Chapter - 4

Materials & Methods
The present study “A study on Mycobacterial species causing lymph node tuberculosis” was carried out in the Department of Microbiology, Blue Peter Research Center (BPRC) - LEPRA Society, Hyderabad. The clinical specimens were obtained from the Institute of Chest Diseases, Irrumnuma, Hyderabad. The period of the study was from January 2004 to January 2006.

A total of 347 enlarged lymph node patients who attended the out patient department of Chest Diseases Hospital, Irrumnuma, Hyderabad and at BPRC, were screened for lymph node tuberculosis based on the clinical suspicion and by diagnostic algorithm defined by RNTCP, as shown below (CTD 1998)(fig 4.1).

**Fig 4.1 Diagnostic algorithm for TB lymphadenitis**

Lymph node enlargement of >2 cm in one or more sites, with or without periadenitis, with or without evidence of Tuberculosis elsewhere; or presence of an abscess with or without discharging sinus.

↓

Prescribe a course of antibiotics for two weeks

↓

If lymph node enlargement persists, suspect TB lymphadenitis
- Pus from discharging sinus / aspirate from lymph node using Fine Needle Aspiration Cytology (FNAC)
- Smear examination for AFB (using pus/aspirate) by Ziehl Nielsen’s method
- Mantoux test for children <14 years

Diagnosis confirmed if the pus / aspirate from FNAC show:
- ZN stain positive for AFB, or
- Granulomatous changes (where facilities available)

If FNAC results are inconclusive, excision biopsy is advisable for smear histo-pathological examination

Start Category III Treatment
Based on the above algorithm, a total of 200 lymph node tuberculosis patients formed the study material and the rest of the patients was excluded from the study.

Patient information, detailed clinical examination, history of previous episodes of TB and history of previous anti TB treatment were carefully obtained (annexure 1). All the patients were subjected to voluntary and confidential HIV testing. (The institutional ethical committee of Blue Peter Research Center approved the study protocol.) (Fig 4.2).
Materials & Methods

Enlarged lymph node → Fine needle aspirate

H & E staining
• Tuberculous Lymphadenitis.
• Granulomatous lymphadenitis
• Non Specific lymphadenitis.

ZN-staining
• Presence of AFB
• Without AFB.

Culture LJ/BacT
2- LJ & 1 BacT Bottle at 37°C

AFB Growth
No AFB Growth
(after incubation of 8 wks)

Rate of Growth
Slow Grower ( > 5 days onwards)
Rapid Growers (1-4 days)

Pigmentation
Photochromogen
Scotochromogen
Non Pigmentation

Growth on PNB
Bio chemical Tests
NRT, Niacin, Tween-80,
Arylsulfatase etc.,

Drug Susceptibility testing. (Isoniazid,
Rifampicin, Streptomycin,
Ethambutol)
(1% proportion method)

Non Tuberculous Mycobacteria/NTM

Mycobacterium tuberculosis- M.Tb
Annexure 1

Patient Details
Code No: Date of Sample Collection:

Name:
Age: Sex:
Address: Weight:

Complaints

History

Present

- Duration of Swelling:
- Presence of Similar Swelling any where Other than the presenting site:
- Swelling associated with Fever / Cough:
- Periodicity of Fever (if any):
- Cough (duration in days):
- Associated Hemoptysis:
- Loss of Appetite:
- Loss of Weight:

Past

- Had any similar Swelling / Complaints:
- Had any past history of Tuberculosis:
Had any other diseases (Please Specify)

Allergy: Tonsillitis: Diabetes: Hepatitis: HIV:

Thyroid: Alcohol: Smoking Habit: Steroid Use:

**Treatment History**

History of Anti-TB treatment

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History of any other treatment:

Family History / History of Contact

Any member of family / neighbors who are either suffering with pulmonary TB / has taken treatment for tuberculosis (Yes / NO):
If YES – Details

Clinical Examination

Features of the Lymph node enlargement

- Anatomical site of swelling :
- Shape :
- Size :
- Number (single /multiple) :
- Discrete / matted :
- Attached with the under lying tissues/ skin :
- Consistency :
- Discharge/ sinuses :
- Any abrasions or injury in the draining area of lymph node :

