2 MATERIALS AND METHODS
2.1 Non-tuberculous Lung Mycobacteriosis in Gujarat

The study is based on the patients admitted in the K.J. Mehta Tuberculosis Hospital, Amargadh (Gujarat) and was carried out for four consecutive years, from July 1983 to June, 1987.

Only residents of Gujarat were included and recent migrants to Gujarat from other States were excluded. Outpatients were not included. Relapses, if admitted again in the study years were included only if non-tuberculosis mycobacteria (NTM) were isolated, but if tubercle bacilli were isolated again, the patient was not included in the study. When several specimens of sputum from the same patient were examined, the organisms from the first culture were included. Thus, sputum specimens from a total of 4,599 patients were examined.

2.1.1 Staining and microscopy for acid-fast bacilli

(Technical guide of IUATLD, 1978)

The Ziehl-Neelsen staining method was used for the microscopic examination of sputum smears to detect acid-fast bacilli.

A smear was prepared from the purulent portion of the sputum and fixed by passing three times through
the flame of the Bunsen-burner. A piece of filter paper was placed over the smear and covered with Ziehl-Neelsen Carbol fuchsin. The slide was gently heated to steaming and allowed to stand for five minutes without further heating. The slide was kept moist by adding more carbol fuchsin, if necessary, without additional heating. After five minutes, the paper strip was removed. The slide was washed with tap water and drained. The smear was decolourized with acid-alcohol solution for three minutes and rinsed with tap water. The smear was decolourized again for 1-3 minutes until no more colour appeared and then rinsed with tap water. The slide was then flooded with methylene blue counterstain for one minute and washed with tap water, drained and allowed to air-dry.

The smear was examined under oil-immersion lens. The acid-fast bacilli are stained red and the background material is stained blue (Fig. 3).

Formulation of Reagents:

A. Carbol fuchsin :
- Saturated alcoholic basic fuchsin ... 10.0ml
  (prepared by dissolving 3.0g basic fuchsin (Merck) in 100ml of 95% of ethyl alcohol)
- 5% aqueous solution of phenol ... 90.0ml
Fig. 3. *M. tuberculosis* in sputum stained with Ziehl-Neelsen stain (x1000).

Fig. 4. Niacin test.
2.1.2 Isolation and cultivation of mycobacteria

Isolation and cultivation of mycobacteria was carried out by employing modified Petroff's method as follows.

Sputum (max. 4ml) was collected in a sterile wide mouth screw capped bottle and equal volume of 4% NaOH was added. The specimen was mixed on the Vortex mixer until it is liquified and then left at room temperature for 15 minutes. After 15 minutes, sterile distilled water was added as diluent to stop the further action of alkali. This was then centrifuged at 3,000 r.p.m. (1800 xg) for 15 minutes, and the supernatant was discarded. Sterile distilled water was added to sediment, mixed and again centrifuged at 3,000 r.p.m. (1800 xg) for 10 minutes. Supernatant was discarded. Sediment was examined microscopically and inoculated with 4.0mm loop into 2 slopes of Lowenstein-Jensen (L.J.) medium which were then incubated at 37°C. Slopes were
observed daily for the first week to pick up any rapidly growing strain and then once a week for eight weeks before discarding them as negative.

Preparation of modified Lowenstain - Jensen Egg medium:

- Mineral salt solution:
  - Potassium dihydrogen phosphate $\text{KH}_2\text{PO}_4$ anhydrous 2.4g
  - Magnesium sulfate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.24g
  - Magnesium citrate 0.6g
  - Asparagine (Loba, India) 3.6g
  - Glycerol (reagent grade) 12.0ml
  - Distilled water 600.0ml

  The ingredients were dissolved by heating, autoclaved at 121°C for 25 minutes, and then cooled to room temperature.

- Malachite green solution: 2% solution of malachite green was prepared in sterile water under sterile conditions.

- Preparation of complete medium:
  - Mineral salt solution 600ml
  - Malachite green solution 20ml
  - Homogenized whole eggs 1000ml

  The complete medium was mixed and distributed in 5ml amounts in sterile 1 oz. (McCartney) bottles. The tubes were
then slanted and coagulated by inspissation at 85°C for 60 minutes. The medium was incubated at 37°C for 48 hours as a sterility check, and then stored in refrigerator.

2.1.3 Screening for non-tuberculous mycobacteria

A group of standard mycobacterial cultures was obtained from Trudeau Mycobacterial Culture Collection, National Jewish Hospital and Research Centre, Denver, Colorado, U.S.A. All the tests for screening and identification of mycobacteria were monitored by including standard mycobacterial culture as known positive and negative controls.

Acid-fast organisms isolated were first examined microscopically using the Ziehl-Neelsen staining method for their acid-fastness, bacterial morphology i.e., approximate length of bacteria, the presence or absence of cross-barring and cord formation (compact grouping of bacilli) (Tsukamura, 1975, 1984).

Screening for non-tuberculous mycobacteria was carried out by the combination of the three tests (Tsukamura, 1981b) described below.
(i) Niacin production test (Konno, 1956; Wayne et al., 1976)

Tests for niacin production were done twice on a 4 week old culture and a 6 week old culture. Test strain was cultivated on two slants of L.J. egg medium for 4 weeks and 6 weeks. The culture slant was flooded with 1.5ml of sterile water. If the growth on the medium was completely confluent, the surface was punctured to bring the liquid in contact with the medium. The slant was laid flat for 60 minutes and then raised to an upright position for 5 minutes to permit water to drain to the bottom. The aqueous extract (0.5ml) was removed to a screw-capped test tube, to which 0.5ml of aniline-ethanol solution (4ml of aniline + 96ml of 95% ethanol) and then 0.5ml of 10% cyanogen bromide (Sisco, India) solution (10g of BrCN in 90ml of distilled water) were added in sequence. The tube was capped and examined for development of a definitive yellow colour within 5 minutes (Fig. 4).

(ii) Test for the growth on p-nitrobenzoic acid (PNB) medium (Tsukamura and Tsukamura, 1964; Wayne et al., 1976)

p-nitrobenzoic acid medium is the L.J. egg medium containing p-nitrobenzoic acid, 0.5mg/ml.
P-nitrobenzoic acid was dissolved in propylene glycol at a concentration of 25mg/ml. Two volumes of this solution were added to 100 volumes of the L.J. egg medium before inspissation. The medium was poured in 5ml quantities in McCartney bottles and made as slopes by inspissation at 85°C for 60 minutes. PNB-medium and plain L.J. medium were inoculated by one loopful of the test organisms incubated at 37°C and observed weekly for the growth.

