CHAPTER - 4

MATERIAL AND METHODS
4. MATERIAL AND METHODS

Patients attending the medicine outpatient department or admitted to the wards of Maharani Laxmi Bai Medical College and Hospital, between Feb 1999 and July 2002 constituted the material for the present study. Patients were divided into 3 groups.

GROUP I: STUDY GROUP

Three hundred patients attending the chest clinic/medicine department with clinically and/or radio-logically suspected pulmonary tuberculosis formed this group.

GROUP II: DISEASE CONTROL GROUP

One hundred patients attending the chest clinic /medicine department with non-tuberculous chest infections were included in this group. They were subjected to the same investigations as the study group.

4.1 METHOD

COLLECTION OF SAMPLES

Three consecutive early morning sputum samples were collected in clean wide mouthed containers, from group II and I patients. They were subjected to Ziehl Nelsen staining for acid-fast bacilli, before and after concentration. Fluorescent microscopy was also done. Samples were
cultured on Lowenstein Jensen (LJ) slopes and drug susceptibility test was done on all positive cultures.

4.2 BACTERIOLOGICAL STUDY

Each sputum sample was processed by the following procedure:

Microscopic examination for acid-fast bacilli by Ziehl Neelsen staining: Smear was prepared from purulent or mucopurulent portion of the sputum and fixed by flaming. It was stained by the standard Ziehl-Neelsen method (Mackie and McCartney, 1989) and examined with 100X oil immersion lens for the presence of acid-fast (red stained) bacilli on a background of non-acid fast (blue stained) material. Hundred fields were examined before accepting a smear as negative.

4.2.1 ZIEHL NEELSEN:

Smears were stained with carbol fuschin (Mackie and McCartney, 1989).

COMPOSITION OF STAINS

i) Ziehl Neelsen (Strong) carbol Fuchin

   a) Basic Fuchin
       10g

   b) Absolute alcohol (ethanol)
       100ml

   c) Solution of phenol (5% in water)
       100ml
ii) Decolorizing solution

   a) Sulphuric acid, 20% solution

iii) Counterstain

   a) Saturated solution of Methylene blue in alcohol 300ml

   b) KOH (0.01 percent in water) 1000ml

PROCEDURE

   • The smear was covered with Carbol fuchsin until stream rises. **Wait for 5-10 minutes.**

   • The stain was washed off in running water.

   • The slides were covered with 20% H₂SO₄ for 1-2 minutes.

   • It was washed off in running water. Step 3 may be repeated till the smear retained a faint pink colour.

   • Flood slides with methylene blue reagent for 1 minute.

   • The slide was washed off in running water and allowed to dry in air.

   • Examine under oil immersion (100x) for presence of acid-fast bacilli. The bacilli are stained red and the background material is stained blue. (Plate No. - 1)
4.2.2 Fluorochrome Staining:

Smears were stained with Auramin ‘O’ (Mackie and McCartney, 1989)

Composition of Stain

i) Auramine ‘O’

a) Auramin O 3g

b) Phenol 30g

c) Distilled water 1 litre

ii) Decolorizing solution:

a) Industrial alcohol (ethanol) 75 percent in water containing 0.5 percent NaCl and 0.5 percent HCl.

iii) Potassium permagnate solution

a) KMnO4 1g

b) Distilled water 1 litre

Procedure

- The heat fixed smear was stained with the auramine solution for 15 minutes.

- It was washed of in running water.
• The smear was decolourized with acid alcohol for about 5 minutes.

• The slide was washed with water.

• The slide was covered with potassium permagnate solution for 30 seconds, washed well with water and allowed to dry.

• Examination was done under fluorescent microscope (Paralens fiber optic Illuminator, Manuf. Becton Dickinson) with 10X objectives first. Tubercle bacilli were seen as yellow luminous rods in a dark field (Plate No. – 2 & 3). When they were detected under low power, the morphology of the bacilli was confirmed by observation under oil immersion objective.

