The Clinical Utility of Polymerase Chain Reaction and Adenosine Deaminase (ADA), for the Diagnosis of Pleural Tuberculosis: Indian Scenario

Paras Singh1,*, Mradula Singh1, Devika Tayal2, Vithal Prasad Myneedu3, Manpreet Bhalla3, Puncham Adlakha1, Rohit Sarin4

1Department of Molecular Medicine, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi-110030, India
2Department of Biochemistry, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi-110030, India
3Department of Microbiology, National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi-110030, India
4National Institute of Tuberculosis and Respiratory Diseases, Sri Aurobindo Marg, New Delhi-110030, India

*Corresponding Author: drparaslrs@gmail.com

Abstract  Background and Objectives: In spite of higher incidence and prevalence of tuberculosis, the diagnosis of extra-pulmonary tuberculosis (EPTB) in various clinical specimens (such as pleural fluid, ascitic fluids, CSF, lymph node aspirate etc), remains true challenge. Current tools for the diagnosis of tuberculosis in various body fluids are suboptimal. Clinicians underestimate these diseases, and use of insensitive conventional analytical method has contributed to the difficulties in managing patient with extra pulmonary tuberculosis. It is important to develop rapid, sensitive and specific test for early diagnosis of extra pulmonary tuberculosis because of the lack of sensitivity & specificity of the conventional methods as AFB smear by ZN technique and culture on LJ media. Pleural tuberculosis (TB) diagnosis often requires invasive procedures such as pleural biopsy. The study was undertaken to evaluate the combined utility of polymerase chain reaction (PCR) for different gene targets (IS6110, MPB64 and protein antigen b; Pab) especially in pleural fluid specimens with adenosine deaminase (ADA) levels in the diagnosis of pleurisy.

Methods: Total 430 clinical specimens (412 extra-pulmonary and 18 pulmonary tuberculosis) were recruited from the outdoor and indoor Department of National Institute of Tuberculosis and Respiratory Diseases, New Delhi during the 2011-2013 periods. All specimens were further processed for AFB smear, culture on LJ media, ADA level and conventional PCR (IS6110 & MPB 64 gene targets). Results: The PCR positivity IS 6110, MPB64 & Pab gene targets was found to be 90.3% (N=28/31) in smear negative & culture positive (as gold standard) extra pulmonary tuberculosis cases. However the total PCR positivity of Pab (43.9%; 181/412) gene target was found to be significantly low as compared to the IS6110 (65.3%; 269/412, χ2=37.058; pc=0.000; Odds ratio 2.401; 95% CI=1.795-3.213) & MPB 64 (63.6%; 262/412; χ2=31.245; pc=0.000; Odds ratio 2.229; 95% CI=1.669-2.978) gene targets in extra pulmonary tuberculosis cases. Further we have analyzed the combined utility of PCR with ADA levels among the body fluids (165 pleural fluid, 15 ascitic fluid, 1 lymph node and 1 cerebrospinal fluid; CSF). Our results indicated that the PCR alone can detect total 72.5% (132/182) TB cases, whereas ADA alone can detect 61.5% (112/182; considering cutoff value >40IU/L or confirmed cases of TB on clinic-radiological findings), M.tuberculosis in body fluids. Further data was compared in between single, two and three gene targets considering cut off value ADA >40IU/L levels in body fluids. Our observation showed that the positivity of tuberculosis cases were significantly higher through three gene targets (N=48/83; 57.8%; Mean of ADA >40IU/L =127.3) as compared to single gene target (N=10/83; 12.1%; Mean of ADA >40IU/L =68.2; χ2=36.27; pc=0.000; Odds ratio 10.011; 95% CI=4.272-24.008) utilizing conventional PCR technology. No significant difference has been observed in other body fluids. The combined evaluation of both techniques (PCR and ADA) raised 14-15 % additional diagnosis of tuberculosis in body fluids (158/182; 86.8%; in pleural fluid= 147/165; 89.1%).

