

# Evaluation of Polymerase Chain Reaction and Pleural Fluid Adenosine Deaminase Levels for the Diagnosis of Tuberculous Pleural Effusion

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## ABSTRACT

### BACKGROUND

We wanted to evaluate the performance of Polymerase Chain Reaction (PCR) and pleural Adenosine Deaminase (ADA) levels for the diagnosis of tuberculous pleural effusion (TPE) as compared to the composite reference standard (CRS) based on clinical findings, smear microscopy, culture, cytology, site specific computerized tomography scan and response to anti-tuberculosis therapy.

### METHODS

A total of 134 pleural fluid samples obtained as a part of routine diagnostic workup from patients suspected of TPE were subjected to real time PCR and determination of pleural ADA levels. Test results of PCR and ADA were compared with CRS. Sensitivity, specificity, positive predictive value, negative predictive value were determined using Statistical Package for the Social Sciences, Version 16.

### RESULTS

60 patients (44.77%) were found to be positive by both PCR and ADA, 22 (16.41%) patients were positive by ADA alone and 9 (6.71%) patients were positive by PCR alone; whereas, 43 (32.08%) patients were negative by both the assays. The overall sensitivity of TB PCR and pleural ADA was found to be 87.34% and 88.61% respectively. Whereas the specificity of PCR and pleural ADA levels for TPE detection was found to be 100 % and 80% respectively.

### CONCLUSIONS

Although we observed almost similar sensitivity of PCR and ADA for diagnosis of TPE, the specificity of PCR was much higher than ADA. PCR and pleural ADA are promising tools in diagnostic workup of TPE. A significant association of TPE with young age males and diabetic patients was observed which remains a concern and signifies the need of further elaborate studies.

### KEY WORDS

ADA, MTB, PCR, Pleural Effusion, CRS, TPE

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## BACKGROUND

Tuberculous pleural effusion (TPE) is a common diagnostic challenge due to its non-specific clinical presentation, paucibacillary nature and lack of diagnostic methods for accurate diagnosis.<sup>[1]</sup> In the developing countries, the burden of TPE is estimated to be 30-80% of total cases of pleural effusion and is the second most common extra-pulmonary manifestation of tuberculosis.<sup>[2]</sup> Common clinical signs associated with TPE are fever, cough and pleuritic chest pain. Detection of *Mycobacterium tuberculosis* in pleural fluid, or pleural biopsy specimens by microscopy and culture, or histopathological demonstration of caseating granulomas in the pleura along with acid fast bacilli is the gold standard for diagnosis of TPE but sensitivity of acid fast bacilli smear is only 5-20%, culture takes 6-8 weeks and histopathological investigation are invasive and requires expertise. <sup>[3,4]</sup> In recent years many studies have investigated the value of Adenosine Deaminase (ADA) in pleural fluid for the early diagnosis of TPE and reported determination of ADA levels as an easy and economical method for diagnosing TPE.<sup>[5-9]</sup> Adenosine deaminase is an enzyme of purine salvage pathway which catalyses the deamination of 2-deoxyadenosine into 2-deoxyinosine and adenosine to inosine.<sup>[10]</sup> ADA helps in proliferation of lymphocytes and maturation of monocytes, thus is a significant marker for cellular immunity.<sup>[11,12]</sup> Stimulation of T cells by mycobacterial antigens results in high levels of ADA; which can be detected in body fluids such as pleural, pericardial and peritoneal fluid thus it is a useful surrogate marker for tuberculosis. Authors in the past have generally associated TPE in cases of lymphocytic pleural effusion with adenosine deaminase level above 40 U/L. <sup>[13]</sup> Elevated levels of pleural ADA with lymphocyte rich effusion is also reported in other infectious diseases, malignant conditions and collagen vascular diseases.<sup>[14-16]</sup> Few studies have also shown significant negative correlation between ADA and factors like old age and smoking, and have not found ADA to be sensitive and specific in clinical practice for TPE.<sup>[17-20]</sup> In recent years, polymerase chain reaction (PCR) has evolved as rapid, sensitive and highly specific test for diagnosis of tuberculosis. Studies evaluating the performance of PCR in diagnosis of TPE have reported variable sensitivities (42-100%) and specificities (85-100%) in the past.<sup>[21]</sup> Though ADA and PCR have been extensively evaluated in the past for diagnosis of TPE; majority of those studies were confined to limited number of samples and have used culture or smear microscopy as reference gold standard but both these tests have suboptimal sensitivity and specificity in TPE. Thus, in the present study we aimed to evaluate the performance of pleural ADA levels and PCR for diagnosis of TPE in comparison with composite reference standard based on clinical findings, smear microscopy, culture, cytology, site specific computerized tomography scan and response to anti-tuberculosis therapy (ATT).

