**ABSTRACT**

The diagnosis of extra-pulmonary tuberculosis (EPTB) remains a significant clinical difficulty, primarily because of the insufficient sensitivity of conventional bacteriologic methods for detecting Mycobacterium tuberculosis in extra-pulmonary specimens. The PCR assays innovated in the developed countries are based on the genetic make-up of strains of Mycobacterial species circulating in those regions. Study also focuses on the distribution pattern of EPTB in different extra-pulmonary specimens as well as in different age groups in males and females. For this study, 1200 extra pulmonary specimens for tuberculosis were collected from suspected patients from different hospitals of Delhi / NCR region. The specimens includes; menstrual blood, pus, urine, endometrial biopsy, endometrial pipelles, ascetic fluid, semen, pleural fluid, products of conception, endometrial tissue, endometrial curetting, body fluid, and tissue, cerebrospinal fluid. The DNA for PCR was extracted by the silica columns. Primers that amplify a 240 bp fragment of MPB64 from Mycobacterium tuberculosis complex were utilized. Out of 1200 specimens, females were more prevalent for the disease as 177 samples showed the positivity and 73 male samples were detected as positive for MTB. Considering age as a one of the parameters for our study, we found that positivity rate of TB detected in females in the age group of 41-60 was higher (i.e. 10.6) and among the males TB was detected higher in the age group of 21-40 (i.e. 13.9). The positivity rate was detected higher in “Menstrual Blood” with the positivity rate of “28.7” and least in “Blood” with the positivity rate of “3.2”. These results demonstrate that PCR based on MPB64 gene target can be a highly specific and sensitive aid in the detection of M. tuberculosis from extra-pulmonary specimens.

**Key words:** Extra-Pulmonary tuberculosis, MPB-64 gene, PCR.

**INTRODUCTION**

More than a century even after the discovery of the tubercle bacillus by Robert Koch, tuberculosis remains one of the major causes of global death from a single infectious agent and constitutes a major health threat worldwide (WHO, 2009). Tuberculosis (TB) continues to be a severe disease that predominantly affects developing countries. India alone contributes more than 25% of the tuberculosis burden, with a population of about 1230 million (Anonymous 1). It is the highest TB-burden country in the world in terms of absolute numbers of incident cases that emerge each year and it contributed one fourth of the estimated global incident TB cases in 2010 (Chow TWP et al., 2002).

Demonstration of acid-fast bacilli (AFB) in a smear prepared from clinical specimens provides a preliminary diagnosis of mycobacterial disease; culture
methods still required an average of 3-4 weeks to recover mycobacterium from clinical specimens (Khan MH, 1996). Since the prevention of tuberculosis relies on the early detection and cure the infectious cases.

In cases of suspected extra pulmonary tuberculosis (EPTB), rapid and accurate laboratory diagnosis is of prime significance, since traditional techniques for detecting acid-fast bacilli have limitations. The disease underestimations by clinicians in terms of the use of insensitive conventional analytical methods have contributed difficulties in managing patients with EPTB (Corbett EL et al., 2006). As AFB staining and mycobacterium culture method are with low sensitivity for detecting EPTB, the development and evaluation of new diagnostic strategies is of extreme priority for tuberculosis control programs (Jonas V et al., 1993). The implementation of EPTB diagnostic methods with high specificity and sensitivity would improve the clinical outcomes for EPTB patients, and should accelerate the application of appropriate public health control measures (Noel AB et al., 1989). The recent demonstration that nucleic acid amplification techniques are rapid and sensitive has modified strategies for the detection of Mycobacterium tuberculosis (Thornton CG et al., 1998).

Since the application of the polymerase chain reaction (PCR) in the diagnosis of tuberculosis as cited by Brisson et al., varying methods for DNA purification and different M. tuberculosis target sequences have been evaluated (WHO, 2010). Among numerous target sequences, PCR-based assays using the mpb64 gene have shown considerable promise in clinical studies. Eisenach et al. initially demonstrated that a polymerase chain reaction (PCR) using primers designed from the IS6110 sequence proved to be a highly sensitive and specific means of diagnosing pulmonary tuberculosis (Horner PJ, Moss FM, 1991). Nolte et al. later showed that IS6110-based PCR assay showed 91% sensitive and 100% specificity in detecting M. tuberculosis in sputum specimens (Selwyn PA, 1991). Because of the difficulties associated with diagnosing EPTB, there has been considerable interest in applying PCR methods.

