Chapter 3
MATERIAL AND METHODS

3.1 SAMPLE COLLECTIONS

Sputum samples were collected from different parts of North-Eastern region with the help of collection centers of the clinic located at various parts. A total of 375 smear positive samples among the collected samples by different collection centres were taken into consideration and processed for further studies. Any further analysis was carried out only after decontaminating all the collected samples. Decontamination process was done by the conventional \( N\)-acetyl-L-cysteine (NACL)-NaOH method (final NaOH concentration, 1%). Whole process was done inside BSL-3 (Biosafety Level) in accordance with CDCs guidelines. The work was carried out in collaboration with Babina Clinic and Diagnostic Research Center, Imphal. Testing was performed on residual portions of routine clinical specimens submitted for culture and DST. Informed consent was not taken for the study as results were unlinked from patient identifiers and no patient information was collected. Only one sample per patient was collected.

3.2 DIGESTION AND DECONTAMINATION

Three methods are widely used for the digestion and decontamination of contaminated specimens: the sodium hydroxide (modified Petroff) method, the simple culture method (modified Kudoh method), and the \( N\)-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method. NALC-NaOH method was used for the study.

3.2.1 \( N\)-Acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) method

The NALC method provides more positive cultures than other methods as it kills only about 30% of the tubercle bacilli in clinical specimens. Time needed for processing a single specimen is approximately 40 minutes, while 20 specimens would take approximately 60 minutes. The mucolytic agent NALC (used for rapid digestion of sputum) enables the decontaminating agent (NaOH) to be used at a lower final concentration of 1%. Sodium citrate is included in the digestant mixture to bind the
heavy metal ions which may be present in the specimen and could inactivate the acetyl-cysteine. The procedure followed was as per recommendation by CDC (2006).

**Materials of NALC-NaOH**

**Stock Solution for NALC-NaOH**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH-Sodium citrate stock solution</td>
<td>(0.1 M)</td>
</tr>
<tr>
<td>Sodium citrate dehydrate</td>
<td>29 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1,000 ml</td>
</tr>
<tr>
<td>4% NaOH solution (1 N)</td>
<td></td>
</tr>
<tr>
<td>NaOH pellets</td>
<td>40 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1,000 ml</td>
</tr>
</tbody>
</table>

Mixed equal volume of NaOH and sodium citrate solution and autoclave at 121°C for 15 min.

**Working NALC-NaOH solution**

Just before use, 0.5 g of N-Acetyl-L-cysteine is added to 100 ml of NaOH-sodium citrate solution.

**Procedure**

1. An equal volume of working NALC-NaOH solution was added in 1 ml of sputum samples in a 50 ml screw cap centrifuge tube.

2. The contents were mixed by vortexing for 30 s.

3. Tubes were incubated for 15 min at room temperature to decontaminate the specimen.

4. The mixtures were then diluted to the 50 ml mark with sterile distilled water.

5. The tubes were centrifuged at 3,000xg for 15 to 20 min.

6. The supernatant poured off into a splash-proof discard container filled with Amphyl, a strong disinfectant.

7. The remaining sediment was then suspended in 1 to 2 ml of sterile 0.85% NaCl.

**3.3 DETECTION TESTS**

**3.3.1 Acid fast Staining**

The acid-fast stain is performed on samples to demonstrate the characteristic of acid fastness in certain bacteria. Clinically, the most important application is to detect *Mycobacterium tuberculosis* in sputum samples to confirm or rule out a
diagnosis of tuberculosis in patients. There are three common acid-fast staining methods, Ziehl-Neelsen (hot), Kinyoun (cold), and Auramine-Rhodamine Fluorochrome (Truant method). Ziehl-Neelsen method was used for the study.

**Ziehl-Neelsen method for acid-fast staining**

In this method heat is used to help drive the primary stain into the waxy cell walls of these difficult-to-stain cells. The use of heat in this method has been the reason that this technique is called the “hot staining” method.

The cell walls of the mycobacteria contain mycolic acids giving the cell walls high lipid content. This characteristic is thought to be the reason (Delisle *et al.*, 2002 and Gerhardt *et al.*, 1981) for difficulty to stain these bacteria. To view these cells in samples staining requires higher concentrations of the dye solution and/or a heating period (Bishop *et al.*, 1976). However, once a stain is introduced into the cell wall, removing it with a decolourizer is even more difficult. Cells that release the primary stain (carbolfuchsin) with decolorizing will be visible after the counterstaining step is complete. Bacteria described as acid fast will appear red when examining specimens using bright-field microscopy. Non-acid-fast cells and field debris will appear blue. As per CDC (2006) recommended procedure, the method was performed for the study.

**Materials**

**Carbolfuchsin stain**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic fuchsin</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Ethanol, 95% (vol/vol)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Phenol, heat-melted crystals</td>
<td>5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>95 ml</td>
</tr>
</tbody>
</table>

Dissolve the basic fuchsin in the ethanol; then add the phenol dissolved in the water. Mix and let stand for several days. Filter before use.

**Decolorizing solvent**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol, 95% (vol/vol)</td>
<td>97 ml</td>
</tr>
<tr>
<td>Hydrochloric acid (concentrated)</td>
<td>3 ml</td>
</tr>
</tbody>
</table>
Counterstain

Methylene blue chloride 0.3 g
Distilled water 100 ml

Procedure

1. Heat fixed an air-dried smear at 80°C for at least 15 minutes.

2. Cut absorbent paper to fit the slide and saturated the paper with the carbolfuchsin stain.

3. Carefully heated the underside of the slide keeping it moist with stain and steaming for 5 minutes.

4. Wash the slide in a gentle and indirect stream of tap water until no colour appears in the effluent.

5. Holding the slide with forceps, wash the slide with the decolorizing solvent.

6. Immediately wash with tap water.

7. Repeat the decolorizing and the washing until the stained smear appears faintly pink and the fluid washing off the slide runs clear.

8. Flood the smear with the methylene blue counterstain for 20 to 30 seconds, and wash gently under indirect stream of tap water.

9. Gently blot or air dried the smear.


Sputum samples showing more than 10 acid fast bacilli (AFB) per microscopic field in the smear were selected for the study.