Interpretation and Conclusion: Our results suggested that Protein antigen b (Pab) gene target showed less sensitivity as compared to IS6110 & MPB64. This study demonstrated the combined utility of both techniques (multigene target PCR with ADA level), enhanced the sensitivity of diagnosis of tuberculosis in body fluids. The study also confirmed the high diagnostic utility of PCR and ADA methods in diagnosis of tuberculosis in various paucibacillary body fluids in Indian scenario.

Keywords  Culture, PCR, ADA, M.Tuberculosis, Body Fluids, Extra Pulmonary Tuberculosis, Pab, IS6110, MPB64

1. Introduction

During the last several decades, the resurgence of tuberculosis (TB) has been documented in both developed
The Clinical Utility of Polymerase Chain Reaction and Adenosine Deaminase (ADA), for the Diagnosis of Pleural Tuberculosis: Indian Scenario

and developing countries, and much of this increase in tuberculosis burden coincided with human immunodeficiency virus (HIV) pandemics. Since then, the disease pattern has changed with a higher incidence of extra pulmonary tuberculosis (EPTB) and disseminated TB [1].

Diagnosis of EPTB can be baffling, compelling a high index of suspicion owing to paucibacillary load in the biological specimens. A poor contribution of conventional bacteriological techniques like negative AFB smear, failure to culture and lack of granulomas on histopathology do not exclude the diagnosis of EPTB.

Pleural tuberculosis accounts for less than 1% of all exudative effusions in western countries, occurring in only 3 - 5% of tuberculosis patients and in developing countries like India, it is responsible for 30 - 80% of all pleural effusions encountered [2].

Nucleic acid amplification methods (PCR) are rapid and sensitive, has modified strategies for the detection of mycobacterial DNA. Several In-house PCR assays and commercial kits have been used for rapid diagnosis of tuberculosis [3-9]. Reported studies showed that highly conserved insertion sequences, IS6110, is most commonly used in the detection of M. tuberculosis. The range of IS6110 copies among isolates varied from 0-19 in the M. tuberculosis genome [10]. However, the sensitivity and specificity of IS6110 sequence in the diagnosis of tuberculosis remains uncertain, and needs to be include other various PCR targets reliable screening test for tuberculosis in clinical specimen targets such as MPB64 (Mpt64), Pab, devR, TRC4, GC repeats, 38 kDa gene etc [3,11,12]. Further the Real time PCR technology has attracted considerable interest because of its high degree of sensitivity and specificity over the conventional methods, particularly with the hope of shortening the time required to detect and identify Mycobacterium tuberculosis in respiratory and non-respiratory samples. Our previous studies have also showed the clinical usefulness of conventional PCR and Real Time PCR in rapid diagnosis of M. tuberculosis in paucibacillary extra pulmonary tuberculosis samples in Indian scenario [8,9]. The molecular tools and methods can be utilized for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimen and also molecular detection of drug resistance [11,13,14,15].

ADA has been more commonly preferred for the diagnostic algorithms in the countries with a moderate to the high incidence of tuberculosis because it is a more inexpensive method that can be accessed more quickly. Many studied have confirmed the high sensitivity and specificity of ADA for early diagnosis of EPTB in body fluids especially in case of negative AFB smears [16-18]. ADA is an enzyme catalyzing the deamination reaction from adenosine to inosine. It is also an essential enzyme of the purine catabolic pathway. ADA presents in all cell types, however, the amount of enzymes differs widely among tissues. The highest ADA levels in humans are found in lymphoid tissues [19]. ADA acts in proliferation and differentiation of lymphocyte, especially T lymphocyte. It also acts in maturation of monocytes transforming them to macrophages. ADA is a significant indicator of active cellular immunity. Furthermore, ADA is a useful surrogate marker for tuberculosis as it can be detected in body fluids such as pleural, pericardial and peritoneal fluid [20]. The levels of ADA increase in tuberculosis because of the stimulation of T cells by mycobacterial antigens. ADA estimation is simple, quick and cost effective, has good sensitivity and can be used for early diagnosis of tuberculosis, especially in case of AFB negative smear from the body specimens in regions with high prevalence of tuberculosis [18].