General Examination
Investigations

- Total WBC count : 
- Differential WBC count : 
- ESR : 
- Hemoglobin : 
- Mantoux Test : 
- Chest X ray : 
- Sputum AFB : 
- Sputum Culture : 
- Any Other (Specify) : 
- H I V sero-positivity :
4.2. Specimen Collection

Fine Needle aspiration of lymph node was done using 22-23gauge needle attached to a 10ml disposable plastic syringe, after fixing the node with the help of left forefinger and thumb, 2-3 passes were made into the lymph node (Witth. Vielh & Orell et al 1999). Aspirates from the involved lymph node were divided into two portions, one portion of the material expressed on to a clean grease free microscope glass slides. Two slides were fixed in 90% Isopropyl alcohol for routine haematoxylin & eosin staining. Two slides were air dried for Ziehl-Neelsen staining. The other aliquot meant for mycobacterial culture was rinsed with 0.5 ml of sterile distilled water, and processed immediately or preserved at 4 °C for not more than 24 hours. In the laboratory, the aliquot for culture was inoculated on two Lowenstein-Jensen slopes and on rapid culture system (MB BacT).

4.3 Laboratory procedures

4.3.1. Procedure for Haematoxylin and Eosin.

- Fixes the smears in 90% ethanol for 15 minutes.
- Immersed the slides in Harris Haematoxylin for 10-15 minutes.
- Washed the slides under running tap water (gentle) for 10 minutes.
- Dipped the smears in 0.5% eosin twice.
- Dehydrated the slides in ascending grade of alcohol (60%, 70%, and 90%).
Blot dried and mounted the slides with DPX (Diastrene Plasticene Xylene).

Observed the smear under scanner (4x), low power (10x), and Highpower (40x). (Bancroft JD 4th edn)

(All the lab procedures were as per the lab manual of Tuberculosis Research Center, Chennai)

4.3.2. Procedure for Ziehl-Neelsen staining.

- Air dried the aspirate on a clean, grease free glass slide.
- Heat fixed the smear.
- Flooded the slide with 1% carbol fuschin,
  - Heated the slide intermittently with stain until vaporization (without boiling).
  - Waited for 5 minutes and washed the slides with running tap water gently.
- Flooded the slide with 25% sulphuric acid for 2-3 minutes.
- Washed the slide under running tap water gently.
- Counter stained with 0.1% methylene blue solution for 30 seconds.
- Washed the slide under running tap water gently and air dried.

Observed the smear under 100x oil immersion. (RNTCP 2005)
4.3.3. Culture of the Aspirate

The LJ Media cultures were examined everyday for one week and thereafter once a week for 8 weeks. Once the growth appeared, it was confirmed by ZN staining and screened for NTM by testing tolerance of the isolates to para-nitrobenzoic acid (PNB) in a concentration of 500 mg/L incorporated in the LJ medium. Species identification of all the NTM was achieved by performing the battery of biochemical tests: i) assessment of photo reactivity of mycobacteria, 2) growth at 25°C, 37°C, and 44°C, 3) aryl sulphatase test, 4) tween -80 hydrolysis, 5) catalase test, 6) tellurite reduction test, 7) growth on MC Conkey agar and 8) resistance to thiophen-2-carbonic acid hydrazide as described below. All the Isolated Mycobacterium tuberculosis were tested for their antibiotic susceptibility testing for the first line drugs (isoniazid, rifampicin, streptomycin and ethambutol) using 1% proportion method.

4.4. Biochemical testing for speciation of Mycobacteria.

4.4.1 Preparation of Standard suspension for biochemical testing

Two thirds loopful of culture taken in 24 SWG nichrome loop with 3mm with internal diameter (to approximately coincide with a moist weight of 4 mg of the organism) was added to 0.2 ml of sterile distilled water in a 7 ml bijou bottle containing 6 glass beads. The bottle was mechanically shaken for one minute at a speed which just lifts the beads
from the bottom of bottle to produce a uniform suspension. Then 0.8 ml of sterile distilled water was added and the bottle was shaken in a vortex and the resultant suspension contained 4mg/ml of the organism. Using 24 SWG nichrome wire loop with a 3 mm external diameter. One loopful of the suspension was taken for performance of the individual tests or for sub culturing onto each slope of the medium. Positive and negative controls were included with each test.