3.4 BIOCHEMICAL TESTS

3.4.1 Niacin accumulation

Niacin (nicotinic acid) plays a vital role in the oxidation-reduction reactions that occur during metabolic processes in all mycobacteria. Although all mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway, *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis (Babady *et al.*, 2012).
Preparation of Lowenstein - Jensen Medium

Lowenstein-Jensen (LJ) is the most common medium used for mycobacterial culture. LJ medium containing glycerol favors *M. tuberculosis* growth. L-Asparagine and Potato Flour are sources of nitrogen and vitamins in Lowenstein-Jensen Medium (Murray *et al.*, 1995). Mono-potassium phosphate and magnesium sulfate enhance organism growth and act as buffers. Glycerol and the egg suspension provide fatty acids and protein required for the metabolism of mycobacteria (Murray *et al.*, 1995). The coagulation of the egg albumin during sterilization provides a solid medium for inoculation purposes. Sodium citrate and malachite green are selective agents to prevent growth of most contaminants and allow early growth of mycobacteria.

Materials

**Lowenstein-Jensen (LJ):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Asparagine</td>
<td>3.6 g</td>
</tr>
<tr>
<td>Monopotassium Phosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>0.24 g</td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td>0.6 g</td>
</tr>
<tr>
<td>Malachite Green</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Potato Flour</td>
<td>30 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>12 ml</td>
</tr>
<tr>
<td>Egg Suspension,</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Dissolve 37.3 g of the medium in 600 ml of purified water containing 12 ml of glycerol. Heat with frequent agitation to completely dissolve the medium. Autoclave at 121°C for 15 minutes. Prepare 1000 ml of a uniform suspension of fresh eggs under aseptic conditions. Avoid whipping air into suspension during the collection and mixing. Aseptically mix the 1000 ml of egg suspension with 600 ml of the sterile Lowenstein-Jensen Medium cooled to 50°C - 60°C, avoiding air bubbles. Dispense the finished medium into sterile screw-cap test tubes. Place the tubes in a slanted position and heat at 85°C for 45 minutes. Inoculate a loopfuls of decontaminated sputum sample to medium.
**Procedure**

1. A water extract is prepared by adding 1 ml of sterile water to the surface of an LJ slant with a growth of mycobacteria species at least three weeks old.

2. An aliquot of this extract is then added to a tube containing a niacin strip, incubated for up to 30 minutes with gentle shaking.

3. A positive reaction (presence of niacin) is read as the development of a yellow colour.

**3.4.2 Catalase test**

The 68 °C catalase test, which measures the heat stability of the enzyme activity, is one of the elements in the identification of *M. tuberculosis* and other tubercle bacilli (Babady et al., 2012). Catalase converts hydrogen peroxide to water and oxygen, generating oxygen bubbles in a liquid solution. Virtually all mycobacteria except certain isoniazid-resistant tubercle bacilli are catalase-positive. The catalase assay for mycobacteria is performed using 30% H$_2$O$_2$ (superoxol) in 10% Tween-80 and the test performed both at 22°C -25°C and 68°C (Vincent et al., 2007).

**Materials**

- **Phosphate buffer**, 0.067 mol/litre, pH 7.0
  - Reagent 1: Dissolve 9.07 g KH$_2$PO$_4$ (monopotassiumdihydrogen phosphate, MW 136.1) in 1000 ml distilled water.
  - Reagent 2. Dissolve 23.68 g Na$_2$HPO$_4$.12H$_2$O (disodium hydrogen phosphate dodecahydrate, MW 358.14) in 1000 ml distilled water.
  - Mix 38.9 ml of reagent 1 with 61.1 ml of reagent 2. Check pH using a pH meter.

- Hydrogen peroxide (H$_2$O$_2$), 30%

- Tween 80 solution, 10%: Mix 10 ml Tween 80 with 90 ml distilled water; autoclave at 121°C for 10 minutes. Store in the refrigerator.

- Substrate: Mix equal volume of 30% hydrogen peroxide and 10% Tween 80. Always prepare the substrate fresh just before the test.
**Procedure**

The entire procedure was carried out in a biological safety cabinet.

1. Dispense 0.5ml of phosphate buffer, 0.067 M, pH 7.0, into two screw-cap tubes.
2. Emulsify several loopfuls of bacterial colonies scraped from solid media into the buffer.
3. Place one tube in the water-bath at 68 °C for 20 minutes. Leave the other tube at room temperature.
4. Allow the heated tube to cool at room temperature.
5. Add 0.5 ml freshly prepared Tween 80 peroxide substrate to each tube and cap loosely.
6. Observe for the formation of bubbles. Retain negative tubes for 20 minutes before discarding. Do not shake the tubes: Tween 80 alone may form bubbles on shaking, resulting in false-positive results.

If oxygen bubbles form in the unheated tube but not in the heated tube, the test strain produces heat-labile catalase and probably belongs to the MTB complex. If oxygen bubbles form in both the heated and unheated tubes, the test strain produces heat-stable catalase and is unlikely to be a member of the *M. tuberculosis* complex. If no oxygen bubbles form in either the heated or the unheated tube, either the test has failed or the strain may be catalase-negative (such as rare isoniazid-resistant strains of *M. tuberculosis*). Repeat the test with a larger quantity of bacilli to eliminate the possibility of a false-negative result.

**3.5 DRUG SUSCEPTIBILITY TESTING**

Determination of resistance to a given drug is performed as an *in-vitro* assay in the laboratory, a process called drug susceptibility testing (DST). Where resources are limited, the WHO recommends a hierarchy of DST that should include at least rifampicin and isoniazid the two most efficacious drugs that define MDR-TB (Rich et al., 2006).

Drug susceptibility testing (DST) is the determination of a strain's susceptibility to drugs used in therapy: a "sensitive" result means that the patient with
that strain has a high probability of treatment success, while a “resistant” result means that there is a high possibility of treatment failure and that the therapy should be changed. Thus, standardized and reliable DST of *M. tuberculosis* provides guidance for the treatment of the patient. Carrying out DST also provides an assessment of the drug resistance rates and trends in a country (or in different areas of a country); standardization is therefore essential to allow data from different laboratories to be compared.

### 3.5.1 Critical concentration of antibiotics in Drug Susceptibility Testing

The critical concentration of antibiotics which is given below (Table 3) were maintained as per prescribed norms for indirect DST assay for proportion method (PM), nitrate reductase assay (NRA) and microscopic observation drug susceptibility (MODS) methods as recommended by WHO.

#### Table 3 Critical concentration of antibiotics in Drug Susceptibility Testing

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Critical Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH)</td>
<td>0.2</td>
</tr>
<tr>
<td>Rifampicin (RIH)</td>
<td>40</td>
</tr>
<tr>
<td>Streptomycin (STR)</td>
<td>4</td>
</tr>
<tr>
<td>Ethambutol (EMB)</td>
<td>2</td>
</tr>
</tbody>
</table>

#### 3.5.2 Antibiotic preparation for DST

The preparation of recommended concentration of drugs was done as per norms prescribed by WHO and CDC.