Our published studies were based on PCR and Real time PCR has lighten the significance of molecular tools in the diagnosis of EPTB [8,9]. The present study evaluated the combined utility of PCR (Insertion sequence (IS6110), MPB64 and Pab gene target) with ADA levels in diagnosis of EPTB in various body fluids.

2. Material and Methods

2.1. Study Design

We conducted this study in National Institute of Tuberculosis and Respiratory Diseases, New Delhi, India during the period of 2011-2013. The inclusion criteria for the recruitment of patients were defined as follows: patients had to present with clinical manifestations suggestive of tuberculosis, i.e., the presence of productive cough, low-grade fever, night sweats, weight loss and chest pain, especially if these symptoms from last ≥ 4 weeks. If the patients presented with less than two of these symptoms and especially if the clinical manifestations were of < 4 weeks in duration, they were excluded from the study. The patients were defined as having pleural tuberculosis on the basis of combination of clinical and radiological findings. Current study was approved by research and ethics committee of the Institute. A detailed clinical history, sex, and age were also collected from the requisition form that accompanied with specimens.

3. Laboratory Procedures

3.1. Collection of the Clinical Specimens

412 EPTB (305 pleural fluid, 21 pus, 31 ascitic fluid, 23 lymph node aspirate, 3 cerebrospinal fluid (CSF), 2 endometrial fluid, 5 peritoneal fluid, 5 Tissue, 9 urine, 8 others) and 18 PTB (16 sputum and 2 bronchial wash) of presumptive tuberculosis were recruited from the outdoor and wards of Department of National Institute of Tuberculosis and Respiratory Diseases, Delhi for this study during the period of 2011-2013 year. A total cohort of 430 patients were consisted of 312 male (age mean 42.4) and 118
female (age mean 32.4) with age range from 16-68 years. Majority of patients were observed between their reproductive ages 20-45 years which represented that overall increase rate of infection in younger one in endemic areas.

These clinical specimens were processed for the investigation of smear, culture on Lowenstein Jenson (LJ) media and PCR to detect *M. tuberculosis* in clinical specimens (Figure 1).

A total of 182 aseptically collected body fluids (i.e. 165 pleural fluids, 15 ascitic fluids, one lymph node and one CSF) were further subjected to adenosine deaminase (ADA) assay (calorimetric technique). Total plasma ADA was measured using Diazyme commercial kit (Diazyme Laboratories, San Diego, CA, USA) which is one of the modified Giusti methods. One unit of ADA is defined as the amount of ADA that generates 1 µmole of inosine from adenosine per minute at 37°C. A positive result for tuberculosis was defined as a value 40 IU/L and above which is based on previous studies of pleural fluid specimens of patients with proven tuberculosis[21].

### 3.2. Processing of the Specimens

All 430 specimens were decontaminated by standard protocol i.e. N-acetyl-L-cysteine (NALC)–sodium hydroxide (NaOH) procedure which included 2% NaOH, 2.9% trisodium citrate, 0.5% NALC[22]. Processed specimens were used for AFB smear, LJ culture (bacteriological identification as a gold standard) and for DNA extraction from all body fluids by QIAamp DNA Mini Kit (Qiagen, Germany).

### 3.3. DNA Amplification for MTB Detection

Amplification of bacterial DNA was performed by following procedures.

### 3.4. In-house PCR

Total 25µl of PCR reaction volume was containing 1x PCR buffer, 0.2 mM dNTPs, 50ng of each primer (IS6110, MPB64 and protein antigen b, 1 unit of *Taq* polymerase (BangloreGenei, India) and 10µl of tested sample DNA along with positive control (*Mycobacterium tuberculosis* (MTB), H37Rv) and negative control lacking DNA (nuclease free water in place of template DNA).

