

Pleural fluid Adenosine deaminase activity – Can it be a diagnostic biomarker?

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Abstract: Aim: To evaluate pleural fluid Adenosine deaminase as the diagnostic marker for tubercular pleural disease.

Patients and Methods: New patients (n=160) with pleural effusion were divided into tubercular (n=92) and non-tubercular (n=68) groups. Non-tubercular group was further divided into patients having exudative effusion (n=46) and patients having transudative effusion (n=22). Patients with exudative effusion included those with adenocarcinoma (n=21), non-tuberculous empyema (n=11), parapneumonic effusion: (n=9) and lymphomas (n=5) while patients having transudative effusion included those with congestive heart failure (n=13), cirrhosis of liver (n=6) and with nephrotic syndrome (n=3). Pleural fluid was aspirated from all patients and tested for ADA.

Results: In tubercular group Mean \pm SD of ADA value was 67.78 ± 37.39 and it ranged between 8.8 – 260.0; in non-tubercular group collectively it was 22.17 ± 15.11 and ranged between 6.0 – 102.0. The performance of ADA for diagnosis of tuberculous pleural effusions in regards to the 95% confidence intervals and cut-off levels above 40.0 IU/L resulted in 88.04% sensitivity, 91.12% specificity, 95.25% positive predictive value, 85.33% negative predictive value, 90.63% diagnostic accuracy, 14.7 positive likelihood ratio, 0.13 negative likelihood ratio and 11.78 diagnostic odds ratio. The prevalence of disease in the studied population was 57.5%.

Conclusion: It is concluded that ADA estimation in pleural fluid is not only simple, inexpensive and rapid but also fairly specific and sensitive method for diagnosing tuberculous etiology in patients of pleural disease. ADA activity in the pleural fluid can be a diagnostic biomarker and for this reason ADA estimation in pleural fluid may find a place as a routine investigation.

Keywords: Tuberculosis, Pleural effusion, Adenosine deaminase, Diagnostic, Biomarker,

I. Introduction:

Tuberculosis is one of the commonest chronic infectious diseases, which is highly endemic killing approximately five lakh patients every year in India [1]. It usually affects lungs but cases of extrapulmonary tuberculosis are not rare. Delay in diagnosis and in initiating treatment results in poor prognosis and sequelae in upto 25% of cases [2].

Pulmonary Tuberculosis (TB) can be confirmed by serial sputum examination and diagnosed easily, but diagnosing extra-Pulmonary TB often becomes difficult since the specificity and sensitivity of available non-invasive methods is low. Pleural tuberculosis is an extrapulmonary form of tuberculosis affecting 15% of the total number of cases. It is the second most frequent clinical presentation of human tuberculosis [1, 2].

Pleural effusion is a common problem in clinics and can result due to a number of diseases. The available tests and procedures for the confirmation of its etiology are ineffective in majority of cases. Thus, there is a need for a sensitive and specific test that is reliable and rapid [3].

Tubercular pleural effusion is usually a result of delayed type hypersensitivity to proteins of the bacterium, but owing to low bacterial load in the pleural space, the aspirated fluids when analyzed for direct evidence of disease show very low acid fast bacilli (AFB) positivity rate. Direct examination of pleural fluid by Zeihl-Neelsen staining requires bacillary densities of 10,000/mL [3] and, therefore, detects AFB in less than 10% of cases. AFB culture is expensive, requires long incubation period and is positive in less than 25% cases. ELISA, PCR, TB IgG / IgM and interferon are very expensive tests and beyond the reach of the masses. Pleural biopsy although considered as the gold standard for diagnosis of pleural disease, is still not a routine medical procedure due to its invasive nature.

Adenosine and deoxyadenosine is converted into inosine and dexyinosine in the purine salvage pathway with the release of ammonia; Adenosine deaminase (ADA) is an enzyme that catalyzes this conversion. It plays important role in differentiating lymphoid cells and is present in abundance in active T-lymphocytes where its concentration is inversely proportional to the degree of differentiation [4]. The enzyme activity increases during mitogenic and antigenic responses of lymphocytes, and some earlier reports have even shown that T-lymphocyte blastogenesis can be inhibited by inhibitors of ADA. Likewise, deficiency of adenosine deaminase is associated with severe defects in the cell-mediated and humoral immunity, predisposing the patient to opportunistic infections. Adenosine deaminase has been proposed as an important marker for pleural TB as its activity correlates with CD4+ T cell infiltration in the pleura and pleural fluid. ADA is now being recognized as a marker of cell-mediated immunity particularly as a marker of T-lymphocyte activation [4, 5]. Elevated levels of ADA in tuberculous effusions have been noted by several authors and because it is an easy little-invasive investigation, it is frequently considered as a diagnostic aid in such cases. ADA levels have also been considered by several researchers to differentiate tubercular disease from non-tubercular effusions with a sensitivity of 90 – 100% and specificity 89 – 100% [5-7].