**Isoniazid**

**Stock solution**

1. Weigh 20 mg of Isoniazid powder.
2. Dissolve completely in 40ml of sterile distilled water to obtain a concentration of 500 µg/ml Isoniazid solution.

**Working solution**

1. Prepare the working solution 1ml of stock solution (500 µg/ml).
2. Add 24 ml of sterile distilled water (=25 ml of 20 µg/ml).
3. Sterilize by filtering through a 0.22 µ membrane filter.
4. Add 1ml per 100 ml of media for final concentration of 0.2 µg/ml.
**Rifampicin**

**Stock solution**
1. Weigh 42.1 mg of rifampicin completely.
2. Dissolve in 5 ml absolute methanol.
3. Add 5 ml of 99% ethanol to get 4000 μg/ml of stock.

**Working Solution**
1. Prepare the working solution 1 ml of stock solution (4000 μg/ml)
2. Add 24 ml of sterile distilled water (=25 ml of 20 μg/ml).
3. Sterilize by filtering through a 0.22 μ membrane filter.
4. Add 1 ml per 100 ml of media for final concentration of 40 μg/ml.

**Streptomycin**

**Stock solution**
1. Weigh 25 mg of streptomycin powder and dissolve in 50 ml of distilled water.
2. Using 0.22 μm syringe filter sterilize the solution to obtain 400 μg/ml of stock solution.

**Working Solution**
1. Prepare the working solution 1 ml of stock solution (400 μg/ml).
2. Add 24 ml of sterile distilled water (=25 ml of 20 μg/ml).
3. Sterilize by filtering through a 0.22 μ membrane filter.
4. Add 1 ml per 100 ml of media for final concentration of 4 μg/ml.

**Ethambutol**

**Stock solution**
1. Weigh 26.7 mg of ethambutol powder and dissolve in 100 ml of distilled water to get 200 μg/ml of drug stock solution.
2. Using 0.22 μm syringe filter sterilize the solution.

**Working Solution**
1. Prepare the working solution 1 ml of stock solution (200 μg/ml).
2. Add 24 ml of sterile distilled water (=25 ml of 20 μg/ml).
3. Sterilize by filtering through a 0.22 μ membrane filter.
4. Add 1 ml per 100 ml of media for final concentration of 2 μg/ml.
3.5.3 Proportion method

An equal quantity of a standardized inoculum of *M. tuberculosis* is seeded on a drug-free and drug-containing medium. The drug free medium is seeded with an inoculum that is 100 times diluted compared with that seeded on the drug-containing medium. Distinct, countable colony-forming units (CFU) should be present on the drug-free medium. On the drug-containing medium, only pre-existing resistant mutants are expected to grow.

Although the proportion of pre-existing mutants based on a mutation rate of 1 in $10^{7-10}$ would be much lower, for ease of interpretation, it is theoretically assumed to be 1%, and this has been determined to predict therapeutic outcome (Canetti *et al.*, 1969). Assuming that 1% of the inoculum on the drug medium are resistant mutants, only these mutants will grow, and by dividing the number of CFU on drug medium by those on drug free medium it is possible to deduce that the isolate is susceptible ($\leq 1\%)$ or resistant ($>1\%)$. Thus to interpret as susceptible, the number of CFU on the drug medium must not exceed those on drug free medium. This is the principle underlying the proportional method of DST in MTB. The LJ medium is recommended by the WHO and the IUATLD for developing countries as it is cheap, easy to read, has low contamination rates and DST results are highly reproducible. The procedure recommended by CDC (2006) was followed for the method.

**Bacterial suspension for inoculation**

1. Approximately 1 mg of representative sample of the bacterial mass visualized as 2/3 loopfuls of 3 mm internal diameter is added to 0.2 ml of sterile distilled water in a 7 ml Bijou bottle containing 10-12 glass beads.

2. This mixture is vortexed for approximately 30 seconds to get uniform suspension.

3. The suspension is then made up to approximately 1mg/ml concentration by adding more distilled water and then kept on the bench for 15-20 min to allow coarser particles to settle down.

4. From this suspension, a ten-fold serial dilution is made by adding 0.2 ml to 1.8ml sterile distilled water.

5. Each serial dilution suspension was inoculated by one standard loopfuls on to the drug-free as well as the drug-containing LJ slopes.
Culture preparation

Lowenstein - Jensen Medium is used with fresh egg and glycerol for the culture of *Mycobacterium* spp.

**Preparation of 1 L of LJ**

**Ingredients:**

Mineral salt solution:

- Potassium dihydrogen phosphate anhydrous (KH$_2$PO$_4$) 2.4 g
- Magnesium sulphate (MgSO$_4$.7H$_2$O) 0.24 g
- Magnesium citrate 0.6 g
- Asparagine 3.6 g
- Glycerol (reagent grade) 12 ml
- Distilled water 600 ml

Dissolve the ingredients in order in the distilled water by heating. Autoclave at 121°C for 30 minutes to sterilize. Cool at room temperature. This solution can be kept indefinitely and may be stored in suitable amounts in the refrigerator.

**Malachite green solution, 2%**

- Malachite green dye 2.0 g
- Sterile distilled water 100 ml

Aseptically dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

**Homogenised whole eggs**

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.
Preparation of complete medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

- Mineral salt solution 600 ml
- Malachite green solution 20 ml
- Homogenised eggs (20-25 eggs, depending on size) 1000 ml

The complete egg medium is distributed in 6-8 ml volumes in sterile 14 ml or 28 ml McCartney bottles or in 20 ml volumes in 20 x 150 mm screw-capped test tubes and the tops are securely fastened. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Interpretation of results

The results are read for the first time on the 28th day. Colonies are counted only on slopes seeded with an inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculum may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculum (10^-6 mg of bacilli) for the control slopes and the high inoculum (10^-4 mg of bacilli) for the drug-containing slopes. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum. Dividing the second figure by the first gives the proportion of resistant bacilli existing in the strain. Below a certain value in the critical proportion, the strain is classified as sensitive; above that value, it is classified as resistant. The proportions are reported as percentages. If, according to the criteria indicated below, the result of the reading made on the 28th day is “resistant”, no further reading of the test for that drug is required: the strain is classified as resistant. If the result at the 28th day is “sensitive”, a second reading is made on the 42nd day: this provides the definitive result. Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs – rifampicin, isoniazid, ethambutol, and streptomycin is classified as resistant to that drug. For calculating the proportion of resistant bacilli, the highest counts obtained on the drug-free and on the drug-containing medium should be taken, regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd.
3.5.4 Nitrate Reductase Assay

Nitrate Reduction Assay is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is routinely used for biochemical identification of mycobacterial species. The presence of nitrite can easily be detected with specific reagents, which produce a colour change. Nitrate reduction assay uses the detection of nitrite as indication of growth when it is used as a drug susceptibility test. The method described by Anandi *et al.* (2009) was followed.