## METHODS

A prospective observational study was conducted in Department of Microbiology in collaboration with

Department of Biochemistry at a tertiary care centre in south coastal Karnataka, India for a period of 2 years from January 2014 to December 2016 after obtaining approval from Institutional Research and Ethics Committee. A total of 134 cases suspected of tubercular pleural effusion requested for investigations including smear microscopy, culture, cytology, histopathology, site specific computerized tomography scan were enrolled for present study and TB PCR and pleural ADA levels estimation was performed. Patient not willing to give consent, missing any of the above-mentioned investigations and were difficult to follow up (Transfer out cases) were excluded from the study.

### DNA Extraction from Pleural Fluid Samples

Pleural fluid samples were centrifuged at 8000 rpm for 10 minutes and DNA was extracted from 200 µl of sediment obtained after centrifugation using a commercial kit from Qiagen (Hilden, Germany) using manufacturer's guidelines. Briefly 200 µl of sediment was mixed with 200 µl of lysis buffer AL followed by addition of 20 µl of proteinase K. The mixture was centrifuged briefly at 10,000 rpm for 1 min and was incubated at 50°C in a water bath for 30 minutes followed by an incubation of 10 minutes at 90°C in a dry bath. The mixture was then mixed with 200 µl of ethanol and transferred to a spin column and centrifuged at 10,000 rpm for 3 minutes; the spin column was then subjected to washing with 500 µl wash buffer 1 and wash buffer 2 followed by centrifugation at 10,000 rpm and 14,000 rpm respectively. Finally, the DNA was eluted by addition of 100 µl of elution buffer and incubation at room temperature for 5 min followed by centrifugation at 10,000 rpm for 3 minutes.

### Real Time PCR for TPE

Detection of TPE was performed by Geno Sens MTB complex kit from Genome Diagnostics Private limited (Solon, India). Briefly 10 µl of DNA was added to PCR master mix containing 12.5 µl R1, 2.5 µl R2 and 0.5 µl R3. The mixture was then amplified in ABI 7500 (Applied Biosystems, USA) using following program; 95°C for 10 minute, 95°C for 15 seconds, 60 °C for 30 seconds and 72°C for 15 seconds for 45 cycle, the results were interpreted by real time graph.

### Determination of ADA Levels in Pleural Fluid

The level of ADA in pleural fluids was determined calorimetrically by enzymatic deamination of adenosine. <sup>[22]</sup> Pleural fluid ADA levels were calculated and expressed in unit per litre (U/L). The following formula was used for calculating adenosine deaminase levels in pleural fluids:  $[(\text{OD Test} - \text{OD Blank}) / (\text{OD Reagent} - \text{OD Reagent Blank})] \times 250$ . The optimum cut off value was calculated by plotting a receiver operator characteristic (ROC) curve (Fig. 1) between true positive rate (Sensitivity) and false positive rate (1-Specificity). All samples having ADA level equal or more than the cut off value were considered positive for TPE.

### Statistical Analysis

Patients details regarding clinical findings, smear microscopy, culture, cytology, histopathology, site specific computerized tomography scan and response to ATT was tabulated in Microsoft excel spread sheet and were used to create a composite reference standard (CRS). Any sample that was found positive for any one component of composite reference

standard and responding to ATT treatment was considered as TPE. ROC curve was plotted between true positive rate and false negative. Test results of PCR and Adenosine deaminase were compared with CRS and sensitivity, specificity, positive predictive value, negative predictive value was analysed using Statistical Package for the Social Sciences 22 (SPSS). T test was performed to compare the performance of tests performed in the study. p value < 0.05 was considered statistically significant for all analysis.