(iii) Test for the growth on hydroxylamine medium (Tsukamura, 1965c, 1970)

The hydroxylamine medium is the L.J. egg medium containing hydroxylamine hydrochloride, \( \text{NH}_2\text{OH.HCl} \), 125μg/ml.

The hydroxylamine hydrochloride (Polypharm, India) was dissolved in distilled water at concentration of 12.5mg/ml. One volume of this solution was added to 100 volumes of the L.J. egg medium before inspissation. The medium was poured in 5ml quantities into McCartney bottles and made as slopes by inspissation at 85°C for 60 minutes. Hydroxylamine medium and plain L.J. medium were inoculated with one loopful of the suspension of the test organisms, incubated with one loopful of the suspension of the test organisms, incubated at
As shown in Table 2.1., if the test organisms showed negative niacin production test and growth on the PNB and/or hydroxylamine medium, organisms were suspected to be of NTM, and were subjected to further identification tests.

2.1.4 Identification of mycobacteria

The following cultural and biochemical tests were performed on the test strains, and the final identification was decided by comparing the overall similarity of the characteristics of the test strains with the characteristics of the named species, as shown in Table 2.2 (Tsukamura, 1975, 1984; Vestal, 1975; Kent and Kubica, 1985; Wayne and Kubica, 1986). 

1. Colony morphology, colony pigmentation and photochromogenicity (Wayne et al., 1974)

These characters were obtained using L.J. egg medium. Two tubes of L.J. Medium were inoculated with small inoculum so that isolated colonies would be obtained. One tube was covered with the black paper to shield it from light and incubated at 37°C. Another tube was incubated at 37°C under exposure to light (30 watts at a
Table 2.1.
Screening of non-tuberculous mycobacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Niacin test</th>
<th>Growth on PNB-medium</th>
<th>Growth on Hydroxy lamine medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

mycobacteria
Table 2.2
Identification of clinically significant mycobacteria

<table>
<thead>
<tr>
<th>CHARACTERISTICS</th>
<th>SPECIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Colony morphology</td>
<td>R S R SR SX S R S S R S SR S R S S S S S R S</td>
</tr>
<tr>
<td>2. Colony pigmentation in dark</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>3. Photomorphogenicity</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>4. Growth in 3 days</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>5. Growth at 30°C</td>
<td>+ + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>6. Catalase Semiquantitative, &gt;45 mm</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>7. Arylsulfatase 3 days</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>8. Nitrate reduction</td>
<td>+ + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>9. Tellurite reduction</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>10. Tween hydrolysis: 7 days</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>11. Iron uptake</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>12. Growth on 5% NaCl</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>13. Growth on TCH</td>
<td>+ + + + + + + + + + + + + + + + + + + + +</td>
</tr>
<tr>
<td>14. Growth on MacConkey's agar</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>15. Growth on hydroxylamine: 250 µg/ml</td>
<td>- - - - - - - - - - - - - - - - - - - -</td>
</tr>
<tr>
<td>16. Pyrazinamidase: 4 days</td>
<td>+ + + + + + + + + - - - - - - - - - - -</td>
</tr>
<tr>
<td>17. Urease</td>
<td>+ + + + + + + + + + + + + + + + + + + + +</td>
</tr>
</tbody>
</table>

1. * indicates majority of strains positive. † indicates majority of strains negative.
2. If the space is blank, sufficient data were not available, or test is of no apparent value. V=Variable results.
4. M. avium is photomorphogenic at 25°C and scotomorphogenic at 37°C.
5. Growth is rapid at 30°C but slow at 37°C.
6. 50% strains show positive reaction.
7. Strains of M. tuberculosis resistant to pyrazinamide are often pyrazinamidase negative.
8. Within M. terrae complex, M. nonchromogenicum usually "*", and M. terrae usually "-".
distance of 40cm). The incubation was continued, with loose caps, until definite growth was observed on unshielded culture. Presence or absence of pigmentation was noted on both shielded and unshielded cultures. The shielded culture was then exposed to continuous light for 8, 24 and 48 hours and any change in the pigmentation was noted (Fig. 5).

<table>
<thead>
<tr>
<th>Record Colony Pigmentation</th>
<th>in the dark</th>
<th>under exposure to light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photochromogenic</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Scotochromogenic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nonphotochromogenic</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Colony morphology, described as rough, smooth, domed, etc., (Vestal, 1975) was recorded.

2. Growth rate (Tsukamura, 1975)

One loopful of the test organism was inoculated into the L.J. egg medium and incubated at 37°C (or at optimum temperature for growth if it was 28°C or 45°C). When the test organism showed abundant growth after 3 days, it was regarded as a rapidly growing Mycobacterium.
Fig. 5. Photochromogenicity of *M. kansasii* (Test strain T1).

Fig. 6. Semiquantitative catalase test.
Fig. 7. Nitrate reduction test.
ARYLSULFATASE TEST

Fig. 8. Arylsulfatase test.

Fig. 9. Pyrazinamidase test.
not so, it was regarded as a slowly growing. Slowly growing mycobacteria show usually the growth of discrete colonies after 7 days or more.

3. Growth temperature (Tsukamura, 1967; Marks, 1976)

One loopful of the test organism was inoculated onto four slants of L.J. egg medium, and these were incubated at 30°C, 37°C, 42°C, 45°C and 52°C. The growth was observed weekly for 4 weeks and recorded. The presence of abundant membraneous growth was recorded as positive.

4. Semiquantitative catalase test (Kubica et al., 1966)

The L.J. egg medium was poured in 5ml quantities into sterile 125 by 16mm screw-cap tubes and inspissated at 85°C for 60 minutes with tubes in an upright position to form a butt of solid medium. Three drops of a bacterial suspension, 20mg wet weight/ml were inoculated to the surface of butt medium and incubated at 37°C with caps loose for 2 weeks. To the surface of 2-week old culture, 1.0ml of Tween-peroxide mixture, prepared by mixing 0.5ml of 30% H₂O₂ plus 0.5ml of a 10% sterile Tween 80 (Sigma) solution was added, and allowed to stand at room temperature for 5 minutes. The height of the column of bubbles above the surface of the medium
was measured in millimetres and recorded. The presence of more than 45mm column of bubbles was regarded as strongly positive, less than 45mm as weakly positive, and no bubbling was regarded as negative (Fig. 6).

