MATERIALS & METHODS

The present study was conducted in the Department of Microbiology of Maulana Azad Medical College and Associated Lok Nayak and GNEC Hospitals, a major tertiary health-care centre in New Delhi.

Patients of age group 2 - 45 years were included for the present study. The study included a total of one hundred (n=100) clinically suspected cases which were suggestive of tuberculous lymphadenitis by cytopathology. The fine-needle aspirates and blood samples were collected from these patients. Informed consent of each patient was taken on a set Clinical Performa.

SELECTION OF PATIENTS

INCLUSION CRITERIA

Clinically suspected cases, which were suggestive of tuberculous lymphadenitis by cytopathology, were taken into considered for the study.

EXCLUSION CRITERIA

Lymphadenopathy due to other causes was excluded, based on cytology, histopathology, hematological techniques (hemogram and ESR), chest radiography, ultrasonography, computerized tomography.

SPECIMEN COLLECTION AND TRANSPORT

Fine needle aspirates (FNA) from the lymph nodes and five milliliters (5ml) of peripheral blood was collected aseptically from each of the cases in sterile containers and in anti coagulated tubes containing EDTA. These samples were then transported quickly to the Mycobacteriology laboratory.

Work done for the study:

The specimens were decontaminated by N-acetyl L-cysteine sodium hydroxide (NALC-NaOH) method. Decontaminated samples were used for conventional laboratory methods like direct smear microscopy, culture on Lowenstein-Jensen (LJ) medium as well as by radiometric (BACTEC) culture.
The positive cultures were further identified by various biochemical methods. Detection of Mycobacterial species was performed by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PRA) analysis by using Different Restriction Enzymes. The standard positive culture of *M. tuberculosis* (H37 Rv) was used as a positive control for the analysis.

**PROCESSING OF THE FINE NEEDLE ASPIRATES SAMPLES**

Processing of FNA/pus samples was done by N-acetyl L-cysteine-Sodium Hydroxide (NACL-NaOH) method (Kubica & Kent, 1985) for the culture and DNA extraction. Extracted DNA was used for the PRA analysis.

i) One ml of sterile phosphate buffer saline (PBS pH 6.8) was added to fine needle aspirates/pus specimen and was transferred to 50 ml of plastic centrifuge tube and then equal volume of digestant (0.25 gm NALC + 4%NaOH & 2.9% Na citrate mixer) was added.

ii) After digestant was added, the tubes were kept for 15 minutes at room temperature for decontamination

iii) Digested and decontaminated specimen was diluted with sterile phosphate buffer (PBS pH 6.8) to the 50 ml mark to minimize the continuing action of sodium hydroxide and to lower the specific gravity of the specimen before centrifuging.

iv) The tubes were then centrifuged at 3000 x g for 15 minutes.

v) After centrifugation, supernatant fluid was discarded and 1 ml sterile PBS was added to the sediment and mixed the sediment by vortex mixer.

vi) This inoculum was used for different conventional laboratory methods.

**PROCESSING OF THE BLOOD SAMPLES**

Blood samples were lysed for the culture and DNA extraction. Extracted DNA were used for the PCR amplification.

i) Blood samples were lysed by adding distilled water and keeping it for 15 minutes at room temperature.

ii) The tubes were then centrifuged at 3000 x g for 15 minutes.
MATERIAL & METHODS

iii) Same steps were repeated to completely lyse the blood cells.

iv) After centrifugation, supernatant fluid was discarded and 1 ml sterile PBS was added to the sediment and mixed the sediment by vortex mixer.

v) This inoculum was used for, direct smear, culture and for extraction of the DNA.

CONVENTIONAL STUDY

1. STAINING OF FINE NEEDLE ASPIRATES

Smears were made from each specimen, after the decontamination (NALC-NaOH) method. The material was spread over 2-3 cm area of clean grease free glass slide with the help of the syringe and allowed to dry. The smears were fixed by passing through the flame for three to four times. Adequate fixing was tested by feeling the slide on the back of palm. Similarly smears were also made from processed blood samples. These smears were stained by Ziehl-Neelsen (ZN) staining.

1.1) Ziehl-Neelsen staining (Kent PT, 1985)

The slides were heat fixed as mentioned earlier. The smears were stained by Ziehl-Neelsen (ZN) staining.

Ziehl-Neelsen staining

(a) Carbol fuchsin

Basic fuchsin- 0.3 gm
95% ethyl alcohol- 10 ml
Phenol crystals- 5 gm
Distilled water- 100 ml

0.3 gm of basic fuchsin was dissolved in 10 ml of 95% ethyl alcohol (solution A). 5 gm of phenol crystals were dissolved in 100 ml of water by gentle heating (solution B). Solution A was mixed with 90 ml of solution B.

(b) Acid alcohol:

Concentrated HCl - 3 ml
95% ethyl alcohol - 97 ml
MATERIAL & METHODS

3 ml concentrated hydrochloric acid (HCl) was dissolved to 97 ml of 95% ethyl alcohol by mixing gently.