RESULTS

A total of 134 pleural fluid samples from patients suspected of TPE were included in present study. The patients comprised of 99 (73.88%) males and 35(26.12%) females. Mean age of the patients was found to be 46.18±17.24 (Mean ± SD) ranging from 17 years to 92 years. A detailed description of the patients is given in Table 1.

On the basis of CRS, 79 (58.95%) patients were found positive for tuberculous pleural effusion whereas 55 (41.05%) were found negative. On ROC curve analysis the optimal cut off value for pleural ADA was found to be 40 U/L (Fig: 1). Area under curve for pleural ADA and standard error was found to be 0.847 (95% CI, 0.774-0.920) and 0.037 respectively. TB PCR and pleural ADA levels were positive for TB in 69 (51.49%) and 81(60.45%) patients respectively. Sixty patients (44.77%) were found positive by both PCR and ADA, 22 (16.41%) patients were positive for ADA alone and 9 (6.71%) patients were positive by PCR alone whereas 43 (32.08%) patients were negative by both the assay. The overall sensitivity of TB PCR and pleural ADA was found to be 87.34% and 88.61% respectively. Whereas the specificity of TB PCR and pleural ADA levels for TPE detection was found to be 100 % and 80 % respectively. A detailed description of performance of TB PCR and pleural ADA for diagnosis of TPE is given in Table 2.

|                                                           | Positive for Tuberculous<br>Pleural Effusion by CRS<br>(79) | Negative for<br>Tuberculous Pleural<br>Effusion by CRS (55) | Odds Ratio<br>(95% CI) p<br>Value |
|-----------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------|-----------------------------------|
| Age,<br>0-50<br>>50                                       | 55(69.62)<br>24(30.38)                                      | 18(32.73)<br>37(67.27)                                      | 4.71(2.25-9.87)<br>0.000025       |
| Gender,<br>Male (%)<br>Female (%)                         | 64(81.01)<br>15(18.99)                                      | 35(63.63)<br>20(36.36)                                      | 2.44(1.11-5.35)<br>.024           |
| Category,<br>New case (%)<br>Previous TB<br>treatment (%) | 61(77.21)<br>18(22.78)                                      | 54(98.18)<br>1(1.82)                                        | .06(.01-0.49)<br>0.00062          |
| HIV status,<br>Positive (%)<br>Negative (%)               | 4(5.06)<br>75(94.94)                                        | 0(0)<br>55(100)                                             | -                                 |
| Diabetes,<br>Positive (%)<br>Negative (%)                 | 9(11.39)<br>70(88.60)                                       | 15(27.27)<br>40(72.72)                                      | 0.34(0.14-0.85)<br>0.018          |

Table 1. A Detailed Description of Patients Enrolled for the Study

|                                     | TB PCR                    |                              | Pleural Adenosine<br>Deaminase |                              |
|-------------------------------------|---------------------------|------------------------------|--------------------------------|------------------------------|
|                                     | Positive (%)<br>69(51.50) | Negative<br>(%)<br>65(48.50) | Positive<br>(%)<br>81(60.40)   | Negative<br>(%)<br>53(39.60) |
| CRS Positive 79( 58.95)             | 69(51.50)                 | 10(7.46)                     | 70(52.24)                      | 9(6.71)                      |
| CRS Negative 55( 41.04)             | -                         | 55(41.04)                    | 11(8.20)                       | 44(32.83)                    |
| Sensitivity (95% CI)                | 87.34 (77.95-93.76)       |                              | 88.61 (79.47-94.66)            |                              |
| Specificity (95% CI)                | 100 (93.5-100)            |                              | 80 (67.03-89.57)               |                              |
| Positive predictive value (95 % CI) | 100                       |                              | 86.42 (78.85-91.57)            |                              |
| Negative predictive value (95% CI)  | 84.62 (86.70-96.36)       |                              | 83.02 (72.27-90.17)            |                              |