4.4.2 Assessment of Photo reactivity of Mycobacterium species

Principle

The appearance of yellow pigment in the colonies of photochromogenic mycobacteria is the result of yellowish orange carotene crystals that are produced by active metabolism of the microorganisms on exposure to bright light. Scotochromogenic species have the capability of producing yellow pigment without exposure to light; however, the type of pigment is unknown. The pigment of young colonies of mycobacteria after growth in the dark or following exposure to light can be an important aid in the identification of certain Mycobacterium species.
Specimen

Primary broth culture of the test organism, diluted sufficiently to produce isolated colonies when inoculated on LJ Media and Middlebrook 7H10 agar plates.

Materials

A. Equipment

1. Biologic safety cabinet.

2. 37°C incubator.

B. Supplies.

1. Sterile screw cap test tubes, 20 x 110 or 20 x 125 mm

2. Sterile Pasteur pipettes

3. Inoculating wires and loops.

C. Medium.

1. Three slants of Lowenstein Jensen medium.

2. Three Middlebrook 7H10 agar plates.

Standards and Controls

1. Positive photochromogen: *M kansasii*.

2. Positive scotochromogen: Stock strains of *M scrofulaceum* or *M gordonae*.


(Standards & Controls were obtained from JALMA, institute for mycobacterial diseases, Agra)
Procedure

1. Inoculate the surfaces of three Lowenstein-Jensen slant media or three Middle book 7H11 agar plates with fluid from a diluted broth culture of the organism to be tested. Wraped two of the tubes or plates with aluminum foil; leave the third exposed to the ambient light in the incubator.

2. Incubated one of the wrapped tubes or plates at 25\(^0\)-30\(^0\)c; the other wrapped tubes or plates at 37\(^0\)c.

3. Light exposed control tube or plate is observed daily for any growth. Once a growth is seen on the light exposed tubes or plates, the wrapped ones are opened to check for growth.

4. If early growth is detected in the wrapped tubes or plates, expose one of each pair to strong light for approximately 5 hours. A 100 W tungsten bulb or fluorescent equivalent is adequate. Loosen the cap of the culture tube during this period of light exposure.

5. Following exposure to light, the tube or plate is returned to the incubator and inspected after 24 to 48 hours for the appearance of yellow pigment.

Interpretation

Mycobacteria that are scotochromogenic produce an equal amount of pigment whether light exposed or left in the dark. \textit{M scrofulaceum}, \textit{M gordonae}, \textit{M flavescens}, \textit{M xenopi} and \textit{M szulgai} (the latter is
scotochromogenic only when incubated at 37° C) compose the scotochromogenic group.

Mycobacteria that are photochromogenic produce yellow pigment only after exposure to light. The more commonly encountered photochromogens include *M kansasii*, *M marinum*, *M simiae* and *M asiaticum*.

Nonchromogenic mycobacteria are incapable of producing pigment either in the dark or after exposure to light. *M tuberculosis*, *M bovis*, *M ulcerans*, *M fortuitum*, *M chelonae*, and classic strains or *M avium* are the more commonly encountered nonchromogens.

(The procedures and their interpretation were strictly as per the laboratory manual of tuberculosis research center, Chennai)
4.4.3. Niacin Accumulation/ Niacin Test.

Principle

All the *Mycobacterial* species produce niacin ribonucleotide; however, virtually all strains of *M tuberculosis*, *M simiae* and some strains of *M chelonae* lack the enzyme to further convert niacin to nicotinamide adenine dinucleotide (NAD). Comparative studies have shown that, *M.tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M.tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests. Cultures grown on egg medium containing asparagines yield the most consistent results in the niacin test and LJ medium is therefore recommended. A culture must be at least three to four weeks old and must have sufficient growth of at least 100 colonies.

Materials

Equipments:

1. Biological Safety Cabinet
2. 37 C incubator.

Reagents

O-toludine, 1.5%

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>O-toludine</td>
<td>1.5 g</td>
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<tr>
<td>Ethanol</td>
<td>100 ml</td>
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</table>
Prepared fresh weekly an amber bottle and store in the dark in the refrigerator.

Cyanogen bromide solution, approx. 10%.: A saturated aqueous solution of cyanogen bromide is approx 10%. Stored at 4°C in the refrigerator.

