using triple sugar Iron Agar (TSIA) medium

Triple sugar Iron agar (TSIA) medium is composed of three sugar; lactose, sucrose and very small amount of (1 percent) glucose, Iron (ferrous sulphate) and phenol red as Indicator. If an organism ferments any of the three sugar or any combinations of them the medium becomes yellow due to production of acid as end product of fermentation.

Inoculate the organism clinging to the tip of the Inoculating needle to a TSIA slant by first streaking the surface of the slant and then stabbing the medium in the butt regions inculcated the Inoculated TSIA tuber at 35°C for 24 hours observed the tubes showing is production of acid or acid and gar with or with are without hydrogen sulfide. The reaction observed on the TSIA slant are used for the differential of intestinal bacilli.
TSIA

Acid Slant
Acid butt

H₂S⁻
Escherichia
Klebsiella
Enterobacter

H₂S⁺
Citrobacter
Arizona
Some

Alkaline slant
Acid butt

H₂S⁻
Shigella
Some
Proteus.Spp

H₂S⁺
Most
Salmonella
Arizona
Citrobacter

Alkaline slant
Alkaline or no change in butt

H₂S⁻
Alkaligenes
Pseudomonas
Acinetobacter
3.9 MOLECULAR CHARACTERIZATION

3.9.1 ISOLATION OF PLASMID DNA

DNA was prepared following lysis by boiling method as described by Sambrook et al., 1989. 1.5 ml of cell culture was taken in microfuge tube and centrifuged at 12,000 rpm for 3 min at 4°C in microfuge. The supernatant was removed completely and the dry pellet was suspended in 350 μl of STET buffer (0.1M NaCl, 10mM Tris-HCl pH 8.0, 0.1 mM EDTA and 0.5% Triton X-100) To the suspended cells 25 μl of freshly prepared lysozyme solution (20 mg/ml in 10 mM Tis-HCl (pH-8.0) was added and mixed well by vortexing for 30 seconds. The tube was incubated in boiling water bath for exactly 40 seconds. The tube was centrifuged at 12,000 rpm for 10 minutes at room temperature and the pellet of cell debris obtained was removed with the sterile toothpick. To the supernatant, 40 μl of 2. M sodium acetate (pH 5.2) and 420 μl of isopropanol was added and mixed will by vortexing. The tubes were incubated at toom temperature for 5 minutes. After 5 minutes incubation, the tubes were centrifuged at 12,000 rpm for 10 minutes at room temperate. The supernatant was discarded and the pellet was dissolved in 200 μl of TE (pH 8.0). To this DNase free pancreatic RNase (20ug/ml) was added and incubated at 37°C for 40 minutes. After incubation the plasmid DNA was extracted with equal volumes of phenol: chloroform. The aqueous phase containing DNA was precipitated by adding equal volume of 7 M ammonium acetate and 2.5 volumes of ethanol and incubating at 4°C for 30 minutes. The tubes were centrifuged at 12,000 rpm for 20 minutes at 4°C. The plasmid DNA pellet obtained was washed with 70%
ethanol and air dried for 5-10 minutes at room temperature. The pellet was now dissolved in minimum of TE (pH 8.0) and used for further experiments.