Several investigators have demonstrated that PCR is a useful tool for identifying tubercle bacilli in cerebrospinal fluid (CSF) (Kashyap RS et al., 2002). PCR analysis of urine specimens has also been shown to be a fast and sensitive technique for identifying M. tuberculosis (Katt MK, 2002). Present study includes the evaluation of diagnostic usefulness of PCR method based on mpb-64 target sequence useful for detecting mycobacterium tuberculosis complex in Indian patients with a presumptive clinical diagnosis of EPTB. Study also focuses on the distribution pattern of EPTB in different age groups in males and females.

**MATERIAL & METHODS**

Total 1200 specimens from patients with a high clinical suspicion of extra-pulmonary tuberculosis were received at Auroprobe Laboratories, Muradnagar, Ghaziabad (U.P), India from Nov 2009 to Nov 2012 for the detection of MTB including Menstrual blood (156), pus (26), urine (198), endometrial biopsy (110), Endometrium (103), Pipelles (67), Ascetic fluid (50), Semen (81), Pleural fluid (65), Product of Conception (69), Endometrial Tissue (43) Endometrial curettng (50), Body fluid (30), Tissue (18), Cerebrospinal Fluid (75) Blood (62). All these samples were smearing negative. Ethical approval was not needed for the current study as all the samples from the subjects were received for clinical diagnosis from Different collection points and we had not disclosed any identification of the subjects.

**DNA Extraction**

For DNA isolation from all clinical samples, 200µl of sample was taken as starting volume. 20µl of Proteinase K (20mg/ml) were added in the entire clinical specimen with the addition of 200 µl of extraction buffer (Lysis buffer 1 and 2). The mixture was vortexed (for 15-20 sec) and incubated at 60°C for 20 minutes thereafter was transferred to 90 °C for 10 minutes. 200µl of chilled absolute ethanol was added to each specimen in order to precipitate the DNA. All the contents were vortexed properly and then transferred into prearranged silica columns and centrifuged at 10,000 rpm for 3 minutes at 4 °C. Now the columns were transferred to fresh collection tubes. 500µl wash buffer [Reconstituted by 60 ml 98% abso (Mol. Grade)] and centrifuged at 10,000 rpm for 3 minutes at 4 °C after which the contents were transferred to fresh collection tubes; the procedure was repeated again followed by dry wash at 13000 rpm for 2 minutes. The columns were transferred to autoclaved microfuge tubes and 60 µl of pre heated milli Q water was added to each column which was then centrifuged at 13000rpm at 4 °C for 5min. The column was discarded and eluted DNA was collected in micro centrifuge tube.

**Polymerase Chain Reaction**

Mannose binding protein-64 (MPB64) gene in the M. tuberculosis complex and was targeted. DNA was extracted with AuPrep DNA extraction kit by silica column based method (Miorner H et al., 1995). PCR was performed for the amplification of MBP64 gene in Mycobacterium tuberculosis complex using primers (Sigma Aldrich), (forward primer 5'-TCCGCTGCCAGTCGTTCC and reverse primer 5'-GTCCTCGCAGTCTGACCA) (Kox LF et al., 1995) . A reaction mixture of 50 µl containing 10X PCR buffer (250 mM Tris HCl, 500 mM KCl), 0.2 mM dNTPS, 25µM primers, Taq DNA polymerase (3units/microlitre) and Mg²⁺ ions (25mM) and milliQ was used. DNA
template was added in the master mix and amplification was done on thermal cycler with initial denaturation at 95°C for 6 minutes, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and primer extension at 72°C for 30 seconds with 35 rounds of repetitive cycles for denaturation, annealing, extension with final extension at 72°C for 7 minutes. A 240 bp amplicon was observed for mpb64 gene on 2% agarose gel. The sensitivity, specificity, positive predictive value and the negative predictive value were calculated using the standard formulae.