We planned the present study to further investigate the role of pleural fluid ADA level in confirmation of the diagnosis of TB and to evaluate; if it can be a diagnostic biomarker.

II. Patients and Methods:

Patients with pleural effusion; attending outdoor/indoor of Respiratory disease of ChatrapatiShivajiSubhartiHospital, Meerut, India between August 2010 to November 2012 were enrolled for the present study. The study was approved by the Ethics Committee of the Institute. Written informed consent was taken from all patients participating in the study.

Fresh pleural effusion patients, with no previous history of medication were included in the study. Detailed present, past, drug and family history was taken, a thorough physical examination, routine and special investigations and radiographic evaluation was done for each patient. Closed pleural biopsy was taken with the help of a Cope's needle and atleast three samples were taken from the same region of thorax. Broncho-alveolar lavage (BAL) was done through a bronchoscope in specific patients.

Thoracentesis was done under aseptic conditions as per standard protocol for all of them and pleural fluid so aspirated was collected in a sterile tube without an anticoagulant for total ADA quantification, routine analysis and cytology.

Patients were classified into various groups as per the following criteria [8]:

1. Presence of first or more than one of the following criteria was adopted to include the patient into tubercular group;
 - A. Bacteriological proof of the presence of Mycobacterium tuberculosis through direct smear / culture of pleural fluid/sputum/BAL.
 - B. Pleural biopsy showing caseating granulomas.
 - C. Clinico-Radiological findings consistent with TB.
 - D. Definite clinical and radiological improvement in one month after specific anti-tubercular treatment.
 - E. History of contact with current disease and positive reaction (>10 mm induration) to 5 tuberculin unit (TU) purified protein derivative (PPD).
2. In case patient did not fulfill the above criteria, they were non-tubercular and were further divided into different subgroups depending upon the following diagnostic criteria;
 - 1) Adenocarcinoma, if their pleural fluid/BAL cytology showed presence of few malignant cells with a tendency to form smoothly contoured cohesive groups composed of large cells with eccentric, malignant appearing nuclei, prominent nucleoli and vacuolated cytoplasm [9].
 - 2) Empyema, if the pleural fluid was pus (thick, opaque, yellowish-white, viscous fluid) loculated or otherwise, was purulent and/or had a positive Gram stain or culture. Pleural infection was indicated by pleural acidosis associated with raised lactate dehydrogenase (LDH) and low glucose levels [10].
 - 3) Parapneumonic, if their pleural fluid was secondary to pneumonia, lung abscess or bronchiectasis in patients with cough, fever, and in cases where radiographic pulmonary infiltrate disappeared with antibiotic treatment [10].
 - 4) Lymphoma, if their pleural fluid/BAL cytology showed presence of discrete monomorphic malignant cells with massive necrosis, pyknosis and fragmentation of nuclei [11].
 - 5) CHF, if the patient had enlarged cardiac shadow on plain X-ray chest with clinical or echocardiographic evidence of cardiac dysfunction, with one or more of the following alterations: pulmonary venous congestion on radiography, peripheral edema, tachycardia or ventricular gallop [12].
 - 6) Cirrhosis liver, if the patient had ascites, splenomegaly, oesophageal varices, portal hypertension, moderate increase in serum bilirubin and SGPT and increased prothrombin time [13].

- 7) Nephrotic syndrome, if the patient had generalized anasarca, proteinuria more than 3.5 gm/day, hypoalbuminemia with altered A/G ratio and hyperlipidemia [14].

Pleural fluid (PF) was classified as [15]:

- a) Exudative if PF/serum protein > 0.5; PF/serum LDH > 0.6 and PF LDH > 2/3 upper normal limit for serum.
- b) Transudative if PF/serum protein < 0.5; PF/serum LDH < 0.6 and PF LDH < 2/3 upper normal limit for serum.

Exclusion criteria:

The patients who presented with any of the following criteria were not included in the study:

- 1) Absolute contraindication or refusal to undergo thoracentesis.
- 2) With recurrent effusion.
- 3) History of previous medication for TB, adenocarcinoma, empyema, parapneumonia, lymphoma, CHF, Cirrhosis liver or nephrotic syndrome.
- 4) On corticosteroid medication.
- 5) With hemolysis in pleural fluid.
- 6) With renal failure, HIV infection or any immunodeficiency disease.
- 7) No diagnosis could be established.