Culture preparation

Lowenstein - Jensen Medium is used with fresh egg and glycerol for the culture of *Mycobacterium* spp.

Preparation of 1 L of Lowenstein - Jensen (LJ)

Ingredients

Mineral salt solution

- Potassium dihydrogen phosphate anhydrous (KH$_2$PO$_4$) 0.4 g
- Magnesium sulphate (MgSO$_4$. 7H$_2$O) 0.24 g
- Magnesium citrate 0.6 g
- Asparagine 3.6 g
- Glycerol (reagent grade) 12 ml
- Distilled water 600 ml

Dissolve the ingredients in order in the distilled water by heating. Autoclave at 121°C for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

Malachite green solution, 2%

- Malachite green dye 2.0 g
- Sterile distilled water 100 ml

Aseptically dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.
Homogenised whole eggs

Fresh hens’ eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

Preparation of complete medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

- Mineral salt solution: 600 ml
- Malachite green solution: 20 ml
- Homogenised eggs (20-25 eggs, depending on size): 1000 ml

The complete egg medium is distributed in 6-8 ml volumes in sterile 14 ml or 28 ml McCartney bottles or in 20 ml volumes in 20 x 150 mm screw-capped test tubes and the tops are securely fastened. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Preparation KNO3 stock

Stock solution: 1 g KNO3 / 5 ml distilled water
Add 1 ml stock KNO3 to 200 ml LJ

Bacterial suspension for inoculation

1. Approximately 1 mg of representative sample of the bacterial mass visualized as 2/3 loopful of 3 mm internal diameter is added to 0.2 ml of sterile distilled water in a 7 ml Bijou bottle containing 10-12 glass beads.
2. This mixture is vortexed for approximately 30 seconds to get uniform suspension.
3. The suspension is then made up to approximately 1 mg/ml concentration by adding more distilled water and then kept on the bench for 15-20 min to allow coarser particles to settle down.
4. From this suspension, a ten-fold serial dilution is made by adding 0.2 ml to 1.8 ml sterile distilled water.

5. Each serial dilution suspension was inoculated by one standard loopfuls on to the drug-free as well as the drug-containing LJ slopes.

**Nitrate reductase assay**

**Nitrate buffer**

Weigh 2 g potassium nitrate and dissolve in 10ml sterile distilled water. Final concentration to be used is 1000 μg/ml. (Note: Sodium nitrate can be used in place of potassium nitrate, depending on availability).

**HCl: Prepare 50% (v/v) concentrated HCl**

Add 10 ml concentrated HCl to 10 ml sterile distilled water.

**0.2% Sulfanilamide**

Weigh 0.1 g of sulphanilamide and dissolve in 50 ml sterile distilled water.

**0.1% N-naphthyethylene-diamine**

Weigh 0.05 g of n-1-naphthylethylenediamine di-hydrochloride and dissolve in 50 ml sterile distilled water.

**Powdered Zinc**

**Procedure**

1. Two millilitres of nitrate buffer in screw-capped tubes were inoculated with two loopfuls of culture.

2. And one tube being a negative control (without inoculation).

3. The contents of the tubes were mixed and incubated in a 37°C incubator for 7 days.

4. After incubation, 1 drop of concentrated HCl, 2 drops of 0.2% sulfanilamide, and 2 drops 0.1% N-naphthyethylene-diamine were added.

5. The solutions were examined for the development of a pink/red colour contrasting with the control.

6. A pinch of powdered zinc was added to all the negative tubes to reduce nitrate to nitrite. The formation of a red colour only after the addition of the zinc, confirmed a negative nitrate test.
Interpretation of results

A strain will be considered resistant if a colour change in the drug containing tube is greater or the same than 1:10 diluted growth control tube.

3.5.5 Microscopic observation drug susceptibility assay (MODS)

The method makes use of two important properties of *M. tuberculosis*: (1) markedly faster growth in liquid media than on solid media, and (2) an easily recognizable and characteristic microscopic cording appearance of that growth in liquid media. Using an inverted light microscope, 24 well plates inoculated with decontaminated sputum samples suspended in supplemented Middlebrook 7H9 medium are examined for micro colonies which can be detected in a median of 7 days, much earlier than macroscopic colony growth can be seen on solid medium. The incorporation of isoniazid and rifampicin in the testing process enables equally rapid MDR TB detection. The simplicity of the technique, the greater sensitivity of liquid over solid media culture for TB detection, the specificity of the characteristic growth of *M. tuberculosis*, the evaluation of drug susceptibility in a short timescale, and the low cost of reagents are the major advantages of the method. The method was carried out as per procedure described by Jorge *et al.*, (2008).

Culture preparation

1. Dissolve 5.9 g of 7H9 medium powder in 900 ml of sterile distilled water containing 3.1 ml of glycerol and 1.25 g of casitone.
2. Mix until completely dissolved, heat if required.
3. Cool and divide the sterile medium into 4.5 ml aliquots in sterile screw capped glass tubes for sample preparation and internal controls. Also aliquot 10.8 ml in sterile screw capped glass tubes for antibiotic solutions.
4. Autoclave at 121°C for 20 min, cool and then add 100 ml OADC (Oleic Albumin Dextrose Catalase) to 900 ml of media along with PANTA (polymyxin, amphotericin, nalidixic acid, trimethoprim, azlocillin) to minimize contamination of MODS culture by oral flora micro-organisms not killed during decontamination process.
5. Nine hundred microlitres of the suspension was then transferred to each of four wells in a 48 well-plate.
6. Next, 100 μl distilled water was added into the control wells. Finally, 100 μl INH 4 μg/ml (Sigma) or 100 μl RIH 10 μg/ml (Sigma) was added to the INH-containing well and RIH-containing well, respectively. The final concentrations of OADC (oleic acid dextrose catalase) and PANTA in each well were 10% and 20 μl/ml, respectively. The drug concentrations in each well were maintained as per given in Table 3.