5. Catalase activity at pH 7/68°C (Kubica et al.,)

0.5ml M/15 phosphate buffer pH 7 was taken in a screw-cap tube with a sterile pipette. Several colonies of the test organism were emulsified into the buffer with a sterile loop. The tube containing emulsified colonies was placed in a water bath at 68°C for 20 minutes. The tube was then removed from heat and cooled to room temperature. 0.5ml of Tween-peroxide mixture (0.5ml of 30% H₂O₂ + 0.5ml of 10% sterile Tween 80 solution) was added to the tube and formation of bubbles appearing on the surface of the liquid was observed for 20 minutes before discarding it as negative.


Substrate M/100 sodium nitrate in M/45 phosphate buffer, pH 7.0, was prepared as follows:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>0.085g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.117g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.485g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100.000ml</td>
</tr>
</tbody>
</table>
Test Solutions:

No. 1 A 1:2 dilution of concentrated HCl (10ml HCl to 10ml of H₂O).
No. 2 0.2g sulfanilamide was dissolved in 100ml H₂O.
No. 3 0.1g n-naphthylethylenediamine dihydrochloride was dissolved in 100ml H₂O.

Substrate and reagents were stored in the dark in the refrigerator.

Procedure: 3 or 4 drops of sterile distilled water were taken in a sterile screw-cap test-tube. One loopful of colonies of the test organisms from the solid medium was emulsified in the water, to which 2 ml of the NaNO₃ substrate solution was added and shaken by hand to mix. It was then incubated at 37°C in a waterbath for 2 hours. After removing from the waterbath, 1 drop of Reagent No.1, 2 drops of Reagent No.2 and 2 drops of Reagent No.3 were added to the tube. Development of pink or red colour showed the presence of nitrate and the test was regarded as positive (Fig. 7).


Stock substrate: 2.6 g phenolphthalein disulfate, tripotassium salt (Koch-Light Laboratories, U.K.) was
dissolved in 50ml distilled water (0.08M solution), sterilized by membrane filtration and stored in refrigerator.

Two flasks, each with 200ml of the sterile Dubos broth medium were prepared. 9.5ml of the 0.8M stock substrate was added to 200ml sterile liquid medium to obtain 0.001M substrate for the 3-day test. 7.5ml of the 0.08M stock substrate was added to 200ml sterile liquid medium to obtain 0.003M substrate for the 2-week test. Each substrate was aseptically dispensed in 2ml amounts into sterile 125 by 16mm screw-cap tubes. As a quality control check, a few drops of sodium carbonate were added to a tube of each uninoculated substrate, which should remain colourless.

The tubes, for 3-day and 2-week test, were inoculated with a loopful of organisms from freshly grown culture, and incubated at 37°C. After 3 days of incubation of the 0.001M substrate, not more than 6 drops of 2N sodium carbonate (Prepared by dissolving 10.6g anhydrous Na₂CO₃ in 100ml H₂O) were added to the tube. This was added to the 0.003M substrate after 2-weeks of incubation. The appearance of a red colour showed a positive reaction (Fig. 8).

8. Tween 80 Hydrolysis (Wayne et al., 1964)

The substrate was prepared by combining 100ml M/15
phosphate buffer, pH 7.0, 0.5ml Tween 80 (Sigma), and 2ml of 0.1% aqueous neutral red stock solution. The substrate was dispensed in 2.0ml amounts into 125 x 16mm screw-cap tubes and autoclaved at 121°C for 10 minutes. The tubes were kept in a refrigerator.

One loopful of the test organisms was inoculated into the substrate and incubated at 37°C. The colouring of the fluid in the tube, not the cells, was observed after incubation for 7 days and for 14 days. The appearance of a pink or red colour was regarded as positive reaction.

9. Tellurite reduction test (Kilburn et al., 1969)

5ml of Middlebrook 7H-9 liquid with Tween 80, in 20 x 150mm screw-cap round bottom tubes, was inoculated with a loopful of test organisms from the fresh culture, and incubated at 37°C for 7 days, when the growth should be heavily turbid. With a capillary pipette, 2 drops of 0.2% potassium tellurite (Prepared by dissolving 0.1g potassium tellurite (BDH) in 50ml distilled water, dispensing in several tubes in 2ml amounts and autoclaving at 121°C for 10 minutes) were added to the tubes which were reincubated at 37°C. On the third day following reincubation, the tube was observed, without shaking, for the presence of black
metallic precipitate of tellurite, which showed a positive reaction.

10. Pyrazinamidase test (Wayne, 1974)

The Wayne pyrazinamide medium was prepared as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dubos broth base (Hi-media, India)</td>
<td>6.5g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0ml</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>0.1g</td>
</tr>
<tr>
<td>Pyruvic acid, sodium salt</td>
<td>2.0g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
</tbody>
</table>

The agar was melted by heating and then the medium was dispensed in 5ml amounts to 16x125mm screw-cap tubes, autoclaved at 121°C for 15 minutes. Medium was allowed to harden upright (not slanted).

Heavy inoculum from a fresh culture slant was placed on the surface of each of the two tubes of medium, and the tubes incubated at 37°C. After 4 days, one tube was removed from the incubator and 1ml freshly prepared 1% ferrous ammonium sulfate solution was added to the tube. The tube was placed in the refrigerator to prevent the growth of contaminants. After 4 hours, the tube was examined for a pink band in the agar. After 7 days' incubation, the second tube was removed from the incubator and developed as described above. The presence of a pink band in the agar was regarded as positive reaction (Fig. 9).
11. Urease test (Steadham, 1979)

The substrate was prepared as follows:

- Peptone: 1g
- Dextrose: 1g
- NaCl: 5g
- KH$_2$PO$_4$: 0.4g
- Urea: 20g
- Phenol red solution: 1ml
- Distilled water: 1 l

The ingredients were dissolved in distilled water. The final pH was adjusted to 5.8 ± 0.1 with NaOH and sterilized by membrane filtration through 0.22 micron pore size filters. This was then dispensed in 1.5ml amounts into 18 x 125mm sterile, screw-cap tubes aseptically, and stored at 5°C for up to 2 months.