(c) Counter stain:

Methylene blue- 0.3 gm
Distilled water- 100 ml

0.3 gm of methylene blue was dissolved in 100 ml distilled water.

Procedure:

i) The carbol fuchsin was poured on a slide so as to cover the fixed smear.

ii) Gentle heat was applied to the underside of the slide, by means of a spirit flame, until the steam arose.

iii) The carbol fuchsin was left on the slide for 5-10 minutes with intermittent heating during that period. Care was taken to ensure that the stain did not dry out or boil. To counteract drying more stain was added to the slide and then reheated and washed with tap water.

iv) Decolorized in several successive portions of acid-alcohol.

v) Washed with tap water.

vi) Counterstained with methylene blue for 1-2 minutes.

vii) Washed with tap water.

viii) Air dried.

ix) Observed under oil immersion lens (X1000).

Examination of smear under microscope:

Acid fast bacilli (AFB) were observed as red, beaded and slightly curved rods against a bluish background.

1.2) Giemsa staining (Koss L, 1979)

The fine needle aspirates smears were made by spreading the aspirates on the slides for Giemsa staining.

Giemsa staining

(a) Giemsa

Giemsa stain - 7.6 gm
95% methyl alcohol - 500 ml
Glycerin - 500 ml

7.6 gm of Giemsa stain was dissolved in 500 ml of 95% methyl alcohol. Then 500 ml of glycerin was added to the solution. The stain is kept at 37 °C for 15 days incubation before use.

Procedure:
i) The fine needle aspirate smears were fixed with absolute methanol for 5 minutes.

ii) Then the Giemsa stain was poured on the smears and was kept for 30 minutes.

iii) The smears were then washed with tap water.

iv) The smears were air dried and were mounted with DPX.

v) Observed under X200-X400 lens for cytological investigation.

Examination of smear under microscope:
The smears were examined under X200-X400 for the presence of granulomas, langhan's giant cells, epithelioid cells and caseous necrosis which are suggestive of tuberculosis.

2) Culture (Kent PT, 1985)
The processed specimens were cultured on the following media

2.1) Lowenstein-Jensen medium

a) Mineral salt solution

Potassium dihydrogen phosphate anhydrous - 2.4 gm
Magnesium sulphate anhydrous - 0.24 gm
Magnesium citrate - 0.6 gm
Asparagine - 3.6 gm

The ingredients were dissolved in the above order in about 300 ml distilled water by heating (solution A).

b) Malachite green solution 2%

Malachite green dye - 2.0 gm
Distilled water - 100 ml
MATERIAL & METHODS

The dye was dissolved in distilled water by grinding the dye with water using a mortar and pestle. The malachite green solution and 12 ml glycerol was added to the above solution A and made up to 600 ml with distilled water. It was autoclaved at 121º C for 15 minutes at 15 lbs pressure to sterilize.

Homogenized whole eggs

Fresh hen’s eggs, not more than seven days old were cleaned by scrubbing thoroughly with a hand brush in water and soap. The eggs were soaked for 30 minutes in soap solution. The eggs were rinsed thoroughly in running water and soaked in 70% ethanol for 15 minutes. Before handling the clean dry eggs the hands were properly washed. The eggs were cracked with the edge of the beaker into a sterile flask and beaten in a sterile manner.

Preparation of whole medium

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

- Mineral salt solution with malachite green - 600 ml
- Homogenized eggs (25 to 30 eggs depending on the size) - 1000 ml

The complete medium was distributed in 6-8 ml volumes in sterile universal containers and the caps were tightly closed and inspissated without delay to prevent sedimentation of heavier ingredients.

The bottles were placed in a slanted position and the medium was coagulated for 50 minutes at 85º C by inspissation each day for three consecutive days. The media were kept in room temperature in between the procedures of inspissation. Any discolored medium or medium containing little holes or bubbles on surface was discarded.

Sterility check

After inspissation a representative sample of the media bottles was incubated at 37ºC for 24 hours as a check for sterility.

Storage

The LJ medium was dated and kept in the refrigerator.
Radiometric culture (Siddiqui Salman H, 1996)

The sediment of sample was inoculated into the BACTEC 12B vial medium as follows:
1. 0.1 ml of PANTA (mixture of different antibiotics namely Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin) was added to the BACTEC 12 B vial medium. The BACTEC 12B vial contains Middlebrook’s 7H9 medium. This medium contains palmitic acid having C\(^{14}\) which is radioactive.
2. 0.5 ml of processed fine needle aspirate sample was inoculated in this vial.
3. These vials were incubated at 37º C.
4. Inoculated media were examined in the BACTEC 460 TB system and Growth Indices (GI) was taken three times a week (after every 2-3 days) for the first three weeks and weekly thereafter for another three weeks (total six weeks). If Growth Index (release of radiolabelled Carbon dioxide) was greater than 30 then it was considered as positive.
5. As soon as culture medium showed positive GI, smear was made and stained by ZN stain for confirmation of acid-fast bacilli (AFB).
6. The culture vials were considered negative, when the GI was observed lesser than 30, even after 8 weeks incubation.