Adenosine deaminase (ADA) assay:

ADA in pleural fluid was assayed by the method of Giusti [16] with slight modifications. The technique is a colorimetric method based on the measurement of ammonia by Berthelot reaction, which is produced when ADA reacts with excess adenosine. The formation of blue indophenol was measured at 628 nm with a spectrophotometer. All solutions for the assay were prepared in house, using triple distilled water and were standardized before subjecting them for ADA estimation. Ammonium sulphate (75 µM) was used as ammonium standard. One IU of total ADA was defined as the amount of enzyme required to release 1 µmol ammonia per minute from adenosine under standard assay conditions. The enzyme is stable for at least 24 hours at 25°C, for 7 days at 4°C, and for 3 months at -20°C [16, 17]. All the samples were stored in a deep freezer at -20°C till analyzed. A positive control sample and two negative control samples for which the ADA value was known were included in each group of pleural fluid sample analyzed. The optimal cut off value of ADA was determined using the receiver operating characteristic (ROC) curve as >40 IU/L.

Statistical Analysis [18, 19]:

All data are expressed as mean and standard deviations. ADA levels were measured in patients with pleural effusions due to TB and also in patients with pleural effusions of non-tuberculous origin as the controls. To compare the differences in ADA levels between the two groups, we performed the Z-test (Double-sample mean) to test the significance at 1% level of significance. Further, Pearson chi-square test without a Yates correction was also applied to find the association between the tubercular and non-tubercular groups at 1% level of significance. Also, Kruskal-Wallis test was used to compare tuberculosis versus nontuberculous groups. A two-tailed p-value less than 0.01 was considered statistically significant. On the basis of the ADA results obtained from pleural TB and control groups, the sensitivity, specificity, accuracy, predictive values, likelihood ratio and diagnostic odds ratio of the test were calculated in order to establish the potential utility of ADA as the diagnostic marker for patients with pleural TB.

III. Results:

Patients (n=160) were divided into two groups i.e., tubercular (n=92) and non-tubercular (n=68) on the basis of their diagnosis. Non-tubercular group was further divided into two groups i.e., patients having exudative effusion (n=46) and patients having transudative effusion (n=22). Patients with exudative effusion included those with adenocarcinoma (n=21), non-tuberculous empyema (n=11), parapneumonic effusion: both simple and complicated (n=9) and lymphomas (n=5) while patients having transudative effusion included those with congestive heart failure (CHF) (n=13), cirrhosis of liver (n=6) and with nephrotic syndrome (n=3) (table 1).

In tubercular group Mean ± SD of ADA value was 67.78 ± 37.39 and it ranged between 8.8 – 260.0; in non-tubercular group Mean ± SD of ADA was 22.17 ± 15.11 and it ranged between 6.0 – 102.0 collectively; Mean ± SD of ADA for exudative effusion was 25.14 ± 16.99 and it ranged between 7.5 – 102.0 while in patients with transudative effusion it was 18.60 ± 7.86 and ranged between 6.0 – 34.0 (Table 1).

In adenocarcinoma patients Mean ± SD of ADA value was 22.90 ± 9.06 and it ranged between 8.8 – 42.0; in non-tubercular empyema patients it was 36.83 ± 28.89 and ranged between 10.0 – 102.00; for parapneumonic patients it was 17.87 ± 9.20 ranging between 7.5 – 32.4 while in lymphoma patients Mean ± SD of ADA value was 21.92 ± 7.00 with ADA values ranging between 16.0 – 34.0 (Table 1).

In CHF patients Mean \pm SD of ADA value was 19.87 ± 6.70 and it ranged between 11.8 – 30.2; in Cirrhosis patients it was 21.36 ± 8.08 and ranged between 12.0 – 34.0; while in nephrotic syndrome patients Mean \pm SD of ADA value was 7.60 ± 1.44 and it ranged between 6.0 – 8.8 (Table 1).

The application of Z-test (double-sample mean) revealed a significant difference between tubercular and non-tubercular groups as well as with tubercular and different subgroups of non-tubercular group at 1% level of significance i.e., ($P < .01$). (Table 1; column 5).

Further, chi-square test statistic also showed a significant difference between tubercular and non-tubercular groups at 1% level of significance i.e., ($P < 0.1$) (chi-square = 93.52, $p < 0.01$). Moreover, Kruskal-Wallis test also showed a significant difference between tubercular and different subgroups of non-tubercular groups at 1% level of significance i.e., ($P < .01$) ($H = 113.71$, $p < 0.01$). (Table 1).