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**Figure 1.** Graphical representation of recruited tuberculosis specimens (N=430) during the period 2011-2013. Extra pulmonary specimens were further categorized as 305 pleural fluid, 21 pus, 31 ascitic fluid, 23 lymph node aspirate, 3 CSF, 2 endometrial fluid, 5 peritoneal fluid, 5 Tissue, 9 urine and 8 others. Pulmonary tuberculosis specimens include 16 sputum and 2 bronchial wash.
The amplification protocol was accomplished on a thermal cycler (9700 Applied Biosystem, thermal cycler, USA) according to the published studies (Figure 2 for Pab PCR)[3,9,23].

3.5. ADA Protocols

ADA levels were estimated using modified Giusti methods [21] which is based on the principles of enzymatic deamination of adenosine to inosine which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP). An automated method (Diazyme commercial kit, Diazyme Laboratories, San Diego, CA, USA) on an autoanalyser Hitachi H-902 was used.

3.6. Statistical Analysis

The statistical calculations were performed using MSTAT software by Chi square ($\chi^2$) and Fischer test. The p values <0.05 were considered to be significant.

4. Results

The combined PCR positivity (IS6110, MPB64 and Pab gene targets) was found to be 90.3% (N=28/31) in smear negative and culture positive (as gold standard) EPTB cases (Table-1). However the total PCR positivity of Pab (43.9%; 181/412) was found to be significantly low as compared to the IS6110 (65.3%; 269/412; $\chi^2=31.245$; pe=0.000; Odds ratio 2.229; 95% CI=1.669-2.978) and MPB 64 (63.6%; 262/412; $\chi^2=31.245$; pe=0.000; Odds ratio 2.229; 95% CI=1.669-2.978) gene targets in EPTB cases as represented in Table - 1. Similarly in pulmonary specimens, the total positivity of Pab (44.4%) is also reduced as compared to IS6110 (72.2% $\chi^2=2.8$, p value=0.09) and MPB64 (55.6%) gene targets, respectively (Table- 2). Further we have analyzed the combined utility of PCR with ADA levels among the body fluids (165 pleural fluids, 15 ascitic fluid, 1 lymph node and 1 CSF) as represented in Table- 3 and Table- 4. The positive result for tuberculosis was defined as a value 40IU/L and above which is based on previous studies of pleural fluid specimens of patients with proven tuberculosis [21, 24-26].Our results indicated that the PCR alone can detect total 72.5% (132/182) TB cases, whereas ADA alone can detect 61.5% (112/182; considering cutoff value >40IU/L or confirmed cases of TB on clinic-radiological findings), MTB in body fluids (Table- 4). Further data was compared in between single, two and three gene targets considering cut off value ADA >40IU/L levels in body fluids specimens. Our observation showed that the positivity of TB cases were significantly increased through three gene targets (N=48/83; 57.8%; Mean of ADA >40IU/L =127.3) as compared to single gene target (N=10/83; 12.1%; Mean of ADA >40IU/L =68.2; $\chi^2=36.27$; pe=0.000; Odds ratio 10.011; 95% CI=4.272-24.008) utilizing PCR technology (Table - 3). No significant difference has been observed in other body fluids. Our observation also suggested that the total mean of ADA level (>40IU/L) was raised in two (Mean =217.7; and three gene targets (Mean =127.3) as compared to the single gene target (Mean= 68.2) in body fluids.

The combined evaluation of both techniques enhanced 14 to 15 % additional diagnosis of tuberculosis in body fluids (158/ 182, 86.8%; pleural fluid = 147/165, 89.1%) whereas positivity raised by 14.3 % (>40 IU/L= 26/182) in PCR negative cases as shown in table-4.