• Smear were also prepared after concentration by Petroff’s method and stained by Ziehl Neelsen method (Mackie and Mclartney 1989).

4.3 Decontamination and Concentration Procedures

4.3.1 Petroff’s Method

Solution: (for preparation, see appendix D)

a) 4%NaOH

b) 1% Phenolphthalein (1 gram in 100ml 95% ethyl alcohol)

c) 8%HCl
PROCEDURE

- The sputum sample was transferred into a universal container.

- An equal volume of 4% sodium hydroxide was added and the container was closed tightly and mixed thoroughly by shaking.

- Leave the mixture at 37°C for 15 to 20 minutes with intermittent shaking.

- Centrifuge at 6000 rpm for 20 minutes.

- Decant the supernatant fluid.

- Add a small drop of indicator and neutralize with 8% HCl.

- Centrifuge again for 3 minutes.

- Discard the supernatant fluid.

- Inoculate the media

- Make smear and stain

4.3.2 ZEPHIRAN- TRISODIUM PHOSPHATE (Z-TSP)

Solution:

a). Zephiran trisodium phosphate- Dissolve 1kg of trisodium phosphate (Na₃PO₄.12H₂O) in 4 liters of hot distilled water. To this solution add
7.5 ml of Zephiran concentrate (17% benzalkonium chloride). Mix well. Store at room temperature.

b). Neutralizing buffer. pH 6.6 (Sterilize by auto claving)

I. M/15 disodium phosphate: In a volumetric flask dissolve 9.47g of anhydrous Na₂HPO₄ in distilled water to make one liter (1000ml).

II. M/15 monopotassium phosphate: In a volumetric flask dissolve 9.07g of KH₂PO₄ in distilled water to make one liter (1000ml).

Mix 37.5ml of (I) with 62.5 ml of (II). pH 6.6, check on pH meter.

**PROCEDURE**

- Add to the specimen a volume of Z-TSP equal to the volume of the specimen

- Agitate vigorously on a mechanical shaker for 30 minutes.

- Let stand for 20-30 minutes without additional shaking.

- Transfer to a screw cap centrifuge tube 50ml.

- Centrifuge at 6000rpm for 20 minutes.

- Decant the supernatant fluid.

- Add 20 ml of the neutralizing buffer. Mix well.

- Centrifuge again for 20 minutes.
• Discard the supernatant fluid.

• With a sterile capillary pipette. Mix the sediment an inoculate 3 drops to each MacCartney tube of media.

4.3.3 N-ACETYL L-CYSTEIN-SODIUM HYDROXIDE (NALC-NAOH)

SOLUTION:

For each 100ml required for the day’s work combine.

a) 50ml sterile 4% NaOH

b) 50ml sterile 2.9% Na citrate

c) 0.5g of N-acetyl-L-Cystein powder.

Sterile pH 6.8 buffed water 30 to 40 ml per specimen.

Sterile water / 0.85% physiological saline water in screw cap tube 45ml in each.

Sterile bovine albumin 0.2% of pH 6.8.

PROCEDURE

• Add to the specimen a volume of NALC-NaOH solution equal to but not more than the amount of the specimen.
• Wait for 15 minutes and no more than 20 minutes before the diluents is added

• Fill each tube with pH 6.8 buffer or water. This should be at least 30ml in a 50ml centrifuge tube and mix.

• Centrifuge at high speed (6000rpm) for 15 minutes.

• Decant the supernatant fluid and add 1 or 2ml of 0.2% bovine albumin or sterile distilled water to the sediment. Shake gently by hand to mix.

• Place 0.1ml (or 2 drops) on the surface of each of 2 tubes of egg base media.

