SUMMARY

The present study was carried out in the Department of Microbiology of Maulana Azad Medical College and Associated and Associated Lok Nayak and GNEC Hospitals Hospitals, a major tertiary health-care centre in New Delhi. A total of 100 cases (N=100), clinically suspected cases of tuberculous lymphadenitis, which were suggestive of tuberculous lymphadenitis by cytopathology, were considered for the study. Biopsy was not required in any of the cases, as the diagnosis could be done by FNAC only.

Clinical samples were processed for direct microscopy, acid fast bacilli (Mycobacterial) culture by conventional culture (LJ medium), mycobacterial culture by radiometric (BACTEC) method and for Polymerase Chain Reaction (PCR). Identification of Mycobacteria was done by various biochemical methods like Niacin test, Catalase test, Nitrate reduction test, Arylsulphatase and Tween 80 hydrolysis. Detection of Mycobacterial species was performed by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PRA) analysis by using Different Restriction Enzymes. Sequencing of few randomly picked amplicons were also done to confirm the mycobacterial species.

A. Study on Fine needle aspirates samples of the patients (n=100)

1. Patients presentations

All the patients in the study group ranged from 2 years to 45 years. Age group 21-30 years contained the maximum number of patients. Majority of the patients were female as compared to male participants (68% v/s 32%).

2. Clinical / Systemic symptoms of the study population (n=100).

Out of 100 patients 73 patients had systemic symptom of weight loss, 72 of them had fever, 66 patients had anorexia and 58 patients had complaint of night sweats.
3. Mantoux results of the study population

Out of 100 cases Mantoux test was found to be reactive (≥10 mm) in 81 patients (81%) and 19 (19%) non-reactive.

4. Radiological results of the study population (n=100)

Out of 100 patients, 76 (76%) were normal on the Chest X-ray and 24 (24%) patients showed impression of having pulmonary tuberculosis. Out of 100 patients, 79 (79%) showed no abnormality in their Ultrasonography of abdomen and 21 (21%) patients showed impression of having abnormalities of lymph node enlargement.

5. Pathological results of the study population (n=100)

5.1. Site of swelling of lymph nodes in the study populations

Out of 100 patients, the most common region of lymph node swelling was cervical region in 66 (66%) cases, followed by submandibular 10 (10%) and supraclavicular 10 (10%), multiple lymph node in 7(7%) , axillary 5 (5%) and inguinal node swelling was observed in 2 (2%) cases.

5.2 Cytopathology results

Out of the 100 patients, (i) 23 (23%) were suggestive of tuberculous lymphadenitis with AFB positivity and (ii) 77 (77%) were suggestive of tuberculous lymphadenitis and AFB negative by cytopathology.

6. Microbiology study

On ZN staining, the bacilli appeared red, slender and beaded rods. 24 (24%) were found to be AFB positive after ZN staining. On examination of Mycobacterial culture on Lowenstein Jensen (LJ) medium, 34 (34%) samples were found AFB positive and (66%) samples were found AFB negative. On examination of BACTEC radiometric culture, 45(45%) samples were found to be AFB positive and 55 (55%) AFB negative. The biochemical tests like niacin production, nitrate reduction, catalase production test, Arylsulphatase test, urease test, pyrazinamidase test and tween 80 hydrolysis tests confirmed the presence of Mycobacterium tuberculosis in the isolates.
7. Molecular study

A total of 100 samples were processed for the DNA extraction to know the utility of the molecular markers. DNA was used for PCR amplification by five different primers as: Hsp65, Hup B, MPB 64, rpoB and 16S-23Sr RNA ITS.

Correlation of PCR with clinical results

When the PCR was compared with presence of clinical/systemic symptoms, out of 100 patients, 89 showed one or more than one clinical/systemic symptoms i.e. fever / weight loss / night sweat / anorexia, along with lymph nodes swelling, among them 41 patients were found to be (41/89) positive by PCR.