3. Identification of growth on LJ media

The processed fine needle aspirates samples were inoculated on two slants of LJ media. The inoculated culture bottles were incubated at 37º C. These were examined within 5-7 days for detection of growth of rapidly growing mycobacteria, any fungal growth and for any contamination or decay of the media. All cultures were examined weekly for 8 weeks, after which they were discarded. As soon as any growth was evident on culture medium, smear was made and stained by ZN stain for confirmation for the acid fast bacilli (AFB) growth. All the growth was then examined for the rate of growth, pigmentation, colony morphology and other properties (Kent PT, 1985)
3.1) Morphological examination

- Pigment production
- Rate of Growth
- Colony Morphology

Biochemical characterization

The following tests were performed to identify the isolate to species level. The colonies were sub cultured on three more slopes of LJ media to identify and confirm the *Mycobacterium tuberculosis complex and non tuberculous mycobacteria (NTM)*.

The colonies positive for Niacin production and Nitrate reduction tests and weak positive for catalase test were labeled as *Mycobacterium tuberculosis*. The colonies negative for Niacin production and Nitrate reduction tests and strong positive for catalase test were labeled as Non-tuberculous mycobacteria (NTM) or *Mycobacteria other than tuberculosis (MOTT)* (Kent PT, 1985). The following biochemical tests were performed.

3.2. Niacin production (Kent PT, 1985)

**Principle**

Although all mycobacteria produce Nicotinic acid, studies have shown that due to the blocked metabolic pathway, *Mycobacterium tuberculosis* accumulates the largest amount and detection of this accumulated niacin is useful for definitive diagnosis of this species.

**Reagents**

A. Cyanogen bromide - 10%
Cyanogen bromide - 10 gm
Distilled water - 100 ml

B. Aniline 4% in alcohol
Aniline - 4 ml
95% Alcohol - 96 ml
Procedure
1. 0.5 – 1.5 ml of sterile water was added to the LJ medium slant containing growth.
2. The container was placed horizontally so that the fluid covered the entire surface of the medium.
3. It was allowed to stand at 37º C for 15 minutes and then 0.5 ml of fluid extract was removed to a small tube.
4. Then 500 µl each of 4% aniline and 10% cyanogen bromide solution was added to the fluid extract.
5. The formation of Canary yellow color immediately after addition of reagents was taken as positive.

Controls

Mycobacterium tuberculosis was used as positive control and Mycobacterium intracellulare- complex as negative control.

3.3. Nitrate Reduction (Kent PT, 1985)

Principle

This test is based on the ability of some mycobacteria to reduce nitrates to nitrites.

Reagents

A. 1:2 dilution of concentrated HCl
B. 0.2% aqueous sulfanilamide solution
C. 0.1% aqueous N- naphthylethylene diamine dihydrochloride
D. 0.01 Molar solution of Sodium nitrate (NaNO₃) in 0.022 Molar phosphate buffer pH 7
E. Powdered Zinc.

Procedure
1. 0.2 ml of sterile distilled water was placed in a screw capped bottle; one loopful of growth was emulsified in it.
2. 2 ml of NaNO₃ solution was added, shaken and incubated in water bath at 37° C for 2 hours.
3. To this one drop of 1:2 dilution of conc. HCl, 2 drops of 0.2% aqueous sulfanilamide solution and 2 drops of 0.1% of aqueous N- naphthylethylenediamine dihydrochloride was added in the order.
4. Bottles were immediately examined for the development of pink to red colour.
5. To all negative tubes, a pinch of powdered zinc was added which reduce nitrates to nitrite. The formation of red colour indicated the true negative results.

Controls
Mycobacterium tuberculosis was taken as positive control and Mycobacterium intracellulare complex as negative control.

3.4. Catalase test (Kent PT, 1985)

Principle
Mycobacteria possess several kinds of catalase enzymes that vary in heat stability. Quantitative differences in catalase activity can be demonstrated by 68° C test for 20 minutes. For these tests cultures on LJ media were used.

Reagents
1. 0.067 M phosphate buffer solution pH 7.0
Solution 1
Na₂ HPO₄ anhydrous 9.47 g
Distilled water 1 litre
Disodium hydrogen phosphate was dissolved in water.
Solution 2
KH₂PO₄ 9.07 g
Distilled water 1 liter
Potassium dihydrogen phosphate was dissolved in water.
61.1 ml of solution 1 was added to 38.9 ml of solution 2.

2. **Hydrogen peroxide 30% solution**

   Stored in refrigerator at 4 °C

3. **Tween-80 - 10%**

   Tween 80 - 10 ml

   Distilled water - 90 ml

   Tween-80 was added to distilled water and autoclaved at 121° C for 15 minutes. It was allowed to cool. It was stored in refrigerator. Complete catalase reagent (Tween- peroxide mixture):

   Immediately before use equal parts of 10% of Tween 80 and 30% hydrogen peroxide were mixed. For each strain 0.5 ml of reagent was used.