A spadeful of growth from a young, actively growing slant was inoculated into the urea broth, and incubated at 37°C. The broth was observed at 1, 3 and 7 days for the colour change from yellow to dark pink, which was regarded as positive. A light pink colour was recorded as negative or doubtful and the test was repeated.

12. Hydroxylamine medium (Tsukamura, 1965c)

L.J. egg medium containing hydroxylamine hydrochloride (NH$_2$OH·HCl), 250 µg/ml and 500 µg/ml, was prepared.
Hydroxylamine hydrochloride was dissolved in distilled water at concentration of 25mg/ml and 50mg/ml. One volume of these solutions was added to 100 volumes of L.J. egg medium before inspissation. The media were poured in 5ml quantities into McCartney bottles and inspissated at 85°C for 60 minutes in slanting position.

The NH$_2$OH-medium and plain L.J. medium were inoculated with one loopful of the test organisms, incubated at 37°C and observed for the growth.


The TCH sensitivity was tested on L.J. egg medium containing TCH, 1 µg/ml.

One volume of a TCH solution, 100 µg/ml, was added to 100 volumes of the L.J. egg medium before inspissation, poured in 5ml quantities in screw-cap tubes and inspissated at 85°C for 60 minutes in slanting position. The TCH medium and plain L.J. egg medium were inoculated with one loopful of the test organisms, inoculated at 37°C and observed weekly for the growth.
14. Sodium Chloride tolerance (Kestle et al., 1967)

Sodium Chloride (NaCl) tolerance was tested on L.J. egg medium containing 5% NaCl.

5g of NaCl was added to 100ml of L.G. egg medium before inspissation. The medium was poured in 5ml amounts in screw-cap tubes and made as slopes by inspissation at 85°C for 60 minutes. The NaCl test medium and plain L.J. medium was inoculated with one loopful of test organisms, incubated at 37°C and observed weekly for the growth.

15. Iron uptake (Wayne and Doubek, 1968)

One loopful of test organism was inoculated onto the L.J. egg medium, and the medium incubated at 37°C ununtil obvious growth appeared. To the cultures growth and to the uninoculated control medium, 5 drops of sterile 20% ferric ammonium citrate (Prepared by dissolving 20g ferric ammonium citrate, green, in 100ml of distilled water, dispensing in small containers and autoclaving it) were added, the medium reincubated for a maximum of 21 days and observed weekly. A positive reaction was seen as the appearance of a rusty brown colour in the colonies and a tan discolouration of the medium.
16. Growth on the MacConkey's agar (Jones and Kubica, 1964)

MacConkey's agar was prepared as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>20g</td>
</tr>
<tr>
<td>Sodium taurocholate (commercial)</td>
<td>5g</td>
</tr>
<tr>
<td>Water</td>
<td>1000ml</td>
</tr>
<tr>
<td>Agar</td>
<td>20g</td>
</tr>
<tr>
<td>Neutral red solution (2% in 50% ethanol)</td>
<td>3.5ml</td>
</tr>
<tr>
<td>Lactose (10% aqueous)</td>
<td>100ml</td>
</tr>
</tbody>
</table>

Peptone and sodium taurocholate (bile salt) were dissolved in water by heating. Then, the agar was added and dissolved by heating. The pH was adjusted to 7.5. Lactose and neutral red solution were added and mixed. This was first heated in autoclave with "free steam" (100°C) for 1 hour; then sterilized at 115°C for 15 minutes. The medium was poured in sterile petri dishes, 20ml per plate.

The surface of the medium was inoculated with one loopful of test organism, the medium plates incubated at 37°C and observed for the growth at 5 days and 11 days.

2.1.5 Thin-layer chromatography of mycobacterial lipids.

The lipid-patterns of 6 non-tuberculous mycobacterial strains, isolated in the present study, 2 of which identified as M. kansasii and other 4 as M. fortuitum on
the basis of cultural and biochemical characteristics, were studied by thin-layer chromatography on silica gel (Jenkins, 1980b). All the cultures were maintained on L.J. egg medium.

Mycobacterial growth from the surface of the L.J. medium was scrapped with a wire loop. Care was taken not to pick up small pieces of the medium. The harvested bacilli were transferred to tarred sample tubes and dried over phosphorous pentoxide in vacuo (60cm Hg).

Extracts: Extracts for lipid analysis were made by adding a freshly prepared mixture of diethyl ether:ethanol:water (17:17:6 v/v) to the dried bacilli in the ratio of 16μl/mg dry weight within the range 10-25 mg. The tubes were sealed with air-tight stoppers and left at room temperature overnight. 10 μl of extract was applied to the chromatogram, 15mm apart, as four superimposed aliquotes each of 2.5 μl allowing natural drying between.

Silica gel plates: Glass plates 20x20cm (previously cleansed in dichromate) were coated with a layer of 0.1mm thick of a slurry of silica gel. The slurry was made by shaking 30g of Merck silica gel H with 65ml distilled water for 30 to 60 seconds in a 150ml stoppered flask. The plates were left until matt and then dried.
for 30 minutes at 100°C in a hot air oven. They were washed in acetone, dried again at 100°C for 30 minutes and finally activated by heating at 120°C for 30 minutes.

Developing solvent: N-propanol: water: ammonia (75:22:3 v/v) was used in 10cm uni-dimensional run.

Location of spots: After running for 10cm the chromatograms were dried at 100°C for 30 minutes, allowed to cool and then sprayed with a mixture of two volumes of 60% sulphuric acid and one of freshly made 0.1% orcinol in water. The plates were then heated at 140°C until the spots appeared.

Spots were recorded on a tracing paper and photographed.

2.1.6 Polyacrylamine gel electrophoresis of mycobacterial cell-proteins

Five strains of non-tuberculous mycobacteria, isolated in the present study from the sputa of the patients with lung disease, two of which identified as *M. kansasii* and other three as *M. fortuitum* on the basis of cultural and biochemical characteristics, were used for the identification by the direct comparison of their
protein-electrophoresis patterns with those of the known standard strains, viz., *M. kansasii* TMC-1201, *M. fortuitum* TMC-1529 and *M. cheloneae* TMC-1542. All the cultures were incubated on L.J. egg medium. The colonies were inoculated into 600ml of Dubos broth (Hi-media) and at 37°C for 2-4 weeks.