All the patients who showed impressions of having pulmonary tuberculosis were positive by PCR for tuberculous lymphadenitis (24/24). 31.6% PCR positivity was achieved among the patients with normal Chest X-rays reports.

Correlation of PCR with cytopathological results

Overall 48% cases were PCR positive for tuberculous lymphadenitis in the study population (48/100). All the patients, who were suggestive of tuberculous lymphadenitis and AFB positive were positive by PCR for tuberculous lymphadenitis (23/23) and patients who were suggestive of tuberculous lymphadenitis and AFB negative by cytopathology among them PCR positivity was found to be in 25 cases (25/77) i.e. 32.5%. We got the maximum PCR positive result by Hsp 65 in 48%, followed by Hup B in 47%, MPB 64 and 16S-23Sr RNA in 45% and rpo B in 44% cases. The sensitivity and specificity of different molecular markers, when compared with cytopathology was found to be 60% to 66.67% and 80% respectively. The PPV and NPV were found to be 81.8% to 83.3% and 57.14% to 61.5% respectively.
Correlation of PCR with microbiological results

All the samples which were positive by direct smear microscopy for AFB (24), all were positive by PCR. There were 76 samples which were negative by direct smear microscopy for AFB, among them 24 were positive by PCR.

All the 34 samples which were positive on LJ medium (34) and by BACTEC (45) were positive by PCR. There were additional 14 samples which did not show growth on LJ medium but were Positive by PCR. The sensitivity and specificity of PCR when compared with LJ medium was found to be 100% and 78.8% respectively in these cases. The PPV and NPV were found to be 70.83% and 100%.

There were 3 (n=3) samples which were negative by BACTEC culture but positive by PCR. The sensitivity and specificity of PCR when compared with BACTEC culture was found to be 100% and 94.54% respectively in these cases. The PPV and NPV were found to be 93.75% and 100%.

The sensitivity of PCR with five different primers namely Hsp 65, Hup B, MPB 64, 16S-23Sr RNA and rpo B when compared with BACTEC culture was found to be 100%, 100%, 100%, 100% and 95.55% respectively and the specificity was 94.45%, 96.36%, 98.18%, 98.18% and 100% respectively. The PPV were found to be 93.75%, 95.74%, 97.78%, 97.78% and 100% respectively and NPV 100%, 100%, 100%, 100% and 96.49% respectively.

To establish PCR assays to differentiate between M. tuberculosis complex and Non-tuberculous mycobacterium (NTM), Polymerase Chain Reaction – Restriction Fragment Length polymorphism (PRA) analysis was used. All the culture positive samples were used for the Polymerase Chain Reaction – Restriction Fragment Length polymorphism (PRA) analysis.

A total of 45 culture positive samples were used for PRA analysis. Few samples were also sent for sequencing to confirm the species.
**Results of Restriction analysis**

1. After the amplification of Hsp 65 gene of 439 base pair, digestion was performed with *BstEII* and *HaeIII* enzyme. The fragments of DNA after *BstEII* enzyme digestion were observed approximately at 240 base pair, 120 base pair and 90 base pair ± 5 base pairs. Similarly the fragments of DNA after *HaeIII* enzyme digestion was observed approximately at 150 base pair, 120 base pair and 70 base pair ± 5 base pairs respectively. The PRA patterns were observed to be similar for *M. tuberculosis* as described by Telenti et al., 1993.

2. After the amplification of rpo B gene of 360 base pair, digestion was performed with *MspI* and *HaeIII* enzyme. The fragments of DNA after *MspI* enzyme digestion were observed approximately at 170 base pair, 80 base pair, 60 base pair and 40 base pair ± 5 base pairs respectively. Similarly the fragments of DNA after *HaeIII* enzyme digestion was observed approximately at 250 base pair and 100 base pair ± 5 base pairs respectively. The PRA patterns were observed to be similar for *M. tuberculosis*, as described by Hyeyoung et al., 2000.