**Procedure**

1. With a sterile pipette, 0.5 ml of 0.0.67M buffer was added aseptically to screw capped test tubes.

2. A loopful of test culture was suspended in the buffer solution, using a sterile loop.

3. The tubes were placed in a previously heated water bath at 68° C for 20 minutes.

4. The tubes were removed from heat and cooled to room temperature.

5. 0.5 ml of freshly prepared Tween- peroxide mixture was added to each tube.

6. The formation of bubbles was observed on the surface of liquid.

7. Formation of bubbles more than 45 mm was taken positive and less than 45 mm was taken as negative.

8. *Mycobacterium tuberculosis* produces less than 45 mm of bubbles.

3.5. **Urease test (Kent PT, 1985)**

**Principle**

The ability of a culture to hydrolyse urea (releasing ammonia) is useful in identifying both scotochromogens and nonphotochromogens.
**MATERIAL & METHODS**

*M.tuberculosis* and *M. scrofulaceum* are positive and *M.avium* complex and *M.gordonae* are negative.

**Reagents**
The following ingredients were dissolved in 1 liter of distilled water as: 1 gm of peptone
1gm of dextrose, 5gm of NaCl, 0.4gm of KH₂PO₄, 20gm of urea, 1ml of 1% phenol red solution of sodium salt, 0.1 ml of Tween 80. The final pH was adjusted to 5.8 with NaOH. The solution was sterilized by membrane filtration.

**Procedure**
1. With a sterile pipette, 1.5 ml of the solution was added aseptically to screw capped test tubes.
2. The growth was then inoculated into the solution and was kept for incubation at 35º C.
3. Results were tested on 1<sup>st</sup>, 3<sup>rd</sup> and 7<sup>th</sup> day.
4. Color change in the broth from yellow to dark pink was considered positive

3.6. *Tween 80 hydrolysis test* (Kent PT, 1985)

**Principle**
The enzymatic hydrolysis of Tween 80 (with few exceptions) is used to separate the potentially pathogenic (negative) from commonly saprophytic (positive) species among the slow growing mycobacteria. The test medium contains Tween 80 and the indicator neutral red in a buffered neutral solution. Contrary to common impression, the colour change in the Tween hydrolysis test is not due to a pH shift related to release of oleic acid from the hydrolytic cleavage of Tween 80.

Normally neutral red is red at pH 7, but when bound by lipids or by Tween 80, the indicator dye takes on the amber or straw color that it commonly has at more alkaline pH. When the bacterial esterases spilt the
Tween 80, it no longer acts to bind the indicator dye and the neutral red the reverts to its color at pH 7, which is red and the test is called positive.

**Reagents**

0.067 M phosphate buffer solution pH 7.0

Solution 1

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

Distilled water \quad 1 \text{ litre}

Disodium hydrogen phosphate was dissolved in water.

Solution 2

\[
\text{KH}_2\text{PO}_4 \quad 9.07 \text{ g}
\]

Distilled water \quad 1 \text{ liter}

Potassium dihydrogen phosphate was dissolved in water.

61.1 ml of solution 1 was added to 38.9 ml of solution 2.

In 100ml of phosphate buffer, 0.5 ml Tween 80 and 2ml of 0.1% aqueous solution of neutral red was added. The solution was then autoclaved at 121º C for 15 minutes.

**Procedure**

1. With a sterile pipette, 2 ml of the solution was added aseptically to screw capped test tubes.
2. The growth was then inoculated into the solution and was kept for incubation at 35º C.
3. Results were tested on 1\textsuperscript{st}, 5\textsuperscript{th} and 10\textsuperscript{th} day.
4. Colour change in the broth from pink to red was considered positive. No colour change up to 10\textsuperscript{th} days was considered negative.

**3.7. Aryl sulfatase test (Kent PT, 1985)**

**Principle**

Arylsulfatases is an enzyme capable of hydrolyzing the bond between the sulfate group and the aromatic ring structure in compounds with the general formula \( \text{R-OSO}_3\text{H} \), where R represents an aromatic ring
When the substrate potassium phenolphthalein disulfate is incorporated into the growth medium, the presence of free phenolphthalein liberated by enzymatic hydrolysis is indicated by red color that develops when alkali is added.

Arylsulfatases activity is limited almost solely to the genus mycobacteria. Since most mycobacteria possess this enzyme, the test conditions must be varied to help identify the various mycobacteria.

The 3 day test facilitates identification of the potentially pathogenic *M.fortuitum complex*, whereas the 14 day test aids in the identification of certain slow growing mycobacteria.

**Reagents**
1. 2.6 gm phenolphthalein disulfate was dissolved in 50 ml distilled water to make 0.08M of enzyme substrate stock solution. The solution was sterilized by membrane filtration.
2. 2N sodium carbonate.
10.6 gm Na₂CO₃ was dissolved in 100 ml distilled water. The solution was sterilized by membrane filtration.
3. In 180 ml of 7H9 Middlebrook’s liquid broth, 20 ml of Albumin, Dextrose, Catalase (ADC) enrichment medium was added after autoclaving.

