

Adenosine Deaminase Assay in Different Body Fluids for the Diagnosis of Tubercular Infection

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Abstract: Diagnosis of tuberculosis from different body fluids remains challenging due to various limitations of the conventional and molecular methods. We studied the role of adenosine deaminase (ADA) assay to diagnose tubercular infection in cerebrospinal fluid, peritoneal fluid and pleural fluid. Fifty three patients with tubercular meningitis, peritonitis and pleuritis were enrolled in this study on the basis of clinical, radiological, cytological, biochemical and somewhere bacteriological evidences. Cases positive by AFB smear, culture or PCR were considered as confirmed TB and other as probable TB cases. Another 28 non-TB cases were included as control. In 53 suspected TB cases ADA was found positive in highest 42 (79.2%) cases, whereas smear and/ culture in 10 (18.7%) and PCR in 18 (33.9%) cases. ADA assay revealed 100% positivity in confirmed TB cases and 14.3% in non TB cases. The sensitivity and specificity of ADA was found 79% and 86% respectively when the cut off value was used ≥ 10 IU/L for CSF and ≥ 40 IU/L for pleural or peritoneal fluid. Mean ADA values of confirmed and probable TB cases were found significantly higher than that of non TB cases (< 0.05). Significance of difference was determined by ANOVA and Kruskal-Wallis test. Thus, adenosine deaminase assay in body fluids has proved to be efficient, reliable and simple method to diagnose tubercular meningitis, peritonitis and pleuritis.

Keywords: Body Fluid, Tuberculosis, Adenosine Deaminase Assay

1. Introduction

Tuberculosis (TB) is a protean disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). Usually TB patients present with pulmonary manifestations. But extrapulmonary tuberculosis (EPTB) is not uncommon in TB endemic countries like Bangladesh. EPTB comprises 10-15% of all TB cases in developing countries [1,2]. Pleural tuberculosis is one of the commonest forms of EPTB which is frequently presented with pleural effusion [3,4]. Likewise, about 78% tubercular peritonitis cases are presented with peritoneal effusion or ascites [5]. Another form of EPTB is tubercular meningitis which has most dangerous sequel if not treated promptly. Pleural and peritoneal tuberculosis is also to be treated as early as possible to minimize morbidity and

mortality.

Diagnosis of TB from body fluids like pleural, peritoneal and cerebrospinal fluid (CSF) is challenging as all these fluid samples possess very few bacilli. Conventional methods like microscopy and culture are widely used for diagnosis, but sensitivity of AFB (Acid fast bacilli) smear is only 5-20% and culture takes about three to six weeks time which may prolong the initiation of treatment. [6-9]. Histological evaluation following laparoscopy and/or biopsy in case of pleural and peritoneal TB brings about better sensitivity, also in smear and culture of the biopsy material [8,10]. But these methods are invasive, require greater expertise and may have sampling error and associated risk of complications [9,10]. Owing to this fact, many newer tests like antigen-antibody detection, antibody in lymphocyte supernatant (ALS) assay, cellular

IFN- γ release assays (IGRAs) and T-Spot have been developed [11]. But reliability of these tests in diagnosing active TB disease is not proven yet. With the advent of molecular technology polymerase chain reaction (PCR) has been developed for rapid detection of *M. tuberculosis*. PCR can reliably detect very low concentration of organism in extrapulmonary samples [5,12]. But requirement of dedicated laboratory areas, rigorous quality control and high cost of the test limits its routine use in resource poor countries. In this regard, adenosine deaminase (ADA) assay has shown to be a useful biochemical test in diagnosing TB from different body fluids, particularly in areas where the disease is prevalent [13-19].

ADA is an enzyme that catalyzes the deamination reaction which converts adenosine to inosine during proliferation and differentiation of lymphocytes. It also plays critical role in maturation of monocytes, thereby converts them into macrophage. So, ADA is being used as a biomarker of T cell activation and the conditions where macrophages are being infected. The level of ADA increases in biological fluids during tubercular infection due to stimulation of T cells by mycobacterial antigens [13].

As a TB-endemic country, a substantial number of EPTB cases are being reported each year with tubercular meningitis, peritonitis and pleuritis in Bangladesh. Major hindrance in the management of these EPTB cases is lack of a rapid, accurate and most importantly convenient diagnostic tool. Notably, under National Tuberculosis Program (NTP) AFB microscopy remains the single available laboratory test to detect TB cases in vast area of Bangladesh. So, the present situation does not permit to adopt newer molecular tests and even AFB culture as a routine method for TB diagnosis. ADA assay has been reported as rapid and cost effective method which can be adopted in even primary healthcare settings in developing countries. Moreover, many studies have confirmed the high sensitivity and specificity of this assay for early diagnosis of extrapulmonary TB, such as tuberculous pleuritis, pericarditis and meningitis [13-19]. To our knowledge no study has been done in Bangladesh by comparing its efficacy with that of conventional methods as well as molecular techniques. In the present study we aimed to evaluate the utility of adenosine deaminase (ADA) assay in detecting *M. tuberculosis* infections in CSF, pleural fluid and peritoneal fluid in comparison to conventional bacteriological methods and PCR.