3. After the amplification of 16SrRNA-23SrRNA inter spacer region gene of 200-330 base pair digestion was performed with *HaeIII, CfoI, TaqI, Avall, Hinfl* and *Ddel* enzyme. The fragments of DNA after *HaeIII* enzyme digestion were observed approximately at 110 base pair, 60 base pair and 50 base pair ± 5 base pairs respectively. No digestion was observed after the digestion with *CfoI, TaqI* and *Hinfl* enzymes and DNA fragments patterns approximately at 140 base pair and 70 base pair ± 5 base pairs respectively were observed after *Avall* and *Ddel* digestion. The PRA patterns were observed to be similar for *M. tuberculosis*, as described by Roth et al., 2000.

To confirm the species after RFLP, few amplicons were sent for sequencing. The generated sequences were assembled and edited using BLAST ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). A distance of 0.00% to less than 1.00% was used as the criteria for species identification. Sequencing results of amplicons confirmed the presence of *Mycobacterium tuberculosis*. 
The biochemical tests like niacin production, nitrate reduction, catalase production test, Arylsulphatase test, urease test, pyrazinamidase test and tween 80 hydrolysis test correlated well with the PRA analysis and sequencing results in the present study. Presence of *Mycobacterium tuberculosis* was confirmed among the tuberculous lymphadenitis patients in our study.

**B. Study on blood samples of the patients (n=100)**

To evaluate the usefulness of PCR performance in blood samples of patients with tuberculous lymphadenitis. The samples were processed for direct microscopy, culture on LJ medium, BACTEC culture and for PCR.

1. **Microbiological study**

   On ZN staining, the bacilli appeared red, slender and beaded rods. 4 (4%) were found to be AFB positive after ZN staining. On examination of Mycobacterial culture on Lowenstein Jensen (LJ) medium, 10 (10%) samples were found AFB positive and (90%) samples were found AFB negative. On examination of Mycobacterial radiometric culture, 19 (19%) samples were found AFB positive and 81 (81%) AFB negative.

2. **Molecular study**

   After the PCR amplification for Hsp 65 gene 16 (16 %) samples were found to be positive and 84 (84 %) were negative.

   Four samples which were positive by direct smear microscopy for AFB, all were positive by blood PCR. There were 96 samples which were negative on direct smear microscopy, among them 12 were positive by blood. The 10 samples which were culture positive on LJ medium were also positive by BACTEC culture and PCR. The sensitivity and specificity of PCR when compared with LJ culture was found to be 100% and 93.3% respectively in these cases. The PPV and NPV were found to be 62.5% and 94%.

   Out of 19 culture positive samples, 63.16% were found to be positive by blood PCR for tuberculous lymphadenitis (12/19) along with this 4 out of 81
culture negative samples, were found to be positive by blood PCR for tuberculous lymphadenitis. Out of 45 fine needle aspirate (FNA) culture positive samples, 35.5% were found to be positive by blood PCR for tuberculous lymphadenitis (16/45). All 55 fine needle aspirate (FNA) culture negative samples, were also negative by blood PCR. When Blood PCR was compared with FNA culture positive case the sensitivity and specificity of PCR was found to be 35.5% and 100% respectively in these cases. The PPV and NPV were found to be 100% and 65.47% (p value = 0.04). When blood PCR and FNA culture were compared the p value was observed to be significant (p value = 0.001). The results indicate that use of blood PCR for diagnosis of tuberculous lymphadenitis is still incipient but holds promise.

The Blood PCR performance was found to be sensitive and specific in diagnosis of tuberculous lymphadenitis when compared to the culture. The sensitivity and specificity of PCR when compared with culture was found to be 63.15% and 95% respectively in these cases. The PPV and NPV were found to be 75% and 91.66%. The results indicate that usefulness of blood PCR for diagnosis of tuberculous lymphadenitis is still incipient, but may be used as an additional diagnostic technique in diagnosis of tuberculous lymphadenitis.