**Procedure**
1. For the, 3rd day test medium 2.5 ml of 0.08M of enzyme substrate stock solution was added to 200 ml 7H9 liquid medium in the flask.
2. 14th day test medium 7.5 ml 0.08M of enzyme substrate stock solution was added to 200 ml 7H9 liquid medium in the flask.
3. Aliquots were made and labelled as tube no. 1 and tube no. 2.
4. The growth was inoculated into the tube no. 1 & 2 and was kept for incubation at 37º C.
5. After 3 days incubation 6 drops of 2 N Na₂CO₃ was added to the tube no. 1.
6. After 14 days incubation 6 drops of 2 N Na₂CO₃ was added to the tube no. 2.
7. Immediate colour change to pink or red after the addition of 2 N Na₂CO₃ was considered positive.

3.8. Pyrazinamidase test (Kent PT, 1985)

Principle
The deamidation of pyrazinamide to pyrazinoic acid and ammonia is helpful in separating weakly niacin positive strains of *M. bovis* from *M. tuberculosis* and the *M. avium complex*. *M. bovis* is negative at 7 days whereas both *M. tuberculosis* and the *M. avium complex* are positive within 4 days.

Reagents
1. 6.5 gm 7H9 Middlebrook broth was dissolved in 1 liter distilled water. Then 0.1 gm of pyrazinamide, 2 gm of pyruvic acid and 15 gm of agar was added to the broth. This solution was then autoclaved.
2. 1% Ferrous ammonium sulphate (0.1gm in 10ml distilled water).

Procedure
1. With a sterile pipette, 5ml of the broth base was added aseptically to screw capped test tubes.
2. The surface of medium was inoculated with growth from the culture and kept for incubation at 37º C.
3. After 4 days, 1 ml freshly prepared 1% ferrous ammonium sulfate was added to the inoculated culture.
4. The test was kept for 30 minutes at room temperature.
5. The medium was then examined for the formation of pink band in the medium.
6. Appearance of pink band in medium was considered to be positive.

The pink band which forms in the subsurface agar and diffuses into the medium, indicates the enzymatic hydrolysis of pyrazinamide to free pyrazinoic acid.
4. Molecular identification of *Mycobacteria*

DNA was extracted from the samples and from the positive cultures. The extracted DNA was used for molecular identification of mycobacteria.

4.1. *Extraction of DNA*

Extraction of DNA from Fine needle aspirates /pus and blood samples and with positive cultures was done by using Lysozyme and Proteinase K method (van Soolingen D, 1992; van Soolingen D, 1994 & Wards BJ, 1995). Confirmations of isolated DNA were analyzed on a 2% agarose gel. The extracted DNA was used for the PCR amplification and PRA analysis. Extraction of the DNA was performed as follow:

1. The processed FNA /culture were taken in the 2 ml micro centrifuge tube, dissolved with 500 μl of 1X Tris-EDTA buffer and was incubated in a water bath at 80 ºC for 20 minutes to inactivate the bacteria.

2. The bacteria were then lysed with 50 μl lysozyme (10 mg/ml) and vortexed before incubation for one hour at 37 ºC. The lysozyme-treated samples were incubated at 65 ºC for 1 to 2 hours in the presence of 70 μl (10 mg/ml) of proteinase K and 10 μl (10%) sodium dodecyl sulphate (SDS).

3. Then 100 μl of 5 M NaCl and 80 μl of cetyl-trimethyl-ammonium bromide sodium chloride (NaCl-CTAB) were added and vortexed until the solution became milky. The solution was kept in water bath at 65ºC for 10 minutes.

4. Chloroform/isoamyl alcohol (24:1) extraction was performed by adding 700 μl of Chloroform: isoamyl alcohol. The solution was vortexed and centrifuged at 13,500 rpm for 5 minutes.
5. The DNA precipitate was obtained by adding 600 µl isopropanol and after storage for 30 minutes to 1 hour at -20 °C. The DNA was collected by centrifugation at 12,000 RPM for 15 minutes.

6. The extracted DNA was washed with 1 ml of 70 % cold ethanol, by putting the ethanol in the tube having the pellet with the help of tips and pipette. The pellet was dissolved in 0.1 X TE buffer.

7. The electrophoresis was performed to check the presence of DNA.

8. Finally, the DNA was stored at 2 °C until PCR was performed.

4.2. Purity of genomic DNA (Ratan A, 1990)

To check the purity of genomic DNA, spectrophotometeric analysis was performed by taking optical density reading ratio at 260 nm and 280 nm.

The absorbance ratio of ~ 1.8 was accepted as “pure” for DNA because this signifies that there was no impurities and proteins present. If the ratio is appreciably lower in this case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

4.3. PCR amplification

PCR amplification was performed from the extracted DNA with different molecular markers for the diagnosis and identification of mycobacterial species.

4.3.1 MPB64 Primers:  
F (5’-TCCGCTGCCAGTCGTCTTCC-3’)
R (5’-GTCCTCGCGAGTCTAGGCCA-3’)

these set of primers were used to amplify a 240 base pair region (460-700 nucleotides) from the gene encoding the MPB 64 kDa protein.