To avoid cross-contamination between samples and contamination of reagents or samples with PCR-amplified products or positive controls all the procedures for sample preparation and PCR included measures were observed as described by Kwok and Higuchi (Kwok S, Higuchi R, 1989). DNA extraction, preparation of reaction mixture and PCR amplification were carried out in three separate laboratory rooms located far away from each other. In all steps filtered tips were used to avoid carry over contamination. All the samples were run in duplicate.

**Statistical analysis**
Statistical analysis was done using SPSS program for window. Statistical significance for comparisons of proportions was determined by the Fisher exact test.

### RESULTS
In current study 1200 smear negative clinically suspected tuberculosis patients samples were analyzed for MTB DNA detection. Of these 409 were male and 791 were from female patients. Of the 766 cases, 356 (46.5%) were found positive for TB by PCR method. Different types of extra pulmonary samples showed different positivity rates as described in Table 1.

It is observed that 28.7% of the menstrual blood samples were found TB positive followed by 26.9% TB positive pus samples while Blood samples were least positive (3.2%).

Table 2 translates the positive mycobacterial DNA in different types of samples in males and females. Overall there was a significant difference of TB positivity in males (17.6%) and females (22.3%).

Effect of age on samples positivity was also being evaluated. It was found that most of the positive samples of males lie in the age group of 41 to 60 years (13.9%) while 34.2% samples were positive in the age group of 21-40 years (Table 2).

**Positivity Rate: Age Groups;** With reference to our first objective data for various types of extra-pulmonary samples is analyzed and thus tabulated accordingly. The data was taken into consideration from Nov, 09 to April, 10 and the data are tabulated as follows.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Type of Samples</th>
<th>Total samples n=1200</th>
<th>Positive</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Menstrual blood</td>
<td>153</td>
<td>44</td>
<td>28.7</td>
</tr>
<tr>
<td>2</td>
<td>Pus</td>
<td>26</td>
<td>7</td>
<td>26.9</td>
</tr>
<tr>
<td>3</td>
<td>Urine</td>
<td>198</td>
<td>49</td>
<td>24.7</td>
</tr>
<tr>
<td>4</td>
<td>Endometrial Biopsy</td>
<td>110</td>
<td>26</td>
<td>23.6</td>
</tr>
<tr>
<td>5</td>
<td>Endometrium</td>
<td>103</td>
<td>24</td>
<td>23.3</td>
</tr>
<tr>
<td>6</td>
<td>Pipelles</td>
<td>67</td>
<td>15</td>
<td>22.3</td>
</tr>
<tr>
<td>7</td>
<td>Ascetic fluid</td>
<td>50</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>Semen</td>
<td>81</td>
<td>17</td>
<td>20.9</td>
</tr>
<tr>
<td>9</td>
<td>Pleural fluid</td>
<td>65</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>Product of Conception</td>
<td>69</td>
<td>13</td>
<td>18.8</td>
</tr>
<tr>
<td>11</td>
<td>Endometrial Tissue</td>
<td>43</td>
<td>8</td>
<td>18.6</td>
</tr>
<tr>
<td>12</td>
<td>Endometrial curetting</td>
<td>50</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>13</td>
<td>Body fluid</td>
<td>30</td>
<td>5</td>
<td>16.6</td>
</tr>
<tr>
<td>14</td>
<td>Tissue</td>
<td>18</td>
<td>3</td>
<td>16.6</td>
</tr>
<tr>
<td>15</td>
<td>Cerebrospinal Fluid</td>
<td>75</td>
<td>4</td>
<td>5.3</td>
</tr>
<tr>
<td>16</td>
<td>Blood</td>
<td>62</td>
<td>2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Age group intervals in years (Total number of specimens) 1200</th>
<th>Positivity rate (%) of TB in males</th>
<th>Positivity rate (%) of TB in females</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>6.0</td>
<td>5.9</td>
</tr>
<tr>
<td>21-40</td>
<td>13.9</td>
<td>9.8</td>
</tr>
<tr>
<td>41-60</td>
<td>8.2</td>
<td>10.6</td>
</tr>
<tr>
<td>&gt;60</td>
<td>6.0</td>
<td>6.7</td>
</tr>
</tbody>
</table>
DISCUSSION