• Make a smear of the undiluted sediment by spreading a drop over an area of 1-2cm² on the microscope slide and stain for acid-fast bacilli.
4.4 Preparation of Media

4.4.1 Preparation of Lowenstein Jensen (L-J) Media

L-J media named after the discoverer, Lowenstein (1930) and Jensen (1932) was prepared as described below:

Mineral salt solution (Solution A)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen ortho phosphate anhydrous</td>
<td>2.4GM</td>
</tr>
<tr>
<td>Magnesium sulphate.7H₂O</td>
<td>0.24gm</td>
</tr>
<tr>
<td>Magnesium citrate</td>
<td>0.6gm</td>
</tr>
<tr>
<td>Aspargine</td>
<td>3.6gm</td>
</tr>
<tr>
<td>Glycerol</td>
<td>12.0ml</td>
</tr>
<tr>
<td>D.W.</td>
<td>600ml</td>
</tr>
</tbody>
</table>

All of the above ingredients were dissolved in DW by heating at 70°C on a hot plate for 10-15 minutes. Solution-A was autoclaved at 121°C (15 lbs pressure) for 20 minutes.

Malachite green solution (solution-B)

20ml of 2% aqueous solution of malachite green was prepared. 0.4gm of the dye was completely dissolved in 20ml of sterile DW.

Homogenized whole egg (solution-C)
Fresh hen's egg about 20-22 depending on size (less than a week old); were cleaned and scrubbed in 5% alkaline soap water using a soft nylon brush. The eggs were kept dipped in the soap water for 30 minutes. Followed by rinsing thoroughly in running tap water (Cruickshank et al, 1975; Vestal, 1977; Finegold and Martin; 1982). Working table and hands were disinfected with 70% alcohol. With a clean absorbent cloth, the eggs were drained and air-dried completely, than soaked in 70% alcohol for 15 minutes. In bacteriological laminar flow safety cabinate, these eggs were broken down into a sterile 1 litre conical flask containing approximately 60-70 glass beads of about 5-7mm in length and 3-5 mm in diameter. The amount of whole egg was homogenized by vigorous mechanical shaking, and filtered into a sterile graduated 1-litre cylinder through 3-4 layers of sterile gauze made of cotton.

Solution a, b and c were mixed in the following proportion:

Solution (a) 600ml
Solution (b) 20ml
Solution (c) 1000ml

Approximately about 10ml of this medium in liquid state was distributed in 30 ml sterile screw capped MacCartney bottles. The medium was sterilized in slanting position in an inspissator at 85°C for one hour daily on 3 consecutive days.
4.4.2 Preparation of Dubos Broth base (DBB):

Modified Dubos medium (Katoch et al, 1989) containing 10% glycerol, 1% glucose, 1% BSA was prepared for incubation of *Mycobacterium*. To prepare 100ml of broth, 0.65gm of ready made dehydrated DBB with tween 80 (from Himedia. Bombay, India) was dissolve in 80ml DW in a 250ml conical flask and heated at 70°C for 5-8 minutes to dissolve the base completely, 10ml of glycerol was mixed properly and pH was adjusted to 6.5. This preparation was autoclaved at 121°C (15 lbs pressure), for 15 minutes. Taking care to avoid contaminintion, 10ml of sterile enrichment medium consisting of glucose and BSA was added to the broth after cooling that down to 50-55°C.

Enrichment media: To prepare 10ml of this enrichment, 1gm glucose and 1gm of BSA were dissolved in 10ml DW. After proper dissolution, it was sterilized by filtration through millipore filter.

4.4.3 Middle Brook 7H9 Broth

To prepare 1 litre (1000ml) of medium

1. Combine 900ml of distilled water with 0.5g tween 80 or 20ml of glycerol as desired. (Do not use tween 80 and glycerol together.