Amplification of 240 base pair region (460-700 nucleotides) was performed in a 25-µl reaction mixture. The reaction mixture for PCR was prepared by adding the reagents stated below:
MATERIAL & METHODS

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X buffer</td>
<td>2.5µl</td>
</tr>
<tr>
<td>50 mM MgCl2</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTP’S</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Primer forward (25 pmol)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer reverse (25 pmol)</td>
<td>1 µl</td>
</tr>
<tr>
<td>5 U of Taq DNA polymerase</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>5 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>11.7 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

The PCR reaction mixture was made in 2 ml eppendorf tube using 10 X buffer, 50 mM MgCl2, 10 mM of dNTP mix, 25 pmol of each primer and 5 units of Taq polymerase and Nuclease free water. The PCR was performed in 0.2 ml PCR tubes which had 5 µl DNA template and 20 µl of reaction mixture.

The amplification cycle was performed by using a Thermocycler of BIO-RAD, USA with the program stated below:

The thermal profile involved initial denaturation for 4 minutes at 94°C and 29 cycles with the following steps: 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C and 30 seconds of extension at 72°C. The final extension was for 10 minutes at 72°C.

4.3.2. Hup B Primers: F  N ( 5’-GGAGGGTTGGGATGAACAAAGCAG-3’ )

R  S (5’-GTATCCGTGTCTTGACCTATTTG-3’) these set of primers was used to amplify a 645, 618 base pair region from the gene encoding the hup B protein (histone like protein).

Amplification of a 645, 618 base pair region from the gene encoding the hup B protein (histone like protein) was performed in a 25-µl reaction mixture. The reaction mixture for PCR was prepared by adding the reagents stated below:
MATERIAL & METHODS

<table>
<thead>
<tr>
<th>Component</th>
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<tbody>
<tr>
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<td>2.5 µl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTP’S</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Primer forward (25 pmol)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer reverse (25 pmol)</td>
<td>1 µl</td>
</tr>
<tr>
<td>5 U of Taq DNA polymerase</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>5 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>11.7 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
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</tbody>
</table>

The PCR reaction mixture was made in 2 ml eppendorf tube using 10 X buffer, 50 mM MgCl₂, 10 mM of dNTP mix, 25 pmol of each primer and 5 units of Taq polymerase and Nuclease free water. The PCR was performed in 0.2 ml PCR tubes which had 5 µl DNA template and 20 µl of reaction mixture. The amplification cycle was performed by using a Thermocycler of BIO-RAD, USA with the program stated below:

The thermal profile involved initial denaturation for 10 minutes at 94°C and 34 cycles with the following steps: 1 minute 30 seconds of denaturation at 94°C, 1 minute 30 seconds of annealing at 60°C, and 1 minute 50 seconds of extension at 72°C. The final extension was for 30 minutes at 72°C.

4.3.3. Hsp65 Primers: F TB11 (5'-ACCAACGATGGTGTTCCAT-3')
R TB12 (5'-CTTGTGCGACCGCATACCT-3') these set of primers was used to amplify to 439 base pair region between positions 398 and 836 from the gene encoding the 65 kDa heat shock protein (hsp 65).

Amplification of a 439 base pair region between positions 398 and 836 from the gene encoding the 65 kDa heat shock protein (hsp 65) was performed in a 25-µl reaction mixture. The reaction mixture for PCR was prepared by adding the reagents stated below:
MATERIAL & METHODS

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>10 X buffer</td>
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</tr>
<tr>
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<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTP’S</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Primer forward (25 pmol)</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer reverse (25 pmol)</td>
<td>1 µl</td>
</tr>
<tr>
<td>5 U of Taq DNA polymerase</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>DNA template</td>
<td>5 µl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>11.7 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

The PCR reaction mixture was made in 2 ml eppendorf tube using 10 X buffer, 50 mM MgCl₂, 10 mM of dNTP mix, 25 pmol of each primer and 5 units of Taq polymerase and Nuclease free water. The PCR was performed in 0.2 ml PCR tubes which had 5 µl DNA template and 20 µl of reaction mixture. The amplification cycle was performed by using a Thermocycler of BIO-RAD, USA with the program stated below:

The thermal profile involved initial denaturation for 5 minutes at 95°C and 35 cycles with the following steps: 1 minute of denaturation at 94°C, 1 minute of annealing at 60°C, and 1 minute of extension at 72°C. The final extension was for 7 minutes at 72°C.

4.3.4. 16SrRNA-23SrRNA inter spacer region Primers

Sp1 (5'ACCTCCTTTCTAAGGAGCACC-3')

Sp2 (5'GATGCTCGCAACCACTATCCA-3') these set of primers was used to amplify to a part of 16S-23S spacer product of 200 base pair to 330 base pair (positions 210 and 190 of the M. tuberculosis spacer sequence).