![Figure 2](image-url). Pab gene-targeted In-house PCR for detection of *Mycobacterium tuberculosis*. Electrophoretic separation of the amplicon into 2% agarose gel is documented across Lanes 1-8. Lanes 1 represent the negative control (nuclease free water), lane-2 to 7 clinical specimens, Lanes 8 PC (*Mycobacterium tuberculosis* H37Rv). The presence of a 419bp amplicon in the lanes 2,3,4,6,8 indicated the presence of the target. Lane 9 50bp M. Lane 5 and 7 represent the absence of target in tested specimens.
### Table 1. Comparative analysis of three gene targets (IS6110, MPB 64, Pab) for *M. tuberculosis* detection through PCR technology in presumptive extra pulmonary specimens

<table>
<thead>
<tr>
<th>Smear/ culture</th>
<th>PCR Gene targets</th>
<th>smear +ve, culture +ve (a=7/412; 1.7%)</th>
<th>smear +ve, culture –ve (b=9/412; 2.2%)</th>
<th>smear –ve, culture +ve (c=31/412; 7.5%)</th>
<th>smear –ve, culture –ve (d=365/412; 88.6%)</th>
<th>Total PCR positive (N=412)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110</td>
<td></td>
<td>7 (100)</td>
<td>7 (77.8)</td>
<td>28 (90.3)</td>
<td>227 (62.2)</td>
<td>269 (65.3)*</td>
</tr>
<tr>
<td>MPB 64</td>
<td></td>
<td>7 (100)</td>
<td>7 (77.8)</td>
<td>28 (90.3)</td>
<td>220 (60.1)</td>
<td>262 (63.6)@</td>
</tr>
<tr>
<td>Pab</td>
<td></td>
<td>7 (100)</td>
<td>6 (66.7)</td>
<td>28 (90.3)</td>
<td>140 (38.4)</td>
<td>181 (43.9)*@</td>
</tr>
<tr>
<td>Total PCR positive (N=412)</td>
<td></td>
<td>7 (100)</td>
<td>7 (77.8)</td>
<td>28 (90.3)</td>
<td>230 (63.0)</td>
<td>272 (66.0)</td>
</tr>
</tbody>
</table>

PCR= Polymerase chain reaction, IS= Insertion sequence, Pab= Protein antigen b
*χ²=37.058; pc=0.000; Odds ratio 2.401; 95% CI=1.795-3.213
@χ²=31.245; pc=0.000; Odds ratio 2.229; 95% CI=1.669-2.978
Total smear positive: n= 16/412 (3.9%)
Total culture positive: n=38/412 (9.2%)
Total smear and culture positive: n=44/412 (10.7%)

### Table 2. Comparative analysis of three gene target (IS6110, MPB 64, Pab) for *M. tuberculosis* detection through PCR technology in presumptive pulmonary specimens

<table>
<thead>
<tr>
<th>Smear/ culture</th>
<th>PCR Gene targets</th>
<th>smear +ve, culture +ve (a=2/18; 11.1%)</th>
<th>smear +ve, culture –ve (b=0/18)</th>
<th>smear –ve, culture +ve (c=3/18; 16.7%)</th>
<th>smear –ve, culture +ve (d=12/18; 66.7%)</th>
<th>Total PCR positive (N=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110</td>
<td></td>
<td>1 (50)</td>
<td>-</td>
<td>3 (100)</td>
<td>9 (75)</td>
<td>13 (72.2)</td>
</tr>
<tr>
<td>MPB 64</td>
<td></td>
<td>1 (50)</td>
<td>-</td>
<td>3 (100)</td>
<td>6 (50)</td>
<td>10 (55.6)</td>
</tr>
<tr>
<td>Pab</td>
<td></td>
<td>1 (50)</td>
<td>-</td>
<td>2 (66.7)</td>
<td>5 (41.7)</td>
<td>8 (44.4)</td>
</tr>
<tr>
<td>Total PCR positive (N=18)</td>
<td></td>
<td>1 (50)</td>
<td>-</td>
<td>3 (100)</td>
<td>10 (55.6)</td>
<td>14 (77.8)</td>
</tr>
</tbody>
</table>