Table 2. Performance of TB PCR and Pleural Adenosine Deaminase Levels for the Diagnosis of Tuberculous Pleural Effusion

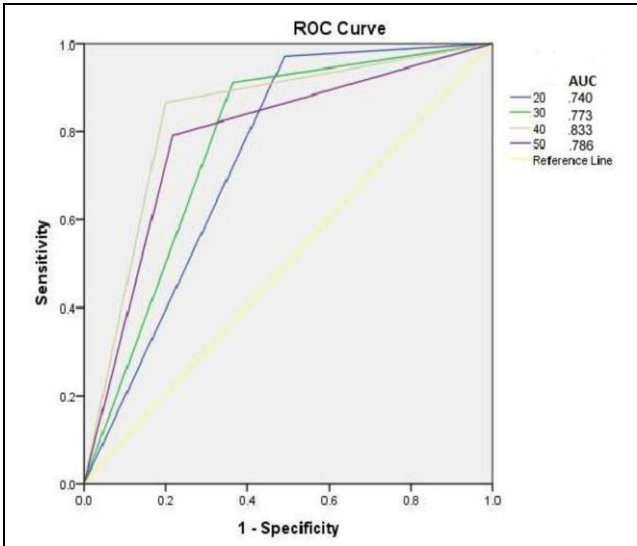


Figure 1. ROC Curve Between True Positive Rate (Sensitivity) and False Positive Rate (1-Specificity) for Determining the Optimal Cut Off Value of ADA

DISCUSSION

Present study was aimed to evaluate the efficacy of pleural ADA levels and PCR for diagnosis of tuberculous pleural effusion in comparison with composite reference standard based on clinical findings, smear microscopy, culture, cytology, site specific computerized tomography scan, and response to ATT. Authors in the past have reported variable sensitivities 42 to 100% and specificities 85 to 100 % using various PCR targets for diagnosis of tuberculous pleural effusion.[21] In the present study the sensitivity and specificity of PCR was found to be 87.34% and 100% respectively which was almost in concordance with the studies from Brazil and Spain which reported sensitivity of 89% and specificity of 100% in their study.[23,24] On ROC curve analysis the optimal cut off for pleural ADA levels was found to be 40 U/L which was reported as the best cut off value in a previously published report. [13] The sensitivity and specificity of pleural ADA levels for diagnosis of TPE was found to be 88.61% and 80 % which was in concordance with the findings of Perez et al and Chen et al. [25, 26] Although the sensitivities of TB PCR (87.34%) and pleural ADA (88.61%) were almost similar; PCR (100%) was found significantly more specific than pleural ADA (80%). Authors in the past have reported high levels of ADA in age groups <35 years and >55 years, parapneumonic effusion, empyema, malignancies and infectious diseases, 11 (8.20%) patients in present study were found to be false positive by ADA, 4 had empyema, 2 had pneumonia, 2 had malignancies whereas 3 were at extremes of age.[17,27]

PCR was unable to detect 11 (8.20%) patients which were positive by CRS low bacterial load or presence of PCR inhibitors may be the most probable reason for false result. [28] Authors in the past have associated TPE with young age. In the present study also 69.62% (55/79) TPE patients below 50 years of age were positive for TPE patients.[29] TPE is commonly seen in males; 81.01% (64/79%) patients in present study were also males. [30] Further we also observed a significant correlation between TPE and newly diagnosed cases and diabetic patients in the present study. No significant correlation can be seen between HIV positive

patients and TPE due to limited sample size which remains one of the major limitations of the study.

## CONCLUSIONS

Although we observed almost similar sensitivity of PCR and ADA for diagnosis of TPE, the specificity of PCR was much higher than ADA. PCR and pleural ADA are promising tools in diagnostic workup of TPE. TPE was found significantly associated with young age, males and diabetic patients which remains a concern and signifies the need of further elaborate study.

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