Quality control
Positive control: *M. tuberculosis*
Negative control: *M intracellulare*

Procedure
1. Flooded the surface of the Lowenstein-Jensen slant over the heavy growth of the test organism with 1 ml of sterile water.
2. Stabed the medium with the tip of the pipette to allow access of the water to the underlying medium.
3. Tilt the tube so the water covers the surface of the slant. Let stand for 20 to 30 minutes.
4. Rotated the tube so that the slant faces downward. Carefully removed 0.6 ml of extract without touching the slant and transferred to the screw cap test tube.
5. Sequentially added 0.25 ml of o-toludine and 0.25 ml of 10% cyanogen bromide.
6. Closed the tube and observed the solution for the formation of a pink color (=positive) within 5 minutes.

7. Added 2-3 ml of 4% NaOH to each tube to neutralize cyanogen bromide before discarding.

Interpretation

The development of pink color indicates a positive test.

Precautions

Cyanogen bromide is a severe lacrimator and toxic, if inhaled. Work in a well-ventilated fume hood when preparing the solution and in a biological safety cabinet while testing cultures. In acid solutions, cyanogen bromide hydrolyses to hydrocyanic acid, which is extremely toxic. Discard all reaction tubes into a disinfectant solution made alkaline by addition of sodium hydroxide.
4.4.4. Nitrate Reduction test for Mycobacteria

Principle

Mycobacteria producing nitroreductase are capable of catalyzing the reduction of nitrate to nitrite. In reaction, oxygen is extracted from nitrate according to the following formula:

\[
\text{NO}_3^- + 2\text{e}^- + 2\text{H}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}
\]

The nitrate produced is detected by the addition of \(\alpha\)-naphthalamine and sulfanilic acid, forming the red diazonium dye, p-sulfobenzene-azo-\(\alpha\)-naphthalamine.

Specimen

Three to four week old culture of the test organism growing on Lowenstein Jensen medium.

Materials

Biological safety cabinet.

37\(^\circ\)C water bath or heating block

Reagents

(1). Nitrate test substrate

0.067M (M/15) phosphate buffer solution, \(pH\) 7.0

\[
\text{Na}_2\text{HPO}_4, \text{ anhydrous} \quad 9.47 \text{ g}
\]

Distilled water \quad 1 liter
Dissolved disodium phosphate in water to provide 0.067 M solution (Solution 1)

\[
\text{KH}_2\text{PO}_4 \quad 9.07 \text{ g} \\
\text{Distilled water} \quad 1 \text{ liter}
\]

Dissolved in water to give 0.067 M KH$_2$PO$_4$ solution (Solution 2)

Mix 61.1 ml of solution 1 with 38.9 ml of solution 2. Check pH.

Substrate: 0.1M sodium nitrate

\[
\text{Sodium nitrate} \quad 0.85 \text{ g} \\
\text{M/45 phosphate buffer, pH 7.0} \quad 100 \text{ ml}
\]

Prepared M/45 buffer by diluting M/15 buffer 1:2 with distilled water. Dissolved the sodium nitrate in the buffer and distributed in 2-ml amounts in screw-capped test tubes. Sterilized at 15 lbs pressure for 15 minutes and stored in the cold.

(2) Hydrochloric acid, 1:1

\[
\text{Hydrochloric acid, concentrated} \quad 10 \text{ ml} \\
\text{Distilled water} \quad 10 \text{ ml}
\]

(3) Sulphanilamide, 0.2%

\[
\text{Sulphanilamide} \quad 0.2 \text{ g} \\
\text{Distilled water} \quad 100 \text{ ml}
\]
(4) Coupling agent

\[
\text{N- (1-naphthyl)-ethylene diamine di-HCl} \quad 0.1 \text{ g} \\
\text{Distilled water} \quad 100 \text{ ml}
\]

(5) Zinc dust

Quality control

Positive control: \textit{M tuberculosis.}

Negative control: \textit{M avium complex}

Procedure

1. Emulsified one loopful of colonies from solid medium into 2 ml of nitrate substrate.

2. Shaken by hand to mix and incubated at 37°C for two hours.

3. Added reagents to mixture in the following order:
   a. One drop of concentrated HCL
   b. Two drops of 0.2% sulfanilamide.
   c. Two drops of 0.1% \textit{N-(1-naphthyl) ethylenediamine dihydrochloride.}

4. Allowed mixture to sit at room temperature for 5 minutes.

5. Read for the development of a pink to red color.
Interpretation

The appearance of a red or pink color is indicative of a positive test. A quantitative reading can be made by comparing with nitrate reduction standards.