PCR= Polymerase chain reaction, IS= Insertion sequence, Pab= Protein antigen b
NT= Not tested
Total smear positive: n= 2/18 (11.1%)
Total culture positive: n=3/18 (16.7%)
Total smear and culture positive: n=3/18 (16.7%)

### Table 3. Comparative analysis of PCR positivity (single, two, three genes) with ADA level in body fluids (pleural fluid, ascitic fluid, lymph node aspirates and CSF specimens) for *MTB* detection

<table>
<thead>
<tr>
<th>Type of specimens</th>
<th>PCR /ADA level</th>
<th>Positivity of PCR (%) with ADA level (IU/L)</th>
<th>Total PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total PCR positive</td>
<td>PCR positive with single, two, &amp; three genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;40  &lt; 40  Total  &gt;40  &lt; 40 &gt;40  &lt; 40 &gt;40  &lt; 40</td>
<td>Single genes  Two genes  Three genes</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td></td>
<td>83 (68.6) 38 (31.4) 121 (73.3)</td>
<td>*10 (12.1) 14 (36.8)  @ 25 (30.1) 11 (28.9)  *@48 (57.8) 13 (34.2)</td>
</tr>
<tr>
<td>Ascitic fluid</td>
<td></td>
<td>2 (20) 8 (80) 10 (66.7)</td>
<td>- - 1 (50.0) 3 (37.5) 1 (50.0) 5 (62.5)</td>
</tr>
<tr>
<td>Lymph node aspirate</td>
<td></td>
<td>1 (100) - 1 (100)</td>
<td>- - - - - 1 (100) -</td>
</tr>
<tr>
<td>CSF</td>
<td></td>
<td>- - - - - -</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>Total (N=182/362)</td>
<td></td>
<td>86 (65.2) 46 (34.8) 132 (72.5)</td>
<td>10 14 26 14 50 18</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>127.7 19.9 90.9 68.2 18.8 217.7 19.2 127.3 17.7</td>
<td></td>
</tr>
</tbody>
</table>

PCR=Polymerase chain reaction, CSF= Cerebral spinal fluid, ADA= Adenosine deaminase

*chi²=37.058; pc=0.000; Odds ratio 2.401; 95% CI=1.795-3.213
@chi²=31.245; pc=0.000; Odds ratio 2.229; 95% CI=1.669-2.978
Total smear positive: n= 16/412 (3.9%)
Total culture positive: n=38/412 (9.2%)
Total smear and culture positive: n=44/412 (10.7%)
5. Discussions

Tuberculosis is one of the most prominent health problems in the world, causing almost 2 million deaths each year. Rapid clinical diagnosis is important in patients who have co-morbidities such as Human Immunodeficiency Virus (HIV) infection. India has the world’s largest burden of tuberculosis (TB), accounting for one-fifth (21%) of the global tuberculosis incidence. Of these tuberculosis cases, a sizeable percentage (10-20%) consists of EPTB, such as pleural effusion, lymphadenopathy, ascites, tuberculous meningitis, and synovial effusion [1,9]. Among them tubercular pleural effusion (TPE) is the most common extra pulmonary form of the tuberculosis. Undiagnosed and mismanaged tuberculosis (TB) continues to fuel the global TB epidemic. The difficulty with the clinical specimens of tuberculous pleural effusion [12]. As it 70% cases with tuberculous pleurisy [26, 38]. The majority of previous studies confirmed the high diagnostic utility of ADA in EPTB cases which includes majority of tuberculous pleurisy, with various range of sensitivity and specificity.