2. Add and dissolve the following salts in the order listed.
<table>
<thead>
<tr>
<th>Ammonium sulphate (20ml of 2.5% solution)</th>
<th>0.5G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid, monosodium salt (20ml of 2.5% solution)</td>
<td>0.5g</td>
</tr>
<tr>
<td>Sodium citrate 2H₂O (1ml of 10% solution)</td>
<td>0.1g</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride (1ml of 0.1% solution)</td>
<td>0.001g</td>
</tr>
<tr>
<td>Biotin (0.25ml of 2.2% of solution)</td>
<td>0.0005g</td>
</tr>
<tr>
<td>Disodium Phosphate (anhydrous)</td>
<td>2.5g</td>
</tr>
<tr>
<td>Monopotassium phosphate (anhydrous)</td>
<td>1.0g</td>
</tr>
<tr>
<td>Ferric ammonium citrate (green) (0.4ml of 10% solution)</td>
<td>0.04g</td>
</tr>
<tr>
<td>Magnesium sulphate 7H₂O (1ml of 1.0% solution)</td>
<td>0.05g</td>
</tr>
<tr>
<td>Calcium chloride 2 H₂O (0.5ml of 0.1% solution)</td>
<td>0.0005g</td>
</tr>
<tr>
<td>Zinc sulphate 7 H₂O (1ml of 0.1% solution)</td>
<td>0.001g</td>
</tr>
<tr>
<td>Copper sulphate 5 H₂O (1ml of 0.1% solution)</td>
<td>0.001g</td>
</tr>
</tbody>
</table>

Adjust the pH of the solution to 6.6 using 10% HCl

3. Autoclave at 121°C for 20 minutes.

4. Allow cooling to less than 50°C and adding the following sterile enrichment solution:

   a) 50% glucose 4ml

   b) Catalase (10:0 mcg/ml) 2ml

   c) 5% bovine albumin 100ml
5. Dispense in freshly sterilized screw cap test tube or other containers as desired (use 5ml amounts in 20mm diameter tubes). Store in the refrigerator to prevent evaporation during storage, tube caps must be securely tightened.

6. Preparation of solutions (a) (b) and (c) for step (4)

a. Prepare 50% of glucose by dissolving 50g glucose in 60ml-distilled water. To this add 1.0 ml of 10% citric acid. Autoclave for 10 minutes at 15 pounds pressure (121°C).

b. Add 0.02ml catalase (technical grad) to 20ml of 8.5% NaCl in distilled water. Sterile by membrane filtration. Prepare fresh each time needed.

c. Mix 5.0g of bovine albumin fraction V and 95ml of 0.85% NaCl in distilled water. Adjust pH to 6.8 with ether 50% NaOH or 16%-19% HCl, which ever is needed. Filter sterilizes the solution. Dispense in freshly sterilized screw cap containers incubate at 37°C overnight to check for sterility. Place in a 56°C water bath for 30 min to inactivate lipase store at 4°C.

The middlebrook. 7H-9 are poured in 5 ml quantities in 20×150mm sterile screw cap test tube incubate for sterility check liquid media may be stored in the refrigerator.
4.5 Biochemical Test

4.5.1 Niacin Production (Venkataraman et al., 1976)

Reagent

a) Sterile distilled water or 0.85% saline.

b) 4% aniline preparation.

   i) Ethyle alcohol (95%)  96ml

   ii) Aniline  4ml

c) Cyanogen bromide preparation

   i) Cyanogen bromide  5g

   ii) D. W.  50ml

Procedure

- To the culture slant, add 1.0 ml sterile distilled water or saline.

- Allow the fluid to remain in contact with the culture medium for 15 minutes. When the culture is in a tube, place in a slanted position so the fluid layers over the colonies.
• Remove 0.5 ml of the liquid extract from the culture and place in a test tube.

• Add 0.5 ml of aniline (equal volume) to the extract. This should be colorless.

• Next, add an equal volume (0.5 ml) of Cyanogen bromide.