Confirmed all negative reaction by adding a pinch of zinc dust. Development of a pink color at this stage indicates that the initial negative reaction was genuine. If no color change occurs after adding the zinc dust, the reaction has proceeded beyond nitrite into other components. Repeat the entire test.

4.4.5. Arylsulfatase Test

Principle

Arylsulfatase is an enzyme that splits free phenolphthalein from the tripotassium salts of phenolphthalein disulfite. The test for the identification of *Mycobacterium species* is performed in a tube containing a substrate of phenolphthalein in oleic acid agar (Wayner). After 3 (or 14) days of incubation of subculture of the unknown species, the appearance of a pink color after addition of sodium carbonate indicates a positive reaction.

Specimen

Mature colony of the unknown *Mycobacterium species* recovered from clinical material grown on an LJ slants or on Middlebrook 7H-10
Materials & Methods

agar. Prepare a suspension of the organism in sterile water and incubate for 3 days (or 14 days).

Materials

A. Equipment.

1. Biologic safety cabinet

B. Media.

1. Arylsulfatase stock Substrate:
Dissolved 2.6 g of phenolphthalein disulfate tripotassium salt in 50 ml of sterile deionized water. Sterilized by membrane filtration (0.22µm-pore size filter).

   a. Stored in the refrigerator at 2^0 - 8^0C.
   b. Shelf life is indefinite if stored properly. Discard if solution becomes cloudy.

2. Arylsulfatase broth: 3 days test.

   a. Aseptically added 2.5 ml of stock substrate to 200 ml of sterile Dubo’s Tween broth.
   b. Aseptically dispensed 2.0 ml amounts into screw cap test tubes (16 x 125 mm).
   c. Store at 2^0-8^0 C.

3. Arylsulfatase broth: 2 week test.

   a. Aseptically added 7.5 ml of stock substrate to 200 ml of sterile Dubo’s Tween broth.
b. Aseptically dispense 2.0 ml amounts into screw cap test tubes (16x25mm).

c. Store at 2°-8° C.

C. Reagents

2N Sodium carbonate (Na$_2$CO$_3$): Dissolved 10.6 g of anhydrous sodium carbonate in 100 ml of distilled water.

Quality Control

A. Three Day test

1. Positive Control: *Mycobacterium fortuitum* (if positive control is negative, repeat tests with a fresh subculture of the positive organism. If the negative controls are positive, repeat the tests with a new lot of media).

2. Negative Control: *Mycobacterium intracellulare*.

3. Uninoculated medium and reagent only: No color.

B. Fourteen Day test.

1. Positive control: *Mycobacterium fortuitum*

2. Negative control: *Mycobacterium intracellulare*.

3. Uninoculated medium and reagent only: No color.
Procedure

1. Inoculate each tube of substrate with a lightly turbid suspension of the test organism in sterile water. Thoroughly emulsified the culture in the broth.

2. Incubated them for 3 days or 14 days (2 weeks) at $35^0$ C in the non CO$_2$ incubator.

3. Following incubation, added 1 ml of the 2N sodium carbonate reagent, mixed and observed for color change.

Procedure Note:

Included an actively growing control organism with each batch of test. For slow growers used a 3 to 5 week subculture, for rapid growers, a 1- 3 week subculture. If the positive control is negative, repeated the tests with a fresh subculture of the positive organism. If the negative controls are positive, repeated the tests with a new lot of media.
4.4.6. Catalase 68°C Test:

Catalase is an enzyme that decomposes hydrogen peroxide ($H_2O_2$) into water and oxygen. Chemically, catalase is a hemoprotein, similar in structure to hemoglobin, except that the four iron atoms in the molecule are in the oxidized ($Fe^{3+}$), rather than the reduced ($Fe^{2+}$), state.

Principle

Catalase splits hydrogen peroxide into water and oxygen. The evolution of oxygen appears as bubbles. Some forms of catalase are inactivated by heating at 68°C for 20 minutes, a valuable identifying feature for certain *Mycobacterium* species. The hydrogen peroxide used for the identification of *Mycobacterium* species differs from that used to detect catalase in other types of bacteria, by using a 30% concentration (Superoxal) in a strong detergent solution (10% tween-80). The detergent helps disperse the hydrophobic tightly clumped mycobacteria from large aggregates to individual bacilli, maximizing the detection of catalase.

Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material, grown on an LJ slant or on Middlebrook7H10 agar.
Materials

1. 68°C water bath or heat block
2. Lowenstein Jensen deeps in 25x150 mm screw cap test tubes.
3. 30% hydrogen peroxide (commercially available as Superoxol)
4. M/15 phosphate buffer (0.067M).

Quality controls

Negative control: M tuberculosis; bubbles at 22-25°C, but not at 68°C.
Positive control: M fortuitum, bubbles at 22-25°C and at 68°C.

Procedure

1. Set up one screw cap tube per specimen to be tested.
2. Labeled each tube with the specimen number.
3. Added 0.5ml of sterile 0.067M phosphate buffer to each tube.
4. Inoculated the buffer with a spadeful of growth from an actively growing subculture of the organism to be tested (2-4 weeks old).
5. Thoroughly emulsified the culture in the buffer.
6. Incubated the tubes in a 68°C water bath for exactly 20 min.
7. Removed the tubes from the water bath. Cooled to room temperature.
8. Added 0.5ml of freshly prepared Tween 80 hydrogen peroxide reagent.
9. All the tubes to sit at room temperature for 20 minutes. Do not shake the tubes.

10. Visually observe for the evolution of bubbles.

Interpretation
The appearance of bubbles indicates a positive test; lack of bubbles is a negative reaction. *M tuberculosis* and other mycobacteria lose their catalase activity when heated to 68°C.

### 4.4.7. Growth on MacConkey Agar.

**Principle**
The ability to grow on special MacConkey agar, formulated without crystal violet, differentiates *M fortuitum* and *M chelonian*, which can grow within 5 days, from other rapidly growing mycobacteria that show only slight growth after 11 days.

**Specimen**
A mature colony of the unknown *Mycobacterium* species recovered from clinical material, growth on an LJ slant or on Middlebrook 7H-10 agar is tested.

**Materials**
MacConkey agar without crystal violet.

**Quality Control**
Positive control: *M. fortuitum*

Negative control: *M. phlei*.

**Procedure**

1. Inoculated a fresh MacConkey’s agar plate with 3 drops of the organism grown for 7 to 10 days in 7H9 broth and streaked for isolation.

2. Incubated at 28°C to 30°C for 11 days without CO₂.

3. At 5 and 11 days, visually observed the surface of the agar plate for the presence of growth.

**Interpretation**

A positive test for *M. fortuitum* will show growth along the entire streak area and possibly a color change in the medium in 5 days. The absence of growth indicates a negative test.

**4.4.8. Tween-80 hydrolysis**

**Principle**

Tween 80 is the trade name of the polyethylene derivative of sorbitan monooleate. Some *Mycobacterium* species possess an enzyme that releases oleic acid from the tween 80. The color change from orange to pink is due to hydrolysis of tween 80, which modifies the optical rotation of light passing through the substrate.
Specimen

Mature colony of the unknown *Mycobacterium* species recovered from clinical material growth on an LJ slant.

Materials & Reagents

1. Biological safety cabinet
2. Screw cap test tubes.
3. M/15 phosphate buffer, pH 7

0.067M (M/15) phosphate buffer solution, pH 7.0

\[ \text{Na}_2\text{HPO}_4, \text{anhydrous} \ 9.47 \text{ g} \]
\[ \text{Distilled water} \ 1 \text{ liter} \]

Dissolved disodium phosphate in water to provide 0.067 M solution (Solution 1)

\[ \text{KH}_2\text{PO}_4 \ 9.07 \text{ g} \]
\[ \text{Distilled water} \ 1 \text{ liter} \]

Dissolved in water to give 0.067 M KH₂PO₄ solution (Solution 2)

Mixed 61.1 ml of solution 1 with 38.9 ml of solution 2. Check pH.

4. Tween-80.

5. Neutral red solution: Dissolved 0.1 gm of neutral red powder in 100 ml of distilled water.
6. Mixed 0.5 ml of Tween-80 with 100 ml of phosphate buffer. Added 2 ml neutral red solution (substrate). Dispensed in 2 ml substrate in screw-capped test tubes and sterilized at 15 lbs pressure for 15 minutes. Stored in amber color tubes in the cold.