This observation showed the importance of using multigene targets in identification of M. tuberculosis directly from clinical paucibacillary body fluids. Similar observation has also been reported earlier on the emphasizes of clinical usefulness of multigene targets such as IS6110, MPB 64, Pab, hsp65, dnaJ, devR, cfp10, TRC, 16S rRNA, ESAT-6 [3,4, 28-33]. Sankar et al [34] has emphasized on the need to develop PCR assays (multiplex format) targeting more than one region of the genome of M. tuberculosis in their study. Our study showed that sensitivity of Pab (43.9% and 44.4%) was significantly low as compared to IS6110 (65.3%) and 72.2%) and MPB 64 (63.6% in EPTB and 55.6% gene targets in both groups i.e. EPTB and PTB, respectively(Table- 1 and Table- 2). These findings showed the Pab is less sensitive PCR gene target. Our observation are in the agreement with previously published study by Sharma et al [33] in tubercular uveitis patients, indicated that the low sensitivity of Pab gene target (22.2%) as compared to the IS6110 (55.55%) and MPB64 (66.66%). Several studies reported that multiplex PCR using amplicons such as hsp65, dnaJ and IS6110, MPB 64 has high sensitivity (60-88%), specificity (81-100%) and remains the best tools and is much better marker for diagnosing EPTB [4,9,35]. This data suggested that the use of one additional PCR gene target (other than IS6110 system) can reduce false negativity of PCR results in the specimens harboring zero copy of IS6110 element which is known to exist in Indian population [10,36].

The second approach, applied in the current study is ADA(Adenosine deaminase) is the most cost –effective, a non invasive, inexpensive and repeatable test or marker for pleural fluid and is routinely used in high prevalence setting[26,37]. High levels of ADA have been found in patients with tuberculous pleurisy [26, 38].

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Table 4. Comparative analysis of ADA levels with PCR in body fluids (pleural fluids, ascitic fluids, lymph node and CSF samples)

<table>
<thead>
<tr>
<th>Type of specimens</th>
<th>PCR /ADA level</th>
<th>ADA level (IU/L) with positive PCR (%)</th>
<th>ADA level (IU/L) with negative PCR (%)</th>
<th>Total ADA Level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;40</td>
<td>&lt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Pleural fluid (n=165/305)</td>
<td></td>
<td>83 (68.6)</td>
<td>38 (31.4)</td>
<td>121 (73.3)</td>
</tr>
<tr>
<td>Combined positivity of PCR with ADA level (N=165)</td>
<td></td>
<td>121=26=147/165 (89.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascitic fluid (n=15/31)</td>
<td></td>
<td>2 (20)</td>
<td>8 (80)</td>
<td>10 (66.7)</td>
</tr>
<tr>
<td>Lymph node aspirate (n=1/23)</td>
<td></td>
<td>1 (100)</td>
<td>-</td>
<td>1 (100)</td>
</tr>
<tr>
<td>CSF (n=1/3)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>1 (100)</td>
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<td>Combined positivity of PCR with ADA level (N=182)</td>
<td></td>
<td>132+ 26=158/182 (86.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>127.7</td>
<td>19.9</td>
<td>90.9</td>
</tr>
</tbody>
</table>

PCR=Polymerase chain reaction, CSF= Cerebral spinal fluid, ADA= Adenosine deaminase
from 80% to 100%, respectively at the different ranges of ADA cut off value from 30 to 70U/L in body fluids[26,37-41]. However, Danielle et al [42] has reported the best cutoff value as >40IU/L in their study. Similarly, in this study ADA cut off value= >40IU/L was used for the ADA level measurement in body fluids i.e. pleural fluid, lymph node aspirate, ascetic fluids, CSF (Table- 3 and 4).