• Observe for a yellow color, which should appear immediately. (See Plate No. - 4)

4.5.2 Nitrate Reduction (Mackie’s and McCartney, 1989)

   a) Medium (Dubos and Davis)

   b) $\text{KH}_2\text{PO}_4$  

   c) $\text{Na}_2\text{HPO}_4 12\text{H}_2\text{O}$  

   d) $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 2\text{H}_2\text{O}$  

   e) $\text{MgSO}_4 7\text{H}_2\text{O}$  

   f) Tween 80, 10 Percent solution  

   g) Casein hydrosylate (20 percent)  

   h) Distilled water  

   i) Bovine albumin solution (9 percent)

   (4.5ml bovine albumin + 45.5ml water)
PROCEDURE

- The cultures were inoculated heavily into 7 ml of test medium (Dubos and Davis medium: 1 litre - 10 percent sodium nitrate solution 10 ml) and incubated at 37°C for 14 days.

- One ml of 0.8 percent sulphanilic acid in acetic acid 5 mol litre was added. Then 1 ml of 0.5 percent alpha-naphthylamine in acetic acid 5 mol litre was added. The mixture was observed for the development of a red color. (See Plate No.-5)

4.5.3 CATALASE TEST (MACKIE AND MCCARTNEY, 1989)

REAGENT

- Equal volumes of 1 percent tween 80 and a 20-volume solution of hydrogen peroxide were mixed.

PROCEDURE:

- Five ml of reagent was poured over the surface of a LJ slope culture. The slope was left in horizontal position for 5 minutes then placed upright and looked for effervescence and formation of bubbles appearing on the surface of the medium. (See Plate No.-7)
4.5.4 ARYL SULPATASE TEST (Mackie and McCartney, 1989).

This test identifies the presence of the enzyme aryl sulphatase in certain atypical mycobacteria.

Medium

a) Potassium phenolphthalein disulphate 0.646g

b) Distilled water 100ml

Ten ml of the above solution was added to 100ml of Middlebrook 7H-9 medium.

PROCEDURE

- 0.1ml of liquid test culture was inoculated into 3ml of medium.

- It was incubated at 37°C for 14 days.

- At the end of incubation 0.5ml of 0.2-mol/litre sodium carbonate solutions was added.

- Development of a red color denoted a positive reaction. (See Plate No. - 6)

4.6 PREPARATION OF DRUG FOR MEDIUM

Drugs may be obtained in the sterile lyophilized form. Instruction accompanying the vial states the potency of the final solution when prepared according to directions. This sterile lyophilized drug
reconstituted with sterile distilled water with aseptic technique requires no further sterilization. To obtain working stock solutions of the required concentration, the drug was diluted with sterile distilled water. A separate sterile pipette was used for each dilution and solutions were mixed well.

4.6.1 Preparation of Streptomycin (SM) Solution

(12.5mg dihydro - Sterptomycin sulphate = 10mg SM)

- Dissolve 125mg of dihydro-streptomycin sulphate in 10ml distilled water. This is a 10,000-mcg/ml solution.

- Sterilize by Millipore filter.

- Add 1 ml to 9ml sterile distilled water. This is 1000 mcg/ml solution.

- Add 0.4 ml of 1000mcg/ml solution to 200ml of LJ medium to obtain a final concentration of 2.0 mcg SM per ml of medium.

- LJ slopes containing 4, 8, 16 & 32 of Streptomycin were prepared.

4.6.2 Preparation of INH (Isoniazid) Solution

(10mg Isoniazid = 10mg INH)

- Dissolve 100mg (100,000 mg) Isoniazid in 10 ml distilled water. This is a 10,000-mcg/ml solution.
• Sterilize by Millipore filter.

• Add 1ml to 9ml sterile distilled water. This is 100-mcg/ml solution.

• Add 1ml of 100mcg/ml to 9ml sterile water. This is 100mcg/ml solution.

• Add 0.4ml of 100mcg/ml to 200ml medium to obtain a final concentration of 0.2mcg INH per ml of LJ medium.