7. Incubate at 37°C for 48 hour to verify sterility (lack of turbidity).

**Bacterial suspension for inoculation**

1. Mix 10 ml sterile DW and 40 μl of 10% sterile Tween 80 in a sterile tube (final Tween 80 concentration = 0.04%).
2. Using a sterile loop, harvest several colonies of *Mycobacteria* from the decontaminated sputum samples and place in a sterile tube containing 100μl water-Tween 80 solution and sterile glass beads.
3. Cap tube tightly and vortex for 2-3 min; (till there are no visible clumps).
4. Let it stand for 5 min.
5. Open tube and add 3 ml of water-Tween 80; cap tightly and vortex again for 20 second (until suspension has uniform turbidity). Let it stand for 30 min.
6. Transfer the supernatant to another sterile tube using a pipette.
7. Adjust turbidity to McFarland Scale 1 (approximately 3 × 10 CFU/ml) with 0.04% water-Tween 80 solution.

**Plate setting for MODS**

1. A 24 well plate is taken and marked for controls and drugs-containing wells.
2. 900 μl of the culture media is dispensed on to the wells and the required drug concentrations are added for drug containing well.
3. The liquid inoculum is then added to make a final volume of 1 ml.
4. The plates are then closed with its lids and sealed with paraffin or with zip lock bags and incubated at 37°C.

**Interpretation of results**

The culture is determined to be susceptible when no growth is observed in the both the wells. If one well in either the drug wells has ≥ 2 cfu growth while the other well has no growth or is intermediate (≤1cfu is termed as intermediate), then the culture is termed as mono-resistant to the drug with growth. If both the drug
containing well of the culture has growth ≥ 2 cfu then it is considered resistant to both the drugs and is confirmed to be MDR. Fungal or bacterial growth indicates contamination (Kent et al., 1985 and Caviedes et al., 2002).

Table 4 Overall MODS culture interpretation

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>Overall culture interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combined well findings (A&amp;B)</td>
<td></td>
</tr>
<tr>
<td>Both wells positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Both wells negative</td>
<td>negative</td>
</tr>
<tr>
<td>Either well indeterminate</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>One well positive, other well negative</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>One well positive, other well indeterminate</td>
<td>Indeterminate</td>
</tr>
<tr>
<td>Either well contaminated</td>
<td>Contaminated</td>
</tr>
</tbody>
</table>

3.5.6 BacT/Alert 3D Drug susceptibility testing

The BacT/Alert 3D system (formerly known as MB/BacT bioMerieux, Marcy L'Etoile, France) is also a non-radiometric, fully automated, continuously monitoring liquid culture system. Growing microorganisms produce CO₂, which induces a colour change in a sensor. It was introduced not only for the primary isolation of mycobacteria, but also for susceptibility testing. It is based on the detection of carbon dioxide (CO₂) released by actively proliferating mycobacteria. The elevated CO₂ concentration lowers the pH in the medium, which in turn produces a colour change in a sensor in the vial, which is detected by a reflectometric unit in the instrument. The BacT/ALERT automatically performs readings every 10 min, and all data are transferred to and saved in the BacT/VIEW data management system. The MB/BacT3D antibiotic supplement is intended to reduce the incidence of breakthrough contamination due to bacteria that may survive the decontamination/concentration process. MB/BacT3D antibiotic supplement must be added to BacT/ALERT3D MP culture bottles prior to inoculation of all non-sterile specimens. The system consists of incubators, data management computers, growth media bottles and reagents. Mycobacteria behave like most other bacteria with respect to carbohydrate metabolism, energy production, and the biosynthesis of low weight metabolites. Glycerol and oleic acid were selected as primary carbon sources in the BacT/ALERT3DMP and BacT/ALERT3D MB culture bottles because of their ability to maximize the amount of carbon dioxide (CO₂) generated by mycobacteria. Once ingested, glycerol and oleic acid are converted to Acetyl-CoA and are oxidized through the Krebs or Tricarboxylic Acid Cycle (TCA). Carbon Dioxide (CO₂) and
free electrons are the major metabolic by-products of oxidation. Although mycobacteria divide slowly; the level of CO₂ generated is similar to the levels seen in most common bacteria. The MB/BacT and BacT/ALERT 3D Mycobacteria Detection Systems utilize a colorimetric sensor and reflected light to monitor the amount of CO₂ dissolved in the culture medium. If microorganisms are present in the test sample, they produce CO₂ as they metabolize the substrates in the culture medium.

The liquid emulsion sensor (LES) is impermeable to most ions including hydrogen ions, and to components of media and whole as well as degraded blood. It is freely permeable to CO₂. As CO₂ diffuses across the membrane and dissolves in the water contained in the sensor, free hydrogen ions are generated:

\[
CO₂ + H₂O \leftrightarrow H₂CO₃ \leftrightarrow H^+ + HCO₃^-
\]

Free hydrogen ions interact with the indicator in the sensor. As CO₂ is produced, the concentration of hydrogen ions increases and the pH falls causing the sensor to change to lighter green or yellow. A Light Emitting Diode (LED) projects light onto the sensor. The light reflected by the sensor is measured by a photo detector, and as more CO₂ is generated, more red light is reflected (lighter colours reflect more light than darker colours). The photodiode converts the reflected light into an electrical signal that is measured every 10 minutes and plotted every hour as a point on a graph. Mathematical algorithms analyse the readings and slope of the curve over time to determine positives and negatives. A representative graph for most bacteria reflects a typical growth curve with a lag phase, log phase, and stationary phase of growth.

The MB/BacT and BacT/ALERT3D Mycobacteria Detection Systems monitor bottles using specific mycobacterial process bottle and mycobacterial blood bottle algorithms. These algorithms employ bacterial and mycobacterial algorithms for the first 4 days of incubation and thereafter use the very sensitive mycobacterial algorithm only. In addition to detecting rapid growers, the bacterial algorithms detect any breakthrough bacterial contamination during the first four days of incubation. Each sample is monitored independently, with its own established baseline. BioMerieux recommends a test protocol of 42 days for all mycobacterial bottles.
Inoculum preparation

1. The strains were cultured in Lowenstein-Jensen medium at 37°C.
2. Cultures less than 4 weeks old were used to prepare a homogeneous suspension at a 1.0 McFarland standard in Middlebrook 7H9 medium.
3. This suspension was inoculated into the BacT/ALERT MP seed bottle.
4. When the seed bottle flagged positive by the system, it was used as the standard inoculum for the subsequent DST, as stated in the protocol provided with the BacT/ALERT MB susceptibility kit.

Drug susceptibility testing was performed according to the BioMerieux protocol.

1. Briefly, 0.5 ml of the lyophilized antibiotic solutions and 0.5 ml restoring fluid were added to the glass BacT/ALERT MP test bottles and the undiluted direct control bottle, respectively. The final drug concentrations in the test bottles were 0.9 mg/liter for RIH, 0.4 and 0.09 mg/liter for INH, and 1.8 mg/liter for EMB.
2. Half a milliliter of the seed inoculum was added to all BacT/ALERT MP test bottles.
3. Bottles were loaded into the BacT/ALERT 3D system simultaneously, and the maximum test time was automatically limited to 15 days.