Amplification of a part of 16S-23S spacer product of 200 base pair to 330 base pair (positions 210 and 190 of the M. tuberculosis spacer sequence) was performed in a 25-µl reaction mixture. The reaction mixture for PCR was prepared by adding the reagents stated below:
### MATERIAL & METHODS

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X buffer</td>
<td>2.5µl</td>
</tr>
<tr>
<td>50 mM MgCl₂</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTP’S</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>Primer forward (25 pmol)</td>
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</tr>
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<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

The PCR reaction mixture was made in 2 ml eppendorf tube using 10 X buffer, 50 mM MgCl₂, 10 mM of dNTP mix, 25 pmol of each primer and 5 units of Taq polymerase and Nuclease free water. The PCR was performed in 0.2 ml PCR tubes which had 5 µl DNA template and 20 µl of reaction mixture. The amplification cycle was performed by using a Thermocycler of BIO-RAD, USA with the program stated below:

The thermal profile involved initial denaturation for 5 minutes at 96°C and 38 cycles with the following steps: 1 minute of denaturation at 94°C, 1 minute of annealing at 59°C, and 1 minute of extension at 72°C.

### 4.3.4. rpo B gene Primers:

**F** RPO (5’TCAAGGAGAAGCGCTACGA-3’)

**R** RPO (5’GGATGTGGATCGGGTCTGC -3’)

These set of primers was used to amplify a region of interest rpo B, product of 360 base pair (bases 902 and 1261 and codons 302 to 420) the sequence of the rpo B gene of *M. tuberculosis*.

Amplification of a region of interest rpo B, product of 360 base pair (bases 902 and 1261 and codons 302 to 420) the sequence of the rpo B gene of *M. tuberculosis* was performed in a 25-µl reaction mixture. The reaction mixture for PCR was prepared by adding the reagents stated below:
### MATERIAL & METHODS

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<td>Total volume</td>
<td></td>
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The PCR reaction mixture was made in 2 ml eppendorf tube using 10 X buffer, 50 mM MgCl₂, 10 mM of dNTP mix, 25 pmol of each primer and 5 units of Taq polymerase and Nuclease free water. The PCR was performed in 0.2 ml PCR tubes which had 5 µl DNA template and 20 µl of reaction mixture. The amplification cycle was performed by using a Thermocycler of BIO-RAD, USA with the program stated below:

The thermal profile involved initial denaturation for 5 minutes at 96°C and 38 cycles with the following steps: 1 minute of denaturation at 94°C, 1 minute of annealing at 59°C, and 1 minute of extension at 72°C.


Detection of amplified product was performed by gel electrophoresis, 5 µl of amplified DNA was electrophoresed on 2% agarose containing ethidium bromide, along with 1 µl of 100 base pair molecular marker.

For preparation of agarose gel, 2 gm of agarose was dissolved in 100 ml of 1X TAE at 100°C and after agarose cools down, 8 µl of ethidium bromide (500 µg/ml) was added. This agarose is poured in the tray having the comb and allowed agarose to solidify. After the solidification the comb is removed and the tray is submerged in electrophoresis unit, having 1X TAE buffer.
The Wearing of gloves is important as ethidium bromide is carcinogenic and mutagenic. The ethidium bromide binds to DNA by intercalating between the bases and its fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light, so that it can be visible under UV trans-illuminator. A mixture of 5 μl amplified DNA, 2 μl of 6X BPB (Bromophenol blue) and 3 μl of distilled water (total 10 μl) was loaded into the 2% agarose gel. The electrophoresis was carried out at a constant voltage of 50 V for 1 hour.

Nucleic acid molecules are negatively charged hence, migrate towards positive pole. The amplicons were analyzed on a 2% agarose gel with 100 base pair ladder size standards and were visualized under UV trans-illuminator.

5. PRA (POLYMERASE CHAIN REACTION- Restriction Fragment Length Polymorphism ANALYSIS

Restriction fragment length polymorphism (RFLP) analysis was performed with the amplicons achieved after the PCR amplification of Hsp 65 gene, 16S-23s rRNA inter spacer region gene and rpoB gene, using different restriction enzymes like BstEII, HaeIII, MspI, CfoI, TaqI, DdeI, AvaII and Hinfl. The amplicons of MPB 64 genes were only used for identification of the member of M. tuberculosis complex and Hup B was only used for identification M. tuberculosis and M. bovis.

Restriction analysis

5.1. After the amplification of Hsp 65 gene of 439 base pair, digestion was performed with BstE II and HaeIII enzyme (Telenti et al., 1993).

For BstE II digestion, 10 μl of PCR product was added directly to a mixture containing 0.5 μl (=5 U) of enzyme, 2.5 μl of restriction buffer (5X buffer B) and 11.5μl of water, and the mixture was incubated for 60 min at 60°C and the fragments were analysed. The fragments of DNA were analysed for different Mycobacterial species, as described by Telenti et al., 1993.
strain of *M. tuberculosis* (H₃₇Rᵥ) was used as a positive control for the analysis.

Similarly for *HaeIII* digestion, 10 µl of PCR product was added directly to a mixture containing 0.5 µl (=5 U) of enzyme, 2.5 µl of restriction buffer (5X buffer B) and 11.5µl of water, and the mixture was incubated for 60 min at 37°C and the fragments were analysed. The fragment of DNA were analysed for different Mycobacterial species, as described by Telenti *et al.*, 1993. The strain of *M. tuberculosis* (H₃₇Rᵥ) was used as a positive control for the analysis.