• LJ slopes containing 0.025, 0.05, 0.1, 0.2, 1 & 5 mcg/ml of INH were prepared.

4.6.3 Preparation Of Rifampicin Solution

(10mg Rifampicin=10mg Rifampicin).

• Dissolve Rifampicin according to manufacturer’s instructions to make a 10,000-mcg/ml solution.

• Sterilize by Millipore filter.

• Add 1ml to 9ml sterile distilled water. This is a 1000mcg/ml solution.

• Add 0.2ml of 100mcg/ml to 200ml of LJ medium to obtain a final concentration of 1.0mcg of Rifampicin per ml of medium.
- LJ slopes containing 6, 12, 24 & 48 mcg/ml of Rifampicin were prepared.

4.6.4 Preparation of Ethambutol (EMB) Solution

(10mg of di-Ethambutol = 10mg EMB)

- Dissolve 100mg di-ethambutol in 10ml-distilled water.

- Sterilize by Millipore filter.

- Add 1ml to 9ml sterile distilled water. This is a 100-mcg/ml solution.

- Add 1.2ml of 1000mcg/ml to 200 ml of medium to obtain a final concentration of 6mcg EMB per ml of medium.

- LJ slopes containing 0.5, 1, 2, 4 & 8 mcg/ml of Ethambutol were prepared.

4.6.5 Preparation of Pyraznamide

(100mg of Pyraznamide = 10mg)

- Dissolve 100 gm Pyraznamide in 10 ml of distilled water.

- Sterilize by Millipore filter.

- Add 0.4ml of 10,000 mcg/ml to 200 ml of medium to obtain a final concentration of 20mcg Pyraznamide of medium.
Pyrazinamide (PZA) Sensitive Test (Proportion Method)

As Pyrazinamide has been shown to exert its in vitro only in acidic medium, sensitivity test of this drug were set up in acidified LJ medium (pH 4.3) Since some strains of mycobacteria fail to grow on acid medium, both normal and acidified LJ media were used as controls of the test. Only one concentration of drug (100ug/ml) was used.

For purposes of calculation, the number of colonies appearing on the drug-containing medium was expressed as proportion of the estimated number of colonies on the acidified drug free medium.

4.6.6 Preparation of Ethionamide (THA) Solution

(610mg Ethionamide hydrochloride = 500mg THA)

- Dissolve 122 mg of Ethionamide hydrochloride in 10ml-distilled water. This is a 10,000-mcg/ml solution.

- Sterile by Millipore filter.

- Add 1ml to 9ml-distilled water. This is a 1000 mcg/ml solution.

- Add 1ml of mcg/ml to 200ml medium to obtain a final concentration of 5mcg THA per ml medium.

- LJ slopes containing 3, 6, 12, 24 & 48 mcg/ml of Ethionamide were prepared.
4.6.7 Preparation of PAS Solution

(5.7g sodium P-aminosalicylate=5g PAS)

- Dissolve 114 mg solution P-aminosalicylate in 10ml of distilled water. This is a 10,000-mcg/ml solution.

- Sterilize by Millipore filter.

- Add 1ml to 9ml sterile distilled water to obtain 1000mcg/ml solution.

- Add 0.4ml of 1000mcg/ml to 200 LJ medium to obtain a final concentration of 2.0 mcg PAS per ml medium.

- LJ slopes containing 0.6, 1, 2, 2.5, 5 & 10 mcg/ml of PAS were prepared.

4.6.8 Preparation of Cyclosirine (CS) Solution

(10mg Cyclosirine= 10mg CS)

- Dissolve 100mg Cyclosirine in 10 ml distilled water. This gives 10,000 mcg/ml.

- Sterilize by Millipore filter.

- Add 0.4ml of 10,000 mcg/ml to 200 ml of medium to obtain a final concentration of 20 mcg CS per ml of medium.
• LJ slopes containing 5, 10, 20, 40 & 60 mcg/ml of Cycloserine were prepared.