**Extracts:** To prepare biologically active cell extracts, mycobacteria have been disrupted by various procedures, including mechanical grinding (Kanai et al., 1960; Counte and Kubica, 1968), by pressure (Castelnuovo et al., 1964) and by sonication (Haas et al., 1972). Although 15 minutes' sonic treatment appeared to be the most suitable method for the preparation of bacillary extracts based on the yield of active components and ease of preparation, no marked qualitative difference between the extracts prepared by different methods was noted (Janicki et al., 1976). In the present study, the extract was prepared as follows:

Cultures were centrifuged at 3,000xg for 30 minutes at 4°C. The supernatant was discarded, and the cells were washed three times in normal saline. Cells were disrupted by mechanical grinding with glass powder in cold. The final product was centrifuged at 10,000xg for 20 minutes at 4°C, the supernatant was decanted and recentrifuged as before and then stored at -20°C.
Electrophoresis: A discontinuous system of sodium dodecyl sulfate-polyacrylamide gel electrophoresis, introduced by Laemmli (1970) for disc gel electrophoresis and later adopted to slab gel by Studier (1973) was used.

Procedure

Assembly of the slab gel apparatus: The slab gel was prepared between two glass plates (thoroughly cleaned and washed with ethanol), one of the two glass plates (14x16x0.3cm) had a 2cm deep and 13.2cm long notch cut out from one of the long edges. The washed, dried plates were placed together with the notch at the top and they were kept apart with three plexiglass spacer (strips), along the side edges and along the bottom edge. The two glass plates and the spacers were tightly assembled with vaseline; the whole system was fixed with clamps. The plates were placed vertically on a flat surface.

Reagents and solutions:
(Double glass distilled water was used throughout).

Solution A: 30% (w/v acrylamide (Sigma) + 0.8% (w/v) N-N'-methylene-bis-acrylamide (Sigma) were prepared in water and stored at 4°C in dark bottles.
Solution B: Tris(Hydroxymethyl)amino methane (Sigma) 18.17g and SDS (20% w/v, Sigma) 2.0ml were dissolved in water and volume was made upto 100ml. The pH was adjusted to 8.8 with 6N HCl.

Solution C: 30% (w/v) acrylamide + 1.6% (w/v) N-N'-methylene-bis-acrylamide were prepared in water and stored at 4°C in bottles.

Solution D: Tris(Hydroxymethyl)amino methane (Sigma) 6.06g and SDS (20%, w/v) 2.0ml were dissolved in water and volume was made upto 100ml. The pH was adjusted to 6.8 with 6N HCl.

Sodium dodecyl sulfate, 20%: 20g SDS was dissolved in 100ml water.

Ammonium persulphate (APS, Merck): 20mg APS was dissolved in 1.0ml water. This was always prepared freshly.

TEMED (N,N,N'-,N'-tetra methyl ethylene diamine, Sigma)

Stock electrode buffer solution: 12.0g Tris and 57.6g glycine (Merck) were dissolved in water and
volume was made upto 1000ml. The pH was adjusted to 8.3.

For electrophoresis run, the buffer was diluted four times and SDS (from 20% stock solution) was added to 0.1%(w/v) final concentration.

Sample stabilizing buffer:

- 2.5ml Solution D,
- 2.5ml 20% SDS (w/v),
- 2.0ml glycerol, and,
- 0.1ml EDTA

were mixed and volume was made upto 18.5ml with water. This was stored in small aliquotes at -20°C. When required, 1.85ml of the sample stabilizing buffer was mixed with 0.05ml betamercaptoethanol (Sigma) and 0.1ml bromophenol blue (0.1% w/v in water).

Fixing solution: 50% (v/v) methanol + 10% (v/v) acetic acid were prepared in water.

Preparation of running and stacking gel:

(a) Running gel: The acrylamide concentration of was 10%, prepared as follows:

- Water - 8.4ml
- Solution A - 6.6ml
- Solution B - 5.0ml
- APS - 0.3ml
Solutions were mixed, 20 μl TEMED was added and the running gel was rapidly poured between the glass plates, up to about 2.5 cm below the notch. The gel was then gently overlayed with water in order to achieve an even surface. Polymerization occurred within 60 minutes at room temperature.

Stacking gel: The stacking gel was prepared by mixing

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>6.34 ml</td>
</tr>
<tr>
<td>Solution C</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Solution D</td>
<td>2.50 ml</td>
</tr>
<tr>
<td>APS</td>
<td>0.15 ml</td>
</tr>
</tbody>
</table>

TEMED, 20 μl, was added before pouring. The overlay of the running gel was removed and 1 or 2 ml of the stacking gel solution was filled. The comb with 9 teeth was inserted and the empty space was filled with stacking gel solution, avoiding the air bubbles. This was not overlayed with water. Polymerization occurred within 30 minutes at room temperature.

Electrophoresis run: The comb and the bottom spacer were carefully removed. The bottom edge was cleaned with filter paper in order to remove residues of vaseline. The glass plates were assembled into the electrophoresis chamber and fixed with clamps. The lower chamber was filled with the electrode buffer; all the air bubbles were removed with a syringe. The top
chamber was filled with the electrode buffer. Samples (100 µl), mixed with an equal volume of sample stabilizing buffer, were applied into each slot with a microsyringe. The electrodes were then connected to the power supply. The anode (+) was the lower electrode and the cathode (-) was the upper one. The electrophoresis was carried out at a constant voltage (50V) for 8-9 hours, until the bromophenol blue tracer migrated almost to the end of the running gel. At the end of the run, the plates were removed from the chamber. The side spacers and the glass plates were removed and the dye-front was marked. The gel was placed in the fixing solution for 20 minutes and then stained with the silver staining method.

Silver stain for proteins in PAGE (Merril et al., 1981)

Reagents:

(A) 10% ethyl alcohol + 5% acetic acid prepared in water.

(B) 0.0034M potassium dichromate (prepared by dissolving 1.0gm in 1000ml water) + 0.0032N nitric acid (0.32ml of 10M per 1000ml).

(C) 0.012M silver nitrate (prepared by dissolving 0.5g in 250ml water).

(D) 0.28M sodium carbonate (prepared by dissolving 29.7g in 1000ml water) + 0.5ml/1000ml of commercial formalin.
(E) 1% acetic acid.