Susceptibility testing interpretation

An organism was determined to be resistant to an antibiotic when the drug-containing bottle had a time to detection that was less than or equal to the sum of the time of detection of the positive direct control plus 3.5 days. If the drug-containing bottle had a time of detection that was more than the sum of time of detection of the positive direct control bottle plus 3.5 days, or remained negative, the organism was interpreted as being susceptible to the drug. If a test bottle flagged positive less than 2 days after inoculation, it was checked for contamination.

3.6 DNA EXTRACTION

Parts of all the decontaminated sputum samples were subjected for DNA isolation following the modified CTAB-NaCl method.
Materials

CTAB/NaCl: Dissolve 4.1 g of NaCl in 80 ml of distilled-deionized water using a magnetic stirrer and stir bar. While stirring, add 10 g CTAB (Hexadecyl trimethyl ammonium bromide). If needed, dissolve the CTAB by heating the solution to 65°C. Allow the solution to cool at room temperature. Adjust the final volume to 100 ml with distilled-deionized water.

1X TE buffer: Add 10 ml of 1 M Tris-HCl (pH 8.0) and 2 ml of 0.5 M EDTA (pH 8.0) to 988 ml of ddH₂O. Filter with 0.5 micron filter and autoclave.

For 500 ml of TE buffer: Made up to 500 ml with sterile distilled water, pH 8.0 with HCl and autoclaved.

Lysozyme: Lysozyme solution: 50 mg/ml. Store in small aliquots at -20°C. Use one aliquot each time, do not freeze and thaw twice.

SDS: Prepare a 10% sodium lauryl sulphate (SDS; also known as sodium dodecyl sulphate) solution by dissolving 50 g SDS in 400 ml of distilled-deionized water. The solution will be cloudy, but adjust the pH to 7.2 using HCl. Bring the final volume to 500 ml with distilled-deionized water. This solution can be stored at room temperature without sterilization. (If the solution remains cloudy or becomes cloudy in the future, warm it to dissolve the SDS before dispensing).

5 M NaCl: Dissolve 29.22 g of NaCl in 100 ml of distilled-deionized water. Store this solution at room temperature.

Proteinase K: 20 mg/ml. Store in small aliquots at -20°C. Use one aliquot each time, do not freeze and thaw twice.

Chloroform: Isoamyl alcohol (24:1): Mix 96 ml chloroform with 4 ml isoamyl alcohol. Store this reagent at 4 degree.

Procedure

1. The decontaminated sample from above process was boiled for 10-15 min cooled to room temperature.
2. A portion of samples was put into 400 µl of 1X TE buffer in a micro-centrifuge tube.
3. Lysozyme was then added at final concentration of 2 mg/ml followed by incubation at 37°C for 2 hour.
4. 70 µl sodium dodecyl sulphate and 5 µl of 10 mg/ml proteinase K were then added.
5. And incubated for 10 min at 65°C.
6. 100 µl of 5 M NaCl and 100 µl CTAB/NaCl were added.
7. Then vortexed until the suspension turned milky and were incubated for 10 min at 65°C.
8. 750 µl of chloroform-isoamyl alcohol (24:1) was added to each tube.
9. The tubes were vortexed and then centrifuged in a micro-centrifuge at 13,000 rpm for 5 min at room temperature.
10. The aqueous supernatant was transferred to a fresh tube and 0.6 volume of isopropanol were added to precipitate the DNA.
11. After 30 min at -20°C and centrifuging for 15 min at 14 000 rpm, the pellet was washed once for 5 min with 1ml of cold 70% ethanol, air-dried and dissolved in 50 µl of 1X TE buffer.
12. To remove contaminating RNA from the preparation, add 1 ml of RNase to the nucleic acid solution. Incubate the tube at 37 ºC for 30 min.

3.7 DNA PURIFICATION

The DNA was purified and concentrated by using the QIAGEN QIAquick PCR Purification Kit (Qiagen, Germany).
1. A total of five volumes (500 µl) of Buffer PB was added to 1 volume of 100 µl DNA extract and mixed well.
2. The mixture was transferred to the QIAquick column and spun at 13000 rpm for 1 minute.
3. The flow-through was discarded and the column was placed into a new 2 ml collection tube.
4. A volume of 750 µl Buffer PE was added to the QIAquick column and centrifuged at 13000 rpm for 1 minute.
5. The flow-through was discarded and the column was placed into a new tube.
6. The column was centrifuged for an additional 1 minute.
7. The collection tube was discarded and the QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
8. A volume of 30 μl Buffer EB (10 mM Tris.Cl, pH8.5) was added to the centre of the QIAquick membrane and stood for 1 minute.

9. Then, the tube was centrifuged at 13000 rpm for 1 minute. The flow-through was collected as purified product.

3.8 LINE PROBE ASSAYS (Genotype MTBDRplus and Genotype MTBDRsl)

The GenoType MTBDRplus test allows for the detection of MTBC and simultaneously its resistance to rifampicin and/or isoniazid by mutations in the rpoB and katG/inhA (high/low isoniazid resistance) genes, respectively. The GenoType MTBDRsl detects MTBC and simultaneously its resistance to fluoroquinolones (e.g. ofloxacin and moxifloxacin) and/or aminoglycosides/cyclic peptides (injectable antibiotics as capreomycin, viomycin/kanamycin, amikacin) and/or ethambutol. The MTBDRplus and MTBDRsl are validated for DNA extracted from both positive cultures and smear-positive pulmonary specimens. Note: these tests should not be used to detect mycobacteria directly from smear-negative materials unless the laboratory independently validates their use. They are licensed for smear-positive sputum only.

Both procedures are identical and are divided into three steps: DNA extraction, a multiplex amplification using biotinylated primers, and hybridisation.

3.8.1 Drug Susceptibility/Resistance Test by Genotype Strip Methods (Hains Life Sciences)

All procedures are identical and are divided into three steps: DNA extraction, a multiplex amplification using biotinylated primers, and reverse hybridization.

In our study, Genotype MTBplus and MTDRsl were performed for drug resistant or susceptibility analysis.