In spite of continuous effort in monitoring and treatment of tuberculosis, the disease remains a major public health issue (Sandgren A et al., 2012). The poor performance of conventional microbiological techniques in extrapulmonary specimens has encouraged the improved use of PCR tests in the laboratory diagnosis of tuberculosis (Foulds J, O’Brien R, 1998).

The diagnosis of extrapulmonary TB is complicated by the difficulty in obtaining adequate material for examination (Hofman V et al., 2003; Morata P et al., 2001). Tuberculous pleuritis, pericarditis, and meningitis have been associated with low number of organisms but high mortality. Microscopic examination of fluid or tissue is rarely positive and culture yield is also low. Therefore, a sensitive, rapid and accurate test would be of tremendous benefit in the diagnosis of extrapulmonary TB. Recently, our PCR assays have been modified and extended to detect Mycobacterium tuberculosis in extra-pulmonary specimens with satisfactory results (Morata P et al., 2001; Laifer G et al., 2004; Cheng VC SK et al., 2004; Hasaneen NA et al., 2003; Kami M et al., 2001; Lemaitre N et al., 2004).

During our study we find that TB is more prevalent in the age group of 21-40 in males with the positivity rate of 14.0 and least in <20 with rate of 5.9. On the other hand, In females TB is more prevalent in age group of 41-60 with the positivity rate of 10.3 and least in age group of 61-80 with positivity rate of 5.7. According to our second objective, different types of samples are considered from different body parts. Then we came to the conclusion that rate is highest in sample “Menstrual Blood” with the positivity rate of “28.7”, then in Pus is “26.9” and least in “Blood” with the positivity rate of “3.2”. According to our lab study we had considered, 1200 extra-pulmonary samples for our different objectives. The incidence of TB varies considerably around the world and most Mycobacterial infections in our country are still caused by MTB members and we confined our study regarding the prevalence of extra pulmonary TB cases. A quick and correct diagnosis is of great importance because of the high morbidity. Unfortunately, conventional bacteriological methods are time consuming, their sensitivity is low, and so treatment occasionally becomes empirical. PCR method has high specificity (92-98%) in identifying M. tuberculosis in various extra pulmonary specimens. Although we present a small number of cases, PCR reached a 100% overall sensitivity in detecting the pathogen. During our useful lab scale work & study we came to conclusion and we analyzed extra pulmonary samples by PCR using MPB 64 as a target gene for amplification and then came to conclusions that males are less prone to TB than females A large study of about 1200 clinical extra pulmonary specimens that includes CSF, EB, EC, ET, MB, Endometrium, Semen, Urine, Pipelles, Body Fluid, Tissue, PF, AF, Blood, Pus and POC showed 250 specimen positive for MTB. Out of 1200 specimens, females were more prevalent for the disease as 177 samples showed the positivity and 73 male samples were detected as positive for MTB. Considering age as one of the parameters for our study, we found that positivity rate of TB detected in females in the age group of 41-60 was higher (i.e.10.6) and among the males TB was detected higher in the age group of 21-40 (i.e. 13.9). The positivity rate was detected higher in “Menstrual Blood” with the positivity rate of “28.7” and least in “Blood” with the positivity rate of “3.2”. However particularly among the females positive cases of extra pulmonary TB were found in MB samples, indicating the increase in incidence of uterine tuberculosis in our country. However, we consider the method as a valid reliable and quick diagnostic tool, with the advantage of taking specimens from areas which are easily approachable.

CONCLUSION

We conclude that MPB-64 based PCR assay is highly sensitive and specific for the diagnosis of M tuberculosis in all types extra-pulmonary and it can be reliably used for rapid identification of TB.

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Conflict of interest: None

REFERENCES