Procedure:

After fixing the gel for at least 20 minutes in normal fixing solution (50% methanol + 10% acetic acid), it was washed three times in Solution A for 10 minutes each time. The gel was then soaked for 10 minutes with agitation in Solution B; the gel turned pale yellow. It was washed four times, each for 30 seconds, in distilled water to remove B, and then soaked in Solution C with agitation for 30 minutes; washed again with distilled water. The gel was then rinsed twice in Solution D with agitation. At this stage, black precipitates formed, which were removed during rinsing. Fresh Solution D was added and agitated until protein bands were developed sufficiently. Then the gel was transferred to 1% acetic acid.

The stained gel was photographed. Tracings from the gels were made with a Ultrascan Leser Densitometer (LKB-Bromma-2202).

2.2 Phage typing of M. tuberculosis strains in Gujarat

1. Mycobacteriophages: Ten mycobacteriophages and their indicator host strains were obtained from Dr. D.G. Groodhuis, the Rijksinstitut voor de Volksgezondheid
en milieuhygiene, Bilthoven, The Netherlands. These phages were designated as Mycobacterial Typing Phages, Human (MTPH) by WHO Study Group, as summarized by Mankiewicz (1972); those used in the present study were MTPH 2 through 5, 7, MTPH 9 through 12 and 15. The phages and their indicator host strains are listed in Table 2.3.

2. Mycobacteria: A total 60 strains of M. M. tuberculosis, isolated from the patients suffering from pulmonary tuberculosis whilst resident of Gujarat and admitted to the K.J. Mehta Tuberculosis Hospital, Amargadh, were phage-typed. Methods of isolation and cultivation of mycobacteria have been described in Section 2.1. The following criteria were employed to confirm the identification of these strains as M. M. tuberculosis (Test procedure as described in Section 2.1).

(i) Acidfastness when stained with Ziehl-Neelsen stain.
(ii) Typical colony morphology, pigmentation and rate of growth at 37°C on L.J. egg medium.
(iii) Failure to grow on L.J. medium at 25°C or at 45°C.
(iv) No growth on L.J. medium containing PNB, 500 μg/ml.
(v) No growth on L.J. medium containing hydroxylamine hydrochloride 125 μg/ml.
Table 2.3.
Typing phages for *M. tuberculosis*, their
designations and bacterial indicator host strains

<table>
<thead>
<tr>
<th>Mycobacteriophage</th>
<th>MTPH Designation</th>
<th>Mycobacterial indicator host strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>DS 6A</td>
<td>2</td>
<td><em>M. tuberculosis, H37 Rv</em></td>
</tr>
<tr>
<td>GS 4E</td>
<td>3</td>
<td><em>M. tuberculosis, H37 Rv</em></td>
</tr>
<tr>
<td>BK 1</td>
<td>4</td>
<td><em>M. smegmatis, ATCC-607</em></td>
</tr>
<tr>
<td>BG 1</td>
<td>5</td>
<td><em>M. tuberculosis, MC-53-85</em></td>
</tr>
<tr>
<td>DNA III 8</td>
<td>7</td>
<td><em>M. tuberculosis, H37 Rv</em></td>
</tr>
<tr>
<td>PH</td>
<td>9</td>
<td><em>M. tuberculosis, H37 Rv</em></td>
</tr>
<tr>
<td>Clark</td>
<td>10</td>
<td><em>M. smegmatis, ATCC-607</em></td>
</tr>
<tr>
<td>Sedge</td>
<td>11</td>
<td><em>M. smegmatis, ATCC-607</em></td>
</tr>
<tr>
<td>Legendre</td>
<td>12</td>
<td><em>M. smegmatis, ATCC-607</em></td>
</tr>
<tr>
<td>D 34. LR-14 *</td>
<td>15</td>
<td><em>M. tuberculosis, LR-14</em></td>
</tr>
</tbody>
</table>

* as designated by Jones et al. 1982.
(vi) Positive niacin production test.
(vii) Positive nitrate reduction test.
(viii) Growth on L.J. medium containing TCH, 1 μg/ml.

Cultures to be phage-typed were grown in Middlebrook-7H9 broth (Hi-media, India) supplemented with Albumin-Dextrose-Catalase enrichment and 0.5% Tween 80, hereafter referred to as 7H9 broth. Broth cultures were shaken daily to maintain dispersed growth (Jones and Greenberg, 1978).

3. Media used for phage-typing (Redmond and Ward, 1966; Rado et al., 1975; Grange et al., 1976)

(i) Nutrient broth (NB3)
Nutrient broth (Hi-media, India). 8g
Sodium Chloride 5g
Calcium Chloride 0.5ml
(1M, prepared by dissolving 2.22gm anhydrous CaCl₂ in 20ml distilled water.)
Distilled water 1000ml

The ingredients were dissolved in distilled water, pH was adjusted to 6.9 - 7.1 by adding 10% NaOH, dispensed in 10ml amounts in screw-cap tubes and sterilized at 121°C for 15 minutes.
(ii) Soft agar (SA 14) overlay:

A. Agar
   Agar
   Distilled water

   Agar was dissolved by heating and 0.1ml of 1M Calcium Chloride solution was added.

B. Nutrient Broth
   0.8g
   Sodium Chloride
   Distilled water

   The pH was adjusted to 7.0 with 4% NaOH. A and B were mixed, distributed in 3.5ml amounts in screw-cap tubes and sterilized at 121°C for 15 minutes.

(iii) RVA 29A agar:

A. Nutrient broth (Hi-media)
   Proteose paptone (Hi-media)
   Sodium pyruate
   Na₂HPO₄
   KH₂PO₄
   Ammonium Chloride
   Sodium Chloride
   Casitone (Hi-media)
   Glycerol
   Zinc Solution*
   Distilled water

   * Zinc solution was prepared by dissolving 10mg ZnSO₄ plus 10mg CuSO₄ in 100ml distilled water.
The ingredients were dissolved in distilled water and sterilized at 121°C for 15 minutes.

B. 2.5g glucose was dissolved in 25ml distilled water and sterilized at 121°C for 15 minutes.

C. 5.5g agar was dissolved in 245ml distilled water by heating, to which

- 1M MgSO$_4$·6H$_2$O 0.5ml
  (2.4077g in 20ml water)
- 1M CaCl$_2$ 0.5ml
  (2.22g in 20ml water)
- 1M FeCl$_3$·6H$_2$O 1.0ml
  (0.4g in 20ml water)

were added and sterilized at 121°C for 15 minutes.