Kit Contents Supplied

Membrane strips coated with specific probes (STRIPS) 96

Primer Nucleotide Mix (PNM)
Contains specific primers, nucleotides, <1% Dimethyl Sulfoxide, dye 4 ml

Denaturation Solution (DEN)
Contains <2% NaOH, dye 2.4 ml

Hybridization Buffer (HYB): contains 8-10% anionic tenside, dye 120 ml
Stringent Wash Solution (STR)
Contains >25% of a quaternary ammonium compound,
<1% anionic tenside, 120 ml
Rinse Solution (RIN): contains buffer, <1% NaCl, <1% anionic tenside 360 ml

Conjugate Concentrate (CON-C)
Contains streptavidin-conjugated alkaline phosphatase, dye 1.2 ml

Conjugate Buffer (CON-D)
Contains buffer, 1% blocking reagent, <1% NaCl 120 ml

Substrate Concentrate (SUB-C)
Contains Dimethyl Sulfoxide, substrate solution 1.2 ml

Substrate Buffer (SUB-D)
Contains buffer, <1% MgCl2, <1% NaCl 120 ml

Tray, evaluation sheet 4 of each

The isolated DNA samples from the process mentioned above were used for multiplex amplification using the following PCR program.

Amplification profile

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>5 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>58</td>
<td>2 min</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>25 s</td>
<td>10 cycles</td>
</tr>
<tr>
<td>53</td>
<td>40 s</td>
<td>30 cycles</td>
</tr>
<tr>
<td>70</td>
<td>8 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>4</td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>

Hybridization

1. Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15 minutes total).
2. Pipette 20 µl DEN (denaturing solution) to each well of tray to be used.
3. Add 20 µl of corresponding amplified DNA sample to each well, and mix well by pipetting up and down several times. Incubate at room temperature for 5 minutes.
4. Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided red pen or pencil.
5. Add 1ml HYB (hybridization solution) to each well and gently shake to homogenize solution.
6. Add 1 strip to each well with colour marker facing up. If strips turn over, reposition them with a fresh pipette tip.
7. Place tray in shaking water bath and incubate for 30 minutes at 45°C.
8. Pour off HYB into the sink, holding it low and close to the drain to avoid amplicon transmission. Remove remaining solution by forcefully tapping tray against paper towels on benchtop.
9. Add 1ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath.
10. Prepare diluted Conjugate and Substrate in 15 ml conical vials by diluting 1:100 with corresponding Con-D and Sub-D. Colours of small tubes (concentrates) correspond to colours of dilution buffer tubes. Wrap Substrate dilution in aluminium foil.
11. Completely remove Stringent Wash Solution. Completely remove STR as previously described for HYB removal.
12. Pour out Wash Solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper.
13. Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform (pour out RIN after incubation).
14. Add 1 ml of diluted Conjugate prepared in step 10 to each strip and incubate for 30 minutes on shaking platform.
15. Remove solution and wash for 1 minute with 1 ml RIN per well on water bath platform. Pour out solution and repeat rinse with 1 ml RIN per well for 1 min.
16. Remove RIN and wash with 1ml distilled water per well.
17. Remove water and add 1 ml of diluted substrate per well and incubate protected from light without shaking for about 4-5 minutes. Look for colour reaction to indicate reaction completion after 4-5 minutes. If colour reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes.
18. Stop reaction by briefly rinsing twice with distilled water.

19. Using tweezers remove strips from the tray and dry them between two layers of absorbent paper. And transfer strips to the Results Sheet provided with the kit.

![Diagram of MTBDRplus and MTBDRsl probe arrangement](image)

**Fig. 3** The Genotype MTBDRplus (Left) and MTBDRsl (Right) probe arrangement on nitrocellulose strip.

**Interpretation of results**

**MTBDRplus**: Each strip in MTBDRplus consist of 27 reaction zones (bands) including six controls (conjugate, amplification, *M. tuberculosis* complex, *rpoB*, *katG* and *inhA* controls), eight *rpoB* wild type (WT) and four mutant (MUT) probes, one *katG* wild type and two mutant and two *inhA* wild type and four mutant probes (Fig. 3 Left).

In order for results to be valid, CC (conjugate control) and AC (amplification control) bands must appear for every sample. The presence of TUB band indicates that *M. tuberculosis* complex is present in the sample. A mutation in the relevant gene (and resistance to the relevant drug) is signified by either an absent wild type band
and/or the presence of a mutant band for each gene cluster. The \( rpoB \), \( katG \) and \( inhA \) each have a control band which must be present in order to interpret the results. \( rpoB \) predicts RIH resistance, \( katG \) predicts high level INH resistance, \( inhA \) predicts low level INH resistance. Resistance to isoniazad \( i.e. \) either \( inhA \) or \( katG \) along with \( rpoB \) establishes MDR. For results to be valid the bands must be of intensity equal to or greater than the intensity of the AC band. In order for a batch of results to be valid, the negative control strip must have a CC and AC band present, but no other bands must be visible. If a positive result is obtained with the negative control, the results of the whole batch must be repeated and measures taken to remove amplicon contamination from all rooms and equipment. For the detection of rifampicin resistance, 8 wild type (WT) probes encoded amino acids 505-533 and 4 mutated probes (D516V, H526Y, H526D and S531L) were used. For detection of high level isoniazid resistance, a \( katG \) WT probe and 2 mutated probes S315T1 and S315T2 were used. For detection of low level isoniazid resistance, 2 WT \( inhA \) probes and 4 mutated probes included C15T, A16G, T8C and T8A were used.

MTBDRs/: Each strip in the MTBDRs/ consists of 22 reaction zones including six controls (conjugate, amplification, \( M.tuberculosis \) complex, \( gyrA \), \( rrs \) and \( embB \) controls). Three \( gyrA \) wild type and six mutant probes, two \( rrs \) wild type and two mutant probes and one \( embB \) wild type and two mutant probes (Fig.3 Right). Results were interpreted similarly as given in MTBDRplus. The \( gyrA \), \( rrs \) and \( embB \) predicts resistance to fluoroquinolones, aminogycosides/cyclic peptide and ethambutol respectively. Resistance to both \( gyrA \) and \( rrs \) establishes XDR.

3.9 STRAIN TYPING

Clinical isolates detected as non Mycobacterial tuberculosis along with our \( M.tuberculosis \) isolates by the different detection test was processed for ARDRA and 16S r RNA sequencing to evaluate the identification of Mycobacterial species. In the phylogenetic analysis, 4 accessions from Genbank were also added.

3.9.1 Amplified Ribosomal DNA Restriction Analysis (ARDRA)

The development of DNA amplification for the direct detection of mycobacteria from clinical samples has been a major goal of clinical microbiology during the last ten years. The application of ARDRA on the 16S – 23S rDNA region
of the mycobacterial genome proved to be a rapid, simple and reliable method for the differentiation of mycobacterial species.