Quality control

Positive control: *M. kansasii*

Negative control: *M. intracellularare* / Uninoculated tubes of substrate.

Procedure

1. Inoculated a loopful of the organism to be tested to the substrate.
2. Incubated at 37°C in the dark with tight caps.
3. Visually read tubes at 24 hours. If negative, read again at 5 and 10 days.
4. Compared the color of the liquid with that in the control tubes.

Interpretation

A positive result is recorded when the liquid, not the cells, turn from light orange to pink or red. *M. kansasii* usually turns positive within 24 hours. Read again at 3, 5 and 10 to 12 days. Record results and discard positives. Discard all tubes at 12 day.
4.4.9. Tellurite reduction

Reagents:

1) Middlebrook’s 7H9 liquid medium:
Omit glycerol and supplement with 0.05% Tween-80 by adding 5 ml of 10% solution per liter of medium. Dispense aseptically in 5 ml amounts in sterile universal containers.

Aqueous 0.2% potassium tellurite solution. Distribute in several tubes in 2 ml amounts. Sterilize and refrigerator.

Procedure:

Inoculated the 7H9 medium with one loopful of culture from L-J medium.

Incubated at 37°C for 7 days.

Removed from incubator, added aseptically 2 drops of the tellurite solution.

Reincubated at 37°C for 72 hours.

Removed from incubator and read without shaking the medium.

A black metallic precipitate of tellurium indicates a positive reaction.

Otherwise, report as negative.

Controls:

Uninoculated medium with reagent as negative control and *M. avium* complex as positive control.
4.4.10. **Sodium chloride tolerance**

**Principle**

Of the slowly growing mycobacteria, only *M triviale* grows in media containing 5% sodium chloride; of the medically significant rapidly growing mycobacteria, only *M cheloneae subsp cheloneae* fail to grow on such media.

**Specimen**

A 3 to 4 weeks old culture of the test organism growing on LJ medium.

**Medium**

To 100 ml of L-J medium add 5 g of sodium chloride and mixed well. Distributed in 5-6 ml amounts in universal containers and inspissated once at 85°C for 50 minutes. For controls use plain L-J medium without NaCl.

**Procedure**

- Inoculated one slope each of control and NaCl medium with one loopful of a standard suspension.
- Incubated at 37°C for 4 weeks.
- Read at weekly intervals.
• A positive test is indicated by growth on both the control and on the NaCl medium. If growth is seen only on the control slope, report as negative.

Control

Use *M. smegmatis* as positive control.

4.4.11. **Resistance to Thiophen-2-carbonic acid hydrazide, Hydroxylamine and *p*-nitro benzoic acid (PNB).**

**Media**

Stock solutions of inhibitors:

1. Thiophen-2-carbonic acid hydrazide (TCH): 1 mg/ml in 50% ethanol.
3. *p*-nitro benzoic acid (PNB): 50 mg/ml in propylene glycol.

**Complete medium**

Prepared 400 ml of L-J fluid. Distributed in 100 ml amounts in flasks. To each 100 ml portion added

- TCH stock solution, 0.1 ml (Final concentration 1 mg/l)
- HA stock solution, 1.0 ml (Final concentration 250 mg/l)
- PNB stock solution, 1.0 ml (Final concentration 500 mg/l)

Dispensed in universal containers and inspissated once.
Procedure

1. Inoculated each slope with one loopful of a standard suspension
2. Also inoculated one slope of plain L-J medium
3. Incubated at 37°C for 3-4 weeks
4. Recorded results as for standard sensitivity tests

Controls

*M.chelonei* is resistant to all four compounds while all the four compounds inhibit *M.tuberculosis*.

**4.4.12. Pyrazinamidase**

**Principle**

The deamidation of pyrazinamide to pyrazinoic acid in 4 days is a useful phenotypic characteristics by which *M marinum* (positive) can be differentiated from *M kansasii* (Negative) and by which weakly niacin-positive strains of *M bovis* (negative) can be distinguished for *M tuberculosis* complex (positive).

**Media/Reagents**

- Dubos Broth base (Difco) 6.5 g
- Distilled water 1 liter
- Dissolve the base in water. Then add,
Pyrazinamide 0.1 g
Sodium pyruvate 2.0 g
Agar 15 g

Heated to melt the agar. Dispensed in 5 ml amounts in screw-capped tubes. Autoclaved at 15 lbs/15 minutes. Allow to solidify upright.