D. Bovin albumin, Fraction V (Sigma) 1.75g
   Oleic acid in NaOH* 2.5 ml
   2.5N NaOH** 0.18ml
   Sodium glutamate 0.25g
   Distilled water 50ml

*Oleic acid 0.12ml was added in 10ml N/20 NaOH.

**1g NaOH was dissolved in 10ml distilled water.

The ingredients were dissolved in distilled water and sterilized by membrane filtration (0.45 micron, Sartorius, W. Germany).
E. Complete medium: After sterilization, A, B and C were kept in a waterbath maintained at 60°C to prevent agar from solidifying. All the three solutions were mixed aseptically and oleic acid-albumin complex (Solution D) was added. Medium was poured in sterile petri plates (9cm diameter) in 15ml amounts and allowed to set.

4. Propagation of phages: Nutrient broth (Hi-media) containing 4% glycerol and 10mg/l Calcium Chloride was employed in 50ml amounts in flasks (Grange et al., 1976). This was inoculated with a 4mm loopful of the host strain, taken from a L.J. culture, and incubated at 37°C for 24 hours (to obtain a culture in early exponential growth phase) before introducing the phage to be propagated. In every case, 1.0ml of stock phage suspension was then introduced aseptically. After inoculation with phage, the culture of M. smegmatis and M. tuberculosis H37Rv were incubated for a further period of 3 and 7 days respectively. After incubation, all phage preparation were transferred to sterile screw-cap tubes in 25ml amounts and centrifuged at 3000 r.p.m. for 15 to 20 minutes to deposit the bacteria. The supernatant was removed aseptically and passed through 0.45micron membrane filter (Sartorius, W.Germany). The filtrates were stored in 4.0ml amounts in screw-cap
tubes at 4°C and titrated individually to determine the routine test dilution (RTD) of phage-suspension before use.

5. Titration: Soft agar (SA14) overlays were melted in a boiling water bath and cooled to 52°C. A 3-day-old 7H9 broth in case of M. smegmatis and 10- to 12-day-old 7H9 broth in case of M. tuberculosis host strain was pipetted in 0.5ml aliquots into tubes containing 3.5ml of soft agar. Each tube was gently swirled to mix bacteria and agar and the mixture was poured onto a RVA 29A agar plate. The petridish was gently rotated to distribute the soft agar evenly over the surface of the hard agar. After the soft agar hardened at room temperature the plates were spotted with the respective phages.

The next day, the phages were diluted in NB3 to give 10 serial 10-fold dilutions. All the dilutions were spotted with a platinum loop (2mm diameter). Two plates were used for each phage. The plates were inverted and incubated at 37°C. The plates seeded with M. smegmatis were examined after two days of further incubation, when the RTD of these phages could be read easily. The plates seeded with M. tuberculosis were incubated up to 21 days (without placing them into plastic bags). The highest of the 10-fold dilutions that induced
an area of confluent lysis was taken as the RTD of the phage.

6. Phage typing: Soft agar (SA14) overlays were melted in a boiling waterbath and cooled to 52°C. A 10- to 12- day - old 7H9 broth culture of the test strain of *M. tuberculosis* was pipetted in 0.5ml aliquote into tubes containing 3.5ml of soft agar, mixed and poured onto the RVA 29A agar plate. Petridish was gently rotated to distribute the soft agar evenly over the surface of the hard agar. After the soft agar hardened at room temperature, the plates were inverted and incubated at 37°C over night. Three plates were prepared for each culture.

The next day, they were spotted with the respective routine test dilution of each phage. The plates were incubated at 37°C until lysis was visible. With each batch of test, a strain of known phage type (*M. tuberculosis*, H*37Rv*, Prototype B) was included as a check on RTDs.

2.3 Antituberculosis Drug Resistance

2.3.1 Prevalence of antituberculosis drug resistance among newly diagnosed patients (Primary drug resistance) and rifampicin-resistance among treatment-failure and
relapse cases of pulmonary tuberculosis

(A) Criteria for eligibility of patients

An individual of any age or sex was considered eligible for the study if he or she-

(i) was the resident of Gujarat. Recent migrants to Gujarat from other States were excluded.

(ii) was attending K.J. Mehta Tuberculosis Hospital, Amargadh, for the first time, because of the symptoms and had radiographic evidence suggestive of pulmonary tuberculosis.

(iii) For primary drug resistance :- had not received any antituberculosis drug previously. Patients with the histories of previous antituberculosis chemotherapy even for a short duration were excluded. Great stress was laid on the importance of obtaining accurate and detailed histories of previous chemotherapy by the Medical Officers of the K.J. Mehta Tuberculosis Hospital, Amargadh. Patients' relatives were also enquired regarding the treatment of their illness. A second interrogation was also undertaken after about 4 to 6 weeks without the knowledge of drug sensitivity test results.

For rifampicin resistance :- had received antituberculosis drugs elsewhere for not less
than three months. Care was taken to avoid the repetition of the same patient on his next visit to the hospital.

(iv) had excreted *M. tuberculosis* only. If the organisms were identified as non-tuberculous mycobacteria, the patient was excluded from the study.

(B) Isolation and identification of mycobacteria (The methods have been described earlier in Sections 2.1.2 and 2.1.3)

(C) Antituberculosis drug sensitivity tests (Canetti et al., 1969)

(i) Medium: Sensitivity tests were done on L.J. egg medium. The drugs were added, in the concentrations noted below, before inspissation. The medium was dispensed in 5ml amounts in McCartney bottles and was inspissated once at 85°C for 60 minutes. The drug-containing media were stored at 4°C. They could be used for at least two months after preparation.

(ii) Inoculum: The sensitivity tests were set up with an inoculum prepared from the growth on primary L.J. medium slope, i.e., indirect sensitivity tests were carried out. The inoculum was prepared as follows:
With a 22 SWG (British Standard Wire Gauge, wire diameter 0.7 cm) Nichrome loop, a representative sweep from the growth (approximately 2 mg moist weight of bacilli) from L.J. medium was taken and discharged into a sterile 7-ml screw-cap tube containing 0.4 ml sterile distilled water and 6 glass beads (3 mm in diameter). The cells were then homogenized on a vortex mixer for 2-3 minutes. To the tube, 1.5 ml sterile distilled water was added and shaken to mix. The density of the suspension was approximately similar to that of BCG. The density was adjusted, if necessary, by the addition of sterile distilled water. Large particles were allowed to settle. With a 3 mm external diameter 27 SWG (wire diameter 0.4 mm) Nichrome loop, a loopful of the suspension was spread on the surface of each slope of the sensitivity test. As a control, a drug-free slope was set up for each strain tested. The standard sensitive strain, *M. tuberculosis* H$_{37}$Rv, was tested in each batch of test.