**PCR amplification of 16S rRNA:** The amplification was done with the same thermal cycler protocol used above except for a different program, which is given below

**PCR reaction mix for strain typing**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/reaction</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR Buffer</td>
<td>10 µl</td>
<td>1x</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>4 µl</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>2 µl</td>
<td>200 µM of each dNTP</td>
</tr>
<tr>
<td>Primer (10 µl each)</td>
<td>2 µl (1+1)</td>
<td>0.5 µl each</td>
</tr>
<tr>
<td>Taq Polymerase (5U/µl)</td>
<td>0.5 µl</td>
<td>2.5 units/reaction</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10 µl</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>21.5 µl</td>
<td></td>
</tr>
</tbody>
</table>

**Primers**

1492r primer (5’-TACGGCTACCTTGTTACGACTT-3’)

27f primer (5’-GAGTTTGATCACTGGCTCAG-3’)

**Amplification profile**

95 °C 5 min 1 cycle

95 °C 1 min

60 °C 1 min 35 cycles

70 °C 1 min

70 °C 8 min

4 °C ∞

Amplified products were confirmed by electrophoresing at 80V for 30 min in 1% agarose gel and documented using Biorad Gel Documentation system.

**For Gel electrophoresis**

Agarose powder

TBE buffer
10x TBE buffer/liter

Tris base 108 g
Boric acid 55 g
0.5 M EDTA, PH8.0 40 ml

Make up the volume upto 1 liter with deionized distilled water.

1X Tris/Borate/EDTA (TBE) Electrophoresis Buffer. Store at room temperature (indefinitely).

Into a spigoted carboy, add 9 liters deionized or distilled water to 1 liter of 10X TBE electrophoresis buffer. Stir to mix.

Bromophenol blue dye
EtBr (1 mg/ml Staining Solution)
Loading buffer 6X

1% agarose gel was prepared by adding 1 g agarose to 100 ml of 1X TBE buffer. The solution was then heated in a microwave oven till the agarose granules completely melted in a uniform consistency.

Procedure: For the detection of amplified product, a 1% agarose gel was prepared and soaked in 1X TBE buffer. 10 µl of the amplified product was added with 6X loading dye and mixed. The mixture was added to the well of the gel. Molecular size marker (Sigma50bp DNA Ladder) with volume of 4 µl was added to first and last well of the gel. The electrophoresis set-up was 60V in 90 minutes. The gel was then stained in 0.5 µg/ml ethidium bromide for 20 minutes with mild shaking and then destained with tap water for 10 mins. The bands were visualized using Biorad Gel Documentation system.

All amplified and restricted product were post stained with EtBr for documentation with Biorad Gel documentation system.

10 µl of the amplified product were subjected to restriction digestion for ARDRA and remaining products were kept for sequencing.

Restriction digestion

Ten microliter of the amplified product amounting to 200 ng of DNA was restricted at 37°C for 1 hour with 2 µl of 10X reaction buffer, 0.5 µg Rsal and 0.2 µl BSA in a final volume of 20 µl. The same process was carried out for HaeIII...
restriction enzyme. To achieve a final image with standardized intensity, all samples were electrophoresed on an agarose gel and restricted DNA concentrations were compared visually to ensure that similar quantities are of DNA were loaded to the agarose gel.

**For Restriction Digestion**

- RE used: BsaI and HaeIII
- Sterile Water: 5.3 μl
- Buffer 10X: 2 μl
- Acetylated BSA 10 μg/μl: 0.2 μl
- DNA ~1 μg: 10 μl of PCR product
- RE 10 U/μl: 0.5 μl

**Gel electrophoresis**

For the detection of this restricted product, a 1% agarose gel was prepared and soaked in 1X TBE buffer. 10 μl of the amplified product was added with 6X loading dye and mixed. The mixture was added to the well of the gel. Molecular size marker (Sigma50bp DNA Ladder) with volume of 4 μl was added to first and last well of the gel. The electrophoresis set-up was 60V in 90 minutes. The gel was then stained in 0.5 μg/ml ethidium bromide for 20 minutes with mild shaking and then de-stained with tap water for 10 mins. The bands were visualized using Biorad Gel Documentation system.

**Data scoring of band patterns**

Presence and absence of bands were manually scored and recorded as a matrix. Bands were scored as 1 for presence and 0 for absence.

**Cluster analysis**

Clustering among individuals were determined by the distance matrix method. Jaccard coefficients were calculated for all pair-wise comparisons between individual samples to provide a distance matrix. A dendrogram was constructed from this matrix based on the hierarchical cluster analysis, which is based on the average linkage between groups, i.e. the un-weighted pair-group method algorithm (UPGMA) as described by Sneath et al. (1973). All clustering analysis were performed in NTSYSpc 2.1 ver.
3.9.2 DNA sequencing and phylogenetic analysis

Samples to be sequenced were sent to Xlcelris Genomic for further processing as the sequencing provision was not available at the study site.

Phylogenetic analysis was conducted with MEGA 5.0. First, pairwise alignment using UPGMA was carried out with Muscle. Alignments were then manually checked for indiscrepencies. The phylogram was conducted using the neighbour-joining method with gaps being treated as complete deletions. To get more resolved tree, gene sequence of four *Mycobacteria* species as listed in Table 5 was downloaded from Genbank and included in the analysis. Bootstrap analyses of 500 replications were also conducted.

**Table 5** List of Mycobacteria species and their genebank numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Genbank Number (Accession Number)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. kansasii</em></td>
<td>M29575</td>
</tr>
<tr>
<td><em>M. simiae</em></td>
<td>X52931</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>M29566</td>
</tr>
<tr>
<td><em>M. chelonae</em></td>
<td>AJ419969</td>
</tr>
</tbody>
</table>

3.10 STATISTICAL ANALYSIS

Calculation of sensitivity, specificity, negative predicative value (NPP), positive predictive value (PPV) and accuracy for comparison of performance of drug susceptibility test of different methods were done using MedCalc software version 13.02 with 95% confidence interval level. Microsoft excel sheet was utilized for generation of graphs for the study.
Terminology and formula for statistical analysis of:

Sensitivity = \((TP/TP+FN) \times 100\)

Specificity = \((TN/TN+FP) \times 100\)

Positive predictive value (PPV) = \((TP/TP+FP) \times 100\)

Negative predictive value (NPV) = \((TN/TN+FN) \times 100\)

Accuracy = \((TN+TP/TP+TN+FN+FP) \times 100\)

NOTE: TP= true positive (disease and tested positive)

TN=true negative (no disease and tested negative)

FP=false positive (no disease and tested positive)

FN=false negative (disease and tested negative)