The performance of ADA for diagnosis of tuberculous pleural effusions in regards to the 95% confidence intervals and cut-off levels above 40.0 IU/L resulted in 88.04% sensitivity, 91.12% specificity, 95.25% positive predictive value, 85.33% negative predictive value, 90.63% diagnostic accuracy, 14.7 positive likelihood ratio, 0.13 negative likelihood ratio and 11.78 diagnostic odds ratio, the prevalence of disease in the studied population was 57.5% (table 2).

In our study there were few cases ($n=4$) in nontubercular group which showed ADA levels more than the cut off value (false-positive cases), they were adenocarcinoma ($n=1$) and empyema from bacterial infections ($n=3$).

IV. Discussion:

Pleural effusion is a common problem in clinics and can result due to a number of diseases. Many times in the absence of reliable test reports diagnosis cannot be confirmed and this results in delay in starting proper treatment ultimately leading to progression of the disease and involvement of other organs and more complications. At times patients are treated on clinical impression rather than on laboratory test results which may lead to overtreatment [10].

The conventional diagnosis of pleural tuberculosis, is usually based on an observation of epithelioidgranulomas in pleural tissue biopsy [10]. Acid-fast staining of pleural fluid is a rapid, inexpensive method for diagnosing pleural tuberculosis, but its sensitivity is as low as 0 to 20%. The isolation of *Mycobacterium tuberculosis* in cultured pleural fluid or tissue biopsy specimens permits a definite diagnosis, but again, the sensitivity is low and results can take as long as 2 to 6 weeks to arrive [10].

New techniques have been reported to facilitate the diagnosis of tuberculous pleuritis; like adenosine deaminase activity, interferon gamma levels and polymerase chain reaction. Surprisingly, using polymerase chain reaction has a relatively low sensitivity in pleural fluid (42% to 81%) and is fairly expensive [20, 21]. The sensitivity of an elevated interferon gamma level appears to be better (89% to 99%), but there have been relatively few studies evaluating its use and the assay is expensive [21].

Reid et al.[18] pointed out serious methodological limitations in the research evaluating diagnostic tests; published in the most prestigious international scientific clinical journals. To prevent errors in this ADA study we applied the methodological criteria recommended by Reid et al and by the Standards for Reporting of Diagnostic Accuracy-STARDD[19]. The standard measures of validity are sensitivity and specificity. Other closely related measures are positive and negative predictive value, diagnostic odds ratio, likelihood ratios and the area under a ROC curve[19].

Our study (table 1) revealed high levels of ADA activity in the pleural fluid of tuberculous patients compared to a control group (chi-square = 93.52, $p < 0.01$). On the basis of the ADA results obtained for the TB and non-TB groups, the sensitivity, specificity, predictive values, likelihood ratio and diagnostic odds ratios of the test were calculated (Table 2), which established the potential utility of ADA as the diagnostic biomarker for patients with pleural TB.

In our study there were few cases ($n=4$) in nontubercular group which showed ADA levels more than the cut off value (false-positive cases), they were adenocarcinoma ($n=1$) and empyema from bacterial infections ($n=3$). Based on this fact we tried to find out the statistical performance so that the question addressed, “Pleural fluid Adenosine deaminase activity – Can it be a diagnostic biomarker?” be answered.

The performance of ADA for diagnosis of tuberculous pleural effusions as regards to the 95% confidence intervals and cut-off levels above 40.0 IU/L resulted in 88.04% sensitivity, 91.12% specificity, 95.25% positive predictive value, 85.33% negative predictive value, 90.63% diagnostic accuracy, 14.7 positive likelihood ratio, 0.13 negative likelihood ratio and 11.78 diagnostic odds ratio (table 2). The prevalence of disease in the studied population was 57.5% (table 2).

Several studies have suggested that elevated pleural fluid ADA level predicts tuberculous pleuritis with a sensitivity of 90-100% and a specificity of 89-100% when measured by the Giusti method. The reported cut off value for ADA in these studies varies from 35.0 to 70.0 U/L[22 - 27].

Bossuyt PM et al and De Oliveira HG et al demonstrated specificity of ADA for the diagnosis of pleural tuberculosis increased to 99.0% in case false positive are included[19, 25]. The specificity of ADA for pleural TB may be increased in clinical practice when false-positive cases remain undiagnosed by other tests. An example in the present study is 3 cases of empyema and one case of adenocarcinoma who had ADA levels above the cut off mark (false positive); but they were diagnosed by other means like clinical examination, imaging and pleural fluid examination.