4.6.9 Preparation Of Ciprofloxacin

(100mg Ciprofloxacin = 100mg)

• Dissolve 100mg Ciprofloxacin in 10 ml distilled water. This gives 10,000 mcg/ml.

• Sterilize by Millipore filter.

• Add 0.24ml of 10,000 mcg/ml to 200 ml of LJ medium to obtain a final concentration of 12 mcg/ml Ciprofloxacin per ml of medium.

• LJ slopes containing 3, 6, 12, 24 & 48 mcg/ml of Ciprofloxacin were prepared.

4.6.10 Preparation Of Ofloxacin

(To prepare 16 mcg/ml)

• Dissolve 100 mg Ofloxacin in 10 ml distilled water. This gives 10,000 mcg/ml.

• Sterilize by Millipore filter.

• Add 0.32 ml of 10,000 mcg/ml to 200 ml of LJ media to obtain a final concentration of 16 mcg/ml Ofloxacin per ml of media.
• LJ slopes containing 4, 8, 16, 32 & 48 mcg/ml of Ofloxacin were prepared.

4.6.11 Preparation of Kanamycin

(1000 mg Kanamycin sulphate = 832 mg KM)

• Dissolve 121.5mg Kanamycin sulphate in 10ml-distilled water. This is a 10,000-mcg/ml solution.

• Sterilize by Millipore filter.

• Add 1ml to 9ml sterile distilled water. This is 100-mcg/ml solution.

• Add 1.0ml of 1000mcg/ml to 200ml LJ medium to obtain a final concentration of 5mcg of KM per ml medium.

• LJ slopes containing 3, 6, 12, 24 & 36 mcg/ml of Kanamycin were prepared.

4.7 Drug Sensitivity Test (Venkataraman et al, 1987 and Canetti, 1963)

All positive cultures of M. tuberculosis were tested against Streptomycin INH, Rifampicin, Ethambutol and Pyrazamide, PAS, Ethionamide, Cycloserine, Ciprofloxacin, Ofloxacin and Kanamycin. by standard resistance ratio method.
Sensitivity tests were done on LJ medium. The drug was added before inspissation. The medium was dispensed in 6ml amounts in screw capped tubes (30ml) and inspissated. The sensitivity test was set up with an inoculum prepared from the growth of the selected positive slope.

4.7.1 Bacterial Suspension

A suspension was prepared by adding approximately one to two colony from the culture were picked up by nichrome wire loop to 0.5ml of sterile distilled water and mixed well, to produce a uniform suspension.

Using a one-drop of this suspension with the help of sterile Pastures pipette) was inoculated on each slope of the sensitivity test media. One control drug free slope and one drug containing slope of each concentration of the drug for each strain was tested. The standard sensitive strain H37Rv was tested with each batch of test. The media batch numbers were recorded in the sensitivity register. All the slopes were incubated at 37°C except one control drug free slope, which was incubate at 25°C incubation and reading of tests.

The incubated slopes were examined for growth after 28 days of incubation. Growth on a slope was defined as the presence of 20 or more colonies and the result was recorded. (Plate No. XII)

+++ Confluent growth
++ Innumerable colonies (more than 100 colonies)

+ 20-100 colonies

1-19 Actual number of colonies

If control slope yielded less than 100 colonies, the test was repeated from the control slope. However, if the control showed no growth or was contaminated, sensitivity test was repeated from the original positive culture or an alternate culture from the same patient, if available.

4.7.2 Interpretation Of Test

The lowest concentration of drug inhibiting growth (the minimal inhibitory concentration of (MIC) was recorded. The ratio of the MIC of the test strain to MIC of the standard strain H37Rv setup with each batch of test was referred to as the resistance ratio (RR).

Definition of resistance ratio

Sensitive Ratio $< 2:1$

Resistance Ratio $> 8:1$

Doubtful Ratio $= 4:1$