Additionally, we have analyzed combined results of PCR positivity (single, two and three gene targets) with ADA level (cut off value= >40IU/L) in body fluids. The positive result for tuberculosis was defined as a value 40IU/L and above which is based on previous studies of pleural fluid specimens of patients with proven tuberculosis [21, 24-26]. Our results indicated more detection of pleural tuberculosis (ADA cut off value= >40IU/L) were observed in pleural effusion cases which were significantly found to be positive through three gene (57.8%) targets as compared to single (12.1%; * χ2=36.27; pc=0.000) or two gene targets (30.1%; @χ2=11.834; pc=0.001) as represented in Table-3. Our results are in concordance with Mishra et al [41] reported that the combined efficacy of PCR with ADA levels increased the sensitivity of test (90-100%) in the diagnosis of tuberculous effusion (TPE) in children.

On the other hand, the combined results of PCR negativity with ADA level (cut off value= >40IU/L) in body fluids were also interpreted. Our findings indicated that the diagnosis of MTB was increased up to (15.8%; 26/165) in pleural fluids while overall diagnosis was increased 14.3% (26/182) in body fluids as represented in table- 4.

Our observation also suggested that the total mean of ADA level (>40IU/L) was raised in two (Mean =217.7; and three gene targets (mean =127.3) as compared to the single gene target (Mean= 68.2) in body fluids which showed the increasing trend towards the diagnosis of tuberculosis by including more number of PCR gene targets with considering cut off value of 40IU/L and above of ADA levels in EPTB specimens. However, the total mean of ADA was found to be 127.7 in body fluids (EPTB cases), at cut off value >40IU/L. No significant difference has been observed in ascitic and other fluids (Table- 3).

The PCR technique alone can detect 72.5% (132/182) whereas ADA alone can detect 61.5% (112/182) in our study. The combined evaluation of both techniques enhanced 14-15% additional diagnosis of tuberculosis in body fluids (158/182; 86.8%; in pleural fluid= 147/165; 89.1%).

Similarly, AFB staining lacks sensitivity (3.9%) and this was also reflected in our study. Body fluids culture showed the presence of M tuberculosis in 9.2% of all cases. This was in accordance with the findings of a previous studies with a culture positivity of <45% and AFB positivity <37% [3,9].

The combination of two methods, with a positive result by either two of the methods considered to be indicative of a positive diagnosis of pleural TB or EPTB, increased the diagnostic sensitivity by individual methods (Table- 3 and Table- 4).

However, false negative cases still remain a big question to any study. This was similar to the findings by Parandaman et al [43], where there were 22% false negative results by PCR with IS6110, MPB64 and Pab primers. Similarly, we could not detect the culture positive (9.7%; 3/31) cases by all PCR. The reasons for the false negativity could be the absence of IS6110 copies or low number of bacilli or the presence of inhibitors or fraction tested does not contain mycobacteria.

The uniqueness of this study is that different tests such as AFB staining, LJ culture, ADA level and PCR assay (multigene target) have been performed separately and evaluated in different body fluids such as pleural fluid, ascetic fluid, lymph node aspirates, CSF. The use of multigene target PCR, which simultaneously diagnoses the mycobacterial species, can be considered an important parameter with ADA level estimation.

6. Conclusions

Our study highlights, the combined utility of both techniques (PCR with ADA level) enhanced the sensitivity of diagnosis of tuberculosis in body fluids specially paucibacillary pleural effusion cases. The study confirmed the high diagnostic utility of PCR (multi gene target) with ADA level in the diagnosis of tuberculosis in various body fluids in Indian scenario which is not diagnosed by conventional test (smear and culture).

Further study needs to be interpreted for different amplification assays or molecular technologies in conjunction with other surrogate markers those of conventional methods and clinical findings to reach the final diagnosis of paucibacillary EPTB.

Abbreviations

AFB= acid-fast bacillus; LJ Culture= Lowenstein-Jensen Culture, PCR= Polymerase chain reaction, ADA= Adenosine deaminase assay, EPTB= Extra-pulmonary tuberculosis, Pab= protein antigen b.

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