3.9.1.1 KADO ANALYSIS

Plasmid was isolated following the methods described by Kado et al., 1981. 100 ml of overnight culture was centrifuged at 8000 PPM for 15 minutes to collect the cells. The collected cell were resuspended in 10 ml of E-buffer (40 mm Tris acetate, 2 mm EDTA; PH 7.9) to this 20 ml of bytic mixture (3% SDS, 50 mm Tris, PH 12.6) was added and mixed well by inverting the tube several times. The contents were incubated at 65° for 45 minutes and extracted with 60 ml of phenol: chloroform solution after cooling the contents to the room temperature. The upper layer was collected after centrifuging for 15 minutes at 12,000 ppm. Required amount of agarose was described in E-Buffer by heating on hot plate to get 0.8% gel. The selection was then cooled to 50-55° C and pooled on a clean sterile gel tray filled with combs. The gel was allowed to solidify at room temperature after solidification the gel along the gel tray was immersed in the E-buffer powered in the electrophoretic kit. The buffer level was adjusted to cover the gel to the depth of 1 mm. An aliquote of 20 ml of Supematant from upper layer of plasmid preparation was mixed with 2ml of 10 X loading buffer (0.25% bromophenol blue, 0.25% oxylene cyanol, 30% glycerol) and carefully loaded into the wells of submerged gel. Eluhophare was carried out at 100 volts till the bromo Phenol blue reaches the and of the gel. The gel was then stained in staining solution (5 μg/ml anthodium bromide in water) for 15 minutes and was put in distaining solution (distilled water) for 15 minutes then the gel was visualized under UV transilluminator and photographed.
3.9.2 PLASMID CURING

Luria Broth with different concentrations of mitomycin C ranging from 1 μg/ml to 8μg/ml was inoculated with an overnight culture and incubated on the shaker for 36 hrs at 30°C. The culture was then plated on the LB agar plates with serial dilutions to obtain single colonies. Single colonies developed on agar plates were tested for methyl parathion hydrolase activity. The colonies, which failed to exhibit enzyme activity were selected for further screening of the plasmid.

3.9.3 TRANSFORMATION

Preparation of competent cells

Competent cells were prepared following the procedure of Mandel and Higa, 1970 with slight modification. The *E.Coli* DH5α cells were grown in a LB broth of 250 ml at 37°C with initial inoculum of 1% till the cell density reached to 0.3-0.4 O.D at 600 nm. The culture was chilled on ice for 30 minutes and centrifuged at 5000 g for 5 minutes. The pellet was suspended will in 25 ml of ice cold 0.1 M CaCl₂ and incubated on ice for 30 minutes. After incubation the cells were sedimented at 5000 g for 5 minutes and gently resuspended in 2.5 ml of ice cold 0.1 M CaCl₂ containing 15% glycerol and stored in aliquots of 200 μl at – 70°C for one day for better transformation efficiency.

Transformation

The frozen competent cells were thawed over ice. The ligation mixture containing the DNA to be transformed was added and incubated on ice for 30 minutes. After 30 minutes, the cells were subjected to heat shock at 42°C for
exactly 90 seconds and immediately chilled on ice for 2 min. Further, 800 µl of LB broth. To this 60 µl of 2% X-gal, 60 µl of 2% IPTG was added and plated on LB agar plates containing 100 µg/ml ampicillin. The plates were now incubated at 37°C for 12 for colonies to appear.

3.9.4 Conjugation

Conjugation experiments were carried out using each isolates as the donor and Escherichia Coli (E.Coli) HMS 174 obtained from the E.Coli Genetics Stock Centre (Yale University. USA) Previous Examination of E.Coli HMS 174 Indicated it was sensitive to all antibiotics examined in this study

Briefly

The isolates which are sensitive to 50 mg ml⁻¹ rifampicin and E.Coli HMS 174, which is Resistant to 50 µg ml⁻¹ rifampicin Were grown overnight at 37°C with shaking in (LB) broth the overnight culture were used to Inoculate fresh LB broth and Incubated at 37°C with shaking until a 0.5 Mc Farland turbidity standard was obtained. Five hundred micro liters of each culture were mixed with 0.5 ml of E.Coli HMS 174 in a Sterile tube and the suspension Incubated at 37°C without shaking for 90 minutes. At 30 minutes intervals, the culture were mixed by gentle inversions conjugation mixer were placed on McConkey Agar Plate containing 50 µg ml⁻¹ rifampicin and either ampicillin (32 µg ml⁻¹) Chloramphenical (32 µg ml⁻¹) or Trimethoprim / Sulfamethoxazole (4 µg ml⁻¹, 16 µg ml⁻¹) and Incubated overnight at 37°C Colonies growing on the plates Indicated conjugational Transfer of antibiotic resistance to Escherichia coli.
3.9.5 AGAROSE GEL ELECTROPHORESIS

0.7% agarose gel was prepared and was used to run the plasmid DNA. PCFF04 control plasmid DNA was loaded at each run. DNA was Visualized after ethidium bromide staining in a UV Transilluminator (biorod gel documentation system USA).