5.2. After the amplification of rpo B gene of 360 base pair, digestion was performed with *Msp I* and *HaeIII* enzyme (Hyeyoung *et al.*, 2000).

For *Msp I* digestion, 10µl of PCR product was digested in a 20 µl reaction volume containing 5 U of *Msp I* and 2µl of the 10X reaction buffer. After 2 hours of incubation at 37°C the fragments were analysed. The fragment of DNA were analysed for different Mycobacterial species, described by Hyeyoung *et. al.*, 2000. The strain of *M. tuberculosis* (H₃₇Rᵥ) was used as a positive control for the analysis.

Similarly, 10 µl of PCR product was digested in a 20µl reaction volume containing 5 U of *HaeIII* enzyme and 2µl of the 10X reaction buffer. After 2 hours of incubation at 37°C the fragments were analysed. The fragments of DNA were analysed for different Mycobacterial species, as described by Hyeyoung *et al.*, 2000. The strain of *M. tuberculosis* (H₃₇Rᵥ) was used as a positive control for the analysis.

5.3. After the amplification of 16SrRNA-23SrRNA inter spacer region gene of 200-330 base pair digestion was performed with *HaeIII, CfoI, TaqI, AvaII, HinfI* and *DdeI* enzyme (Roth *et al.*, 2000).

For *HaeIII, CfoI, TaqI, Ava II, HinfI* and *DdeI* digestion, 10 µl of PCR product was added directly to a mixture containing 0.5 µl (=2 U) of enzyme, 2.5 µl of restriction buffer (5X buffer B) and 11.5µl of water and the mixture was incubated for 60 min at 37°C and the fragments were analysed.
The fragments of DNA were analysed for different Mycobacterial species, after the digestion with HaeIII, as described by Roth et al., 2000. The strain of *M. tuberculosis* (H$_{37}$R$_{v}$) was used as a positive control for the analysis. The patterns of DNA fragments were analysed for different Mycobacterial species, after the digestion with CfoI, TaqI, Hinfl, Ava II and Ddel enzymes. The strain of *M. tuberculosis* (H$_{37}$R$_{v}$) was used as a positive control for the analysis.

To confirm the species after RFLP, few amplicons were sent for sequencing. Nucleotides BLAST analysis (NCBI) was performed to confirm the species and the results of sequencing. The PCR sequencing was done with forward and reverse primers, the nucleotide sequences and Electropherogram results were achieved using Finch TV software. The identification of Mycobacteria was completed by comparing the nucleotide sequence with the library of known sequences at the databases.

The generated sequences were assembled and edited using BLAST (www.ncbi.nlm.nih.gov). A distance of 0.00% to less than 1.00% was used as the criteria for species identification.

5.4. Detection of Amplified Product (Telenti et al, 1993)

Detection of DNA fragments after the digestion were performed by gel electrophoresis, 10 μl of DNA fragment was electrophoresed on 3% agarose containing ethidium bromide, along with 1 μl of 50 base pair molecular markers.

For preparation of agarose gel, 3 gm of agarose was dissolved in 100 ml of 1X TAE at 100°C and after agarose cools down, 8 μl of ethidium bromide (500 μg/ml) was added. This agarose is poured in the tray having the comb and allowed agarose to solidify. After the solidification the comb is removed and the tray is submerged in electrophoresis unit, having 1X TAE buffer.

The Wearing of gloves is important as ethidium bromide is carcinogenic and mutagenic. The ethidium bromide binds to DNA, by
intercalating between the bases and is fluorescent meaning that it absorbs invisible UV light and transmits the energy as visible orange light, so that it can be visible under UV trans-illuminator. A mixture of 10 μl amplified DNA, 2 μl of 6X BPB (Bromophenol blue) and 3 μl of distilled water (total 15 μl) was loaded into the 3% agarose gel. The electrophoresis was carried out at a constant voltage of 50 V for 1 hour.

DNA fragments are negatively charged hence, migrate towards positive pole. The amplicons after the digestion with restriction enzymes were analyzed on a 3% agarose gel with 50 base pair and 100 base pair ladder size standards and were visualized under the UV trans-illuminator.

6. DNA Sequencing (GCC, BIOTECH PVT LTD, Kolkata, India)

DNA sequencing was done by dideoxy-terminator cycle sequencing method and analyzed by 3500 genetic analyzer by Applied biosystems sequencing instrument.

ANALYSIS OF THE RESULTS

The findings of the PCR were correlated with the findings of other diagnostic techniques. AFB culture was considered as a Gold Standard for the Statistical Analysis i.e. Sensitivity and specificity of different assay methods were calculated. The results were analyzed with SPSS version 16. The p values <0.05 were considered significant. Kappa coefficient was used to analyze the agreement between the diagnostic criteria. The k value consideration as values between 0.2 to 0.4 (moderate), 0.4 to 0.6 (fair), 0.6 to 0.8 (good) and above 0.8 (excellent). The Fisher Exact test is used when the minimum expected count was observed to be less than 5. This is discussed in details in our results.