Gupta et al[28] observed that ADA levels in non tuberculous disease rarely exceeded the cut-off value; set for tuberculous disease. They[29] have further observed that ADA estimation is not only a fairly sensitive and specific test (more than 90%), helpful in differentiating tubercular from non-tubercular etiology; both in pulmonary and extra-pulmonary disease but is also simple, inexpensive and rapid. For these reasons this test may help in early diagnosis, improve the prognosis and reduce spread of disease and sequelae.

Although Tuon et al[30] in their meta-analysis concluded that, ADA cannot distinguish between bacterial meningitis and TBM, but their observations are based on studies having heterogeneity, publication bias and nonstandardized method for ADA estimation and so further studies are needed to obtain reliable conclusions.

Gupta et al[8,31] had further found the sensitivity of this test to be 94.73%; specificity 90.47%, positive predictive value is 90.00% and negative predictive value 95.00% and they had concluded that ADA estimation in CSF is not only simple, inexpensive and rapid but also fairly specific method for making a diagnosis of tubercular meningitis in children and may be established as a marker for diagnosis of the same.

Our findings are similar to other researchers[28-30, 32]. The high sensitivity and specificity for adenosine deaminase, can contribute to a diagnosis of pleural tuberculosis, and, in many cases, render a pleural biopsy unnecessary. Furthermore a pleural ADA assay is inexpensive, rapid, and simple to perform and is of great value for the diagnosis of tubercular pleuritis and the early initiation of specific treatment.

V. Conclusion:

It is concluded that ADA estimation in pleural fluid is not only simple, inexpensive and rapid but also fairly specific and sensitive method for diagnosing tuberculous etiology in patients of pleural disease because of the accuracy parameters demonstrated in this study. ADA activity in the pleural fluid can be a diagnostic biomarker and for this reason ADA estimation in pleural fluid may find a place as a routine investigation.

Acknowledgement:

We are thankful to Mr. RupeshTiwari, statistician in the department of Preventive Medicine, Subharti Medical College, Meerut, India, for extending technical help in doing all statistical calculations.

Table 1: Distribution of patients; pleural fluid ADA activity and range with Probable values of Z-test; compared with tuberculosis.

Study Group	Number of Patients	Mean ± SD ADA (IU/L)	Range of ADA (IU/L)	Probable values of Z-test; compared with tuberculosis
Tuberculosis	92	67.78 ± 37.39	8.8 – 260.0	-----
Non-tuberculosis	68	22.17 ± 15.11	6.0-102.0	0.0000 (P<.01)*
Exudative	46	25.14 ± 16.99	7.5-102.0	0.0000 (P<.01)*
Adenocarcinomas	21	22.90 ± 9.06	8.8-42.0	0.0000 (P<.01)*
Empyemas non-tubercular	11	36.83 ± 28.89	10.0 – 102.00	0.0057 (P<.01)*
Parapneumonic simple and complicated	09	17.87 ± 9.20	7.5 – 32.4	0.0000 (P<.01)*
Lymphomas	05	21.92 ± 7.00	16.0 – 34.0	0.0000 (P<.01)*
Transudative	22	18.60 ± 7.86	6.0 – 34.0	0.0000 (P<.01)*
CHF	13	19.87 ± 6.70	11.8 – 30.2	0.0000 (P<.01)*
Cirrhosis	06	21.36 ± 8.08	12.0 – 34.0	0.0000 (P<.01)*
Nephrotic syndrome	03	7.60 ± 1.44	6.0 – 8.8	0.0000 (P<.01)*

(p<.01)* - shows significant difference at $\alpha = .01$ level of significance.

Table 2: Performance of pleural fluid ADA activity for diagnosis of tuberculous pleural effusions at cut-off levels above 40.0 IU/L.

Statistical indices of diagnostic accuracy	Results	95% CI
Sensitivity (%)	88.04	82.61 – 92.11
Specificity (%)	91.12	84.55 – 96.30
Positive predictive value (%)	95.25	89.48 – 99.38

Negative predictive value (%)	85.33	80.43 – 90.17
Positive likelihood ratio	14.7	13.23 – 15.80
Negative likelihood ratio	0.13	0.11 – 0.31
Diagnostic odds ratio	11.78	10.98 – 13.18
Accuracy (%)	90.63	85.09 – 97.12
Prevalence (%)	57.5	53.63 – 63.33

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