**Agarose gel Electrophoresis**

Plasmid DNA of clinical strains was prepared by the procedure described by kado and Liu and crude extract was used directly for Electrophoresis which was performed in agarose gel (0.7% wt/vol) for 90 min at 50 Volts. The size of the plasmids was determined by using PcFF04 (85 kb) and PcFF14 (180 kb) as standard and the plasmid DNA an control standards 90 minutes at 50 volts. DNA was visualized after ethidium bromide staining in a UV Transilluminator (biorad gel documentation system USA).

3.9 Culture Media

**Pseudomonas Enrichment broth (Kelly etal. 1983)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nacl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Mg so4</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NH₄H₂P0₄</td>
<td>1.0g</td>
</tr>
<tr>
<td>Acetamide CH₃ CONH₃</td>
<td>20.0 g</td>
</tr>
<tr>
<td>D/W</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Dissolve the actamide and other ingredients in the distilled water and autoclave at 121°C for 10 minutes incubate swabs or specimens in these medium for 18 h at 37°C and this sub culture to pseudomonas Isolation agar (PTA).
Pseudomonas Isolation agar (PTA)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactopeptone</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.4 g</td>
</tr>
<tr>
<td>Potassium sulphate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Irgasan, 2,4,4 Trichlorohydrazinyl ether</td>
<td>2 - 0.025 g</td>
</tr>
<tr>
<td>Bacto agar</td>
<td>13.6 g</td>
</tr>
<tr>
<td>D/W</td>
<td>980 ml</td>
</tr>
</tbody>
</table>

Dissolve 45 g Medium in the water and add 20 ml Bacto glycerol. Heat to boiling to dissolve the medium completely. Autoclave for 15 minutes at 121°C. Final PH 7.0 ± 0.2 at 25°C

Mueller – Hinton Agar (Bauer et al 1966)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion</td>
<td>300 ml</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>17.5 g</td>
</tr>
<tr>
<td>Stach</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>10.0 g</td>
</tr>
<tr>
<td>D/W</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

Emulsify the starch in a small amount of cold water, pour into the beef infusions and add the casein hydrolyysate and the agar make up to the volume to 1 liter with D/W. Dissolve the constituents by heating gently at 100°C. With agitations filter if necessary adjust the PH to 7.4. Dispense in screw caped bottle and sterilize by autoclaving at 121°C for 20 minutes pour plates.
**Nutrient Agar**

- Peptone: 5.0 g
- Beef extract: 3.0 g
- Nacl: 5.0 g
- Agar: 15.0 g
- D/W: 1000 ml

**Blood Agar**

- Peptone: 5.0 g
- Beef extract: 3.0 g
- Nacl: 5.0 g
- Defibrinated Sheep blood: 5-10%
- Agar: 15.0 g
- D/W: 1000 ml

**Chocolate Agar (heated blood agar)**

It is prepared by heating 10% of sterile blood in sterile nutrient agar. Melt the agar, cool it in a water bottle at 75°C, add the blood and allow the Medium to remain at 75°C, mixing the blood and agar by gentle agitation from time to time until the blood became chocolate – brown is colour, within about 10 minutes then pour as slopes or plates.
Mannitol Salt agar (PH.7.4)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>75.0 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.025 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>D/W</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Cystine Lectose Electrolyte deficient medium (CLED)
(Mackey & Sandys 1966)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Meat extract powder</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.128 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.00 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