1% Ferrous ammonium sulphate: Place 0.1 g of ferrous ammonium sulfate in sterile screw cap test tubes. Add 10 ml of sterile deionized water immediately before use, and allow the crystals to dissolve.

Quality control

Positive control: *M intracellulare*

Negative control: *M kansasii*

Procedure

1. Inoculated 2 tubes of the medium with a few drops of a heavy bacterial suspension.
2. Incubated at 37°C.
3. After 4 days, removed one tube and added 1 ml of freshly prepared ferrous ammonium sulphate solution.
4. Placed the tubes in the refrigerator for 4 hours.
5. Examined the tubes for a pink band in the agar. Used a white background and incident light.
Note: If the 4-day test is positive, it is not necessary to do the 7-day test.

Interpretation

After 4 hours, examine the tubes for a pink band in the reagent layer on the surface of the agar (positive reaction), using incident room light against a white background.

4.5. Antibiotic susceptibility testing

4.5.1. 1% PROPORTION METHOD

BACTERIAL SUSPENSION

A suspension is prepared by adding approximately 4 mg moist weight of a representative sample of the bacterial mass visualized as 2/3 loopful of 3mm internal diameter 24 SWG wire loop into 0.2 ml of sterile distilled water in a 7 ml Bijou bottle containing 10-12 glass beads (3 mm diameter). This is vortexed for 30 seconds to produce a uniform suspension. To this 3.8 ml of sterile distilled water is added to give a suspension containing approximately 1mg/ml (S1). This suspension is kept on the bench for 15-20 minutes to allow the coarser particles to settle down. From this suspension a 10-fold dilution is made by carefully adding 0.2 ml to 1.8 ml sterile distilled water (S2, 10⁻¹). Two further serial dilutions 10⁻² (S₃) and 10⁻³ (S₄) are prepared in a similar manner. One standard loopful (3 mm diameter, 27 SWG) is inoculated on to drug-free as well as drug-containing LJ slopes as indicated below:
Materials & Methods

<table>
<thead>
<tr>
<th>Suspension</th>
<th>Control drug-free</th>
<th>S*</th>
<th>H</th>
<th>R</th>
<th>E</th>
<th>PNB</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₁ (1 mg/ml)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>S₂ (10⁻¹)</td>
<td>x x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>S₃ (10⁻²)</td>
<td>x x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>S₄ (10⁻³)</td>
<td>x x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

* Dihydrostreptomycin is used in the proportion method.

The standard strain *M. tuberculosis*, H₃⁷Rv is tested with each new batch of medium. The recommended drug concentrations are 4 mg/l for dihydrostreptomycin, 0.2 mg/l for isoniazid, 40 mg/l for rifampicin and 2 mg/l for ethambutol. It is necessary to convert the required amount of the substrate salt to the free base (e.g. streptomycin or dihydrostreptomycin which is supplied as the sulphate and for ethambutol which is supplied as the dihydrochloride). Also, the potency of the product should be taken into consideration while preparing the drug media.

**Incubation And Reading**

Incubate the slopes at 37°C.
Read the growth at 28 days and again at 42 days.

Record growth as

- **++** Confluent growth
- **+** More than 100 colonies
- 1-100 cols. The actual number of colonies

When the number of colonies on a given dilution is less than 5, count the number of colonies with the next larger inoculum, or estimate if more than 100. (Make no attempt to estimate the number of colonies if the growth is **+++**)

Interpretation of Test.

Interpretation of all tests is based on the 42-day readings. However, if a strain shows clear-cut resistance based on the 28-day reading, no further reading is needed and the report may be sent as such. Strains that are susceptible at 28 days must be read again at 42 days and the report is based on the later reading only. For each strain, express the number of organisms resistant to each drug concentration as a percentage of the number of organisms growing on the drug-free slope. Make the selection of slopes for estimating the growth on the drug-free and drug containing media in the following order of preference:

Drug-free slope:

- 20-70 colonies
5–19 colonies

More than 70 colonies

Drug-containing slope:

5-100 colonies in the same row or the row nearest to the control slope

1-4 colonies in the same row or the row nearest to the control slope

No colonies in the farthest row

More than 100 colonies, if there are no other acceptable counts.