(iii) Incubation and reading of tests: The slopes were incubated at 37°C. A reading was taken at two weeks for a preliminary indication of resistant strains, but the definitive reading was made at four weeks.
(iv) Procedures with different drugs:

(1) Isoniazid

A stock solution of 10,000 µg/ml isoniazid was prepared by dissolving 100mg of isoniazid in 10ml distilled water and sterilized by membrane filtration (porosity 0.45 micron, Sartorius, W. Germany). The L.J. medium of 0.2 µg/ml and 1.0 µg/ml isoniazid concentrations was prepared as follows:

0.06ml of the stock isoniazid solution was added, before inspissation, to the 600ml L.J. medium. This was 1.0 µg/ml isoniazid medium. 100ml of 1.0 µg/ml medium was 5-times diluted by adding 400ml plain L.J. medium to give 0.2 µg/ml isoniazid medium.

If growth of more than 20 colonies was observed on the L.J. medium containing 0.2 µg/ml isoniazid, the strain was classified as resistant.

(2) Streptomycin

A stock solution of 10,000 µg/ml streptomycin was prepared by dissolving 125mg of dihydrostreptomycin sulfate in 10ml distilled water and sterilized by membrane filtration (12.5mg dihydrostreptomycin sulfate = 10mg
The L.J. medium of 4.0 μg/ml and 8.0 μg/ml streptomycin concentrations was prepared as follows:

0.48ml of the stock streptomycin solution was added to 600ml of L.J. medium. This was 8.0 μg/ml medium. 200ml of 8.0 μg/ml medium was 2-times diluted by adding 200ml plain L.J. medium to give 4.0 μg/ml streptomycin medium. The H₃₇Rv strain was tested in each batch and was inhibited by 4.0 μg/ml streptomycin. If growth of more than 20 colonies was observed on 8.0 μg/ml streptomycin medium, the test strain was classified as resistant.

(3) Ethambutol

A stock solution of 10,000 μg/ml ethambutol was prepared by dissolving 100mg of ethambutol in 10ml distilled water and sterilized by membrane filtration. The L.J. medium of 4.0 μg/ml ethambutol concentration was prepared by adding 0.2ml of the stock solution to the 500ml of L.J. medium. If growth of more than 20 colonies was observed on 4.0 μg/ml ethambutol medium, the test strain was classified as resistant.
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(4) Thiacetazone

A stock solution of 10,000 \( \mu \text{g/ml} \) thiacetazone was prepared by dissolving 100mg thiacetazone in 10ml of triethylene glycol (Trigol, BDH). The triethylene glycol was warmed at 37°C before pipetting to reduce its viscosity. The solution was self-sterilizing. The L.J. medium of 2.0 \( \mu \text{g/ml} \) thiacetazone concentration was prepared by adding 0.1ml of stock solution to 500ml of L.J. medium. If growth (20 colonies or more) on 2.0 \( \mu \text{g/ml} \) thiacetazone medium was observed, the test strain was classified as resistant.

(5) Rifampicin

A stock solution of 10,000 \( \mu \text{g/ml} \) rifampicin was prepared by dissolving 100mg of rifampicin in 10ml of propylene glycol. This required heating at 85°C for at least 20 minutes with thorough mixing on a vortex mixer. The L.J. medium containing 50 \( \mu \text{g/ml} \) rifampicin was prepared by adding 2.5ml of the stock solution to the 500ml of the L.J. medium.

If the growth (20 colonies or more) was observed on the 50 \( \mu \text{g/ml} \) rifampicin medium, the
test strain was classified as resistant (Tsukamura, 1972).

(6) Pyrazinamide

A stock solution of 10,000 µg/ml pyrazinamide was prepared by dissolving 100mg of pyrazinamide in 10ml distilled water and sterilized by membrane filtration.

600ml of L.J. medium was acidified by adding 1N HCl very gradually and with continuous shaking to the medium before inspissation until the medium was at pH 5.5 as measured with a glass electrode.

The L.J. medium with 100 µg/ml pyrazinamide concentration was prepared by adding 3ml of the stock solution to 300ml of acid L.J. medium. The remaining acid L.J. medium was used as drug-free control medium.

Pyrazinamide sensitivity tests, were set up with a 1:10 dilution of the standard inoculum. If the growth (10% or more colonies in comparison with the control) was observed on the 100 µg/ml pyrazinamide medium, the test strain was classified as resistant if the
test strain was sensitive at four weeks, slopes were further incubated at 37°C and readings were taken at six weeks as pyrazinamide resistant strains might show delayed growth. If there was no growth on drug-free acid L.J. medium, the sensitivity result was not considered and the test was repeated.

2.3.2 Pyrazinamidase activity of M. tuberculosis
-A test of sensitivity to pyrazinamide

The study was performed on 378 strains of M. tuberculosis which were isolated from the patients admitted to K.J. Mehta Tuberculosis Hospital, Amargadh. Information about previous treatment was obtained from all the patients.

(1) Pyrazinamide sensitivity test
Sensitivity tests to pyrazinamide were performed according to the method described in Section 2.3.1 (C).

(2) Level of pyrazinamide resistance
183 pyrazinamide resistant strains, including pyrazinamide-resistant mutants from predominantly pyrazinamide sensitive cultures showing growth on L.J. medium containing 100 μg/ml pyrazinamide, were further inoculated on the medium containing 200 μg/ml and 300 μg/ml pyrazinamide to isolate mutants with the highest
levels of pyrazinamide resistance.

200 μg/ml and 300 μg/ml pyrazinamide media were prepared by adding 6ml and 9ml stock solution respectively to 300 ml of acid L.J. medium as described in Section 2.3.1 (C).

(3) Pyrazinamidase test

Pyrazinamidase activity was determined by the Wayne method as described in Section 2.1.3. The presence of a pink band (any degree of pink colour) indicates pyrazinamidase activity (Fig. 9).