Suspend the ingredients in the water brings to the boil to dissolve, sterilize for 15 minutes at 121°C and mix well before pouring.
Xylose lysine deoxycholate (XLD) Agar (Taylor’s 1965)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.75 g</td>
</tr>
<tr>
<td>Lactose</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>7.5 g</td>
</tr>
<tr>
<td>L-Lysine Hcl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium deoxycholate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Sodium Thiosulphate</td>
<td>6.8 g</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>0.08 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>D/W</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

McConkey Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>20.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.0</td>
</tr>
<tr>
<td>Bile salts</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Neutral red</td>
<td>0.075</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Prepare and sterilize as instructed by the manufacturer. When the medium has cooled to 50 – 55° C, mix well and dispense in sterile Petri dishes.
**Luria Bertani (LB) Medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10.09</td>
</tr>
<tr>
<td>Agar (NaCl)</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Distilled waters</td>
<td>100 mg</td>
</tr>
</tbody>
</table>

**3.11 Reagents**

**Crystal violet (gram stain)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystal Violet</td>
<td>20 g</td>
</tr>
<tr>
<td>Ammonium Oxalate</td>
<td>09 g</td>
</tr>
<tr>
<td>Ethanol or method, absolute</td>
<td>95 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 liter</td>
</tr>
</tbody>
</table>

1. Weigh the crystal violet on a piece of clean paper (Pre weighed). Transfer to a clean, brown bottle pre marked to hold 1 liter.

2. Add the absolute ethanol or Methanol (technical grade is suitable) and mix until the dye is completely dissolved.

**Caution:** ethanol and Methanol are highly flammable therefore use will away from an open flame. Weigh the Ammonium Oxalate and dissolve in about 200 ml of distilled water add to the stin make of to the 1 liter mark with distilled water and mix well.

**Caution:** (Ammonium Oxalate is a toxic chemical therefore handle with care.)

3. Lable the bottle and store at room temperature the stain is stable for several months.

**For use:** Filter a small amount of the stain into a dropper bottle or other stain dispensing container.
**Iodine for staining**

To make about 20 ml

- Potassium iodide 2 g
- Iodine 1 g
- Distilled water 20 ml

1. Weigh the Potassium Iodide and dissolve completely in the water.

2. Weigh the Iodine, and add to the Potassium Iodide, solution. Mix well to dissolve.
   
   **Caution:** (Iodine is injurious to Health if Inhaled or allowed to come in contact with the eyes therefore handle with care in a well ventilated room)

3. Transfer to a clean brown bottle and store in the dark at room temperature. The reagent is stable for several months.

**Kovac’s (indole) regents**

To make about 40 ml:

4. Dimethylaminobenzaldehyde 2 g
   
   (Para -- Dimethylaminobenzaldehyde)

- Isoamyl alchohl (3-methyl - - butanol) 30 ml
- Hydrochloric acid, concentrated 10 ml
Preparation

1. Weigh the dime thylaminobenzaldehyde, and dissolve in the Isoamyl alcohol.

   **Caution:** (Isoamyl alcohol is a Highly flammable and toxic chemical, therefore handle with care well away from an open flame.)

2. Add the concentrated Hydrochloric acid, and mix well.

   **Caution:** (Concentrated Hydrochloric acid corrosive, therefore handle with care. Do not mouth – pipette)

3. Transfer to a clear brown bottle.

4. Label the bottle and mark it flammable store at 2-8\(^0\) C Renew monthly

**Ethyl alcohol (95%)**

<table>
<thead>
<tr>
<th>Ethyl alcohol (100%)</th>
<th>95.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/W</td>
<td>5.0 ml</td>
</tr>
</tbody>
</table>

**Safranin**

<table>
<thead>
<tr>
<th>Safranin (2.5% solution is 95% ethyl alcohol)</th>
<th>10.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/W</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>