2. Materials and Methods

This cross sectional study was conducted in the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh in the year 2011. Clinical samples were collected from Dhaka Medical College Hospital and National Institute of Diseases of the Chest and Hospital (NIDCH), Dhaka.

2.1. Selection of Cases

Total 53 suspected TB cases either tuberculous pleuritis,

peritonitis or meningitis was included in the study that fulfilled the following criteria. Notably, samples were collected before starting the anti-TB drugs.

- Strong clinical suspicion of TB based on history and radiological evidences.
- Cerebrospinal fluids having raised protein levels (≥ 0.45 g/L) and/or decreased glucose (≤ 2.5 mmol/L or less than 2/3 of blood sugar) and/or lymphocytic pleocytosis ($\geq 50\%$ lymphocytes).
- Pleural and peritoneal fluids having raised protein levels (3 g/dl) and/or lymphocytic pleocytosis ($\geq 50\%$ lymphocytes)
- Revealed no growth in routine bacteriological culture media.

Another 28 cases of different clinical entity other than TB were taken as non TB control. CSF was collected from patients diagnosed as pyogenic meningitis (6), benign intracranial hypertension (2) and hydrocephalus (2), Pleural fluid from pleural empyema (5), nephrotic syndrome (2) and pleural malignancy (3); peritoneal fluid from chronic liver disease (5) and nephrotic syndrome (3). Diagnosis of the control cases were confirmed clinically and by appropriate laboratory tests.

2.2. Categorization of Cases

All the CSFs, pleural fluids and peritoneal fluids were undergone AFB microscopy, culture in Lowenstein-Jensen medium and PCR using IS6110 primer. On the basis of bacteriological evaluation suspected TB cases were then grouped into two categories.

- Confirmed TB cases: Positive for *M. tuberculosis* either in AFB smear, culture or PCR.
- Probable TB cases: Fulfilled the above mentioned inclusion criteria but negative in bacteriological tests.

2.3. Laboratory Procedures

2.3.1. Sample Processing

About 2-3 ml CSF, 20-30 ml Pleural fluid and 100-200 ml peritoneal fluid were collected from each patient by trained physician under aseptic condition. One ml of each specimen was subjected to biochemical, cytological and routine bacteriological tests. Remaining specimens were then centrifuged for 15 minutes (CSF at 10000 rpm and pleural/peritoneal fluid at 3000 rpm) and deposits were used for staining, culture and PCR assay. Supernatant was preserved at -20°C for ADA assay.

2.3.2. AFB Smear and Culture

For detection of *M. tuberculosis* Ziehl-Neelsen staining and then culture in Lowenstein-Jensen medium was performed.

2.3.3. ADA Assay

Adenosine deaminase level was determined from the supernatants of the centrifuged specimens. A commercial adenosine deaminase assay kit (Diazyme laboratories, USA) was used which follows the principle of modified Giusti method [13,14,20]. Cut-off value for tuberculosis was

considered as ≥ 10 IU/L in CSF and ≥ 40 IU/L in pleural or peritoneal fluid [6,7,13].

2.3.4. Detection of *M. Tuberculosis* DNA

(i). Extraction of DNA

The pellet was re-suspended in 135 μ l of lysis buffer [Prepared by mixing 20 μ M Tris/HCl (pH 8.3), 1mg/ml proteinase K, 0.5% Tween 20 and 10 ml sterile distilled water] and then incubated at 56°C for 3 hours. At the end of incubation lysate was centrifuged at 12000 rpm for 15 min and resultant pellet was resuspended with 100 μ l distilled water. This suspension was boiled at 95°C for 30 minutes in a water bath and then sonicated in an ultrasonication bath for 15 min. Then centrifuged at 10,000 rpm for 5 minutes; supernatant was recovered and stored at 4°C [21].

(ii). Amplification of DNA by PCR

PCR was performed to amplify 123 bp of template targeting IS6110 element. Following primers were used-

IS-F 5'-CCT GCG AGC GTA GGC GTC GG-3' and

IS-R 5'- CTC GTC CAG GCG CGC TTC GG-3'

The 25 μ l reaction mixtures contained 5X PCR buffer; 1.5mM of MgCl₂; 10 mM dNTPs 0.2mM of primers, 1 units of Taq DNA Polymerase and 5 μ l of the DNA. Conditions followed were initial denaturation at 94°C for 5 minutes, followed by 31 cycles at 94°C for 45 sec, 68°C for 45 sec and 72°C for 2 minutes and final extension at 72°C for 10 minutes.

To eliminate false positive or false negative results each run of the PCR assay included one positive control (DNA of H37RV strain) and one negative control (PCR grade water).

(iii). Detection of Amplified DNA by Gel Electrophoresis

The amplified products were subjected to electrophoresis on a 1.5% agarose gel containing 0.5 μ g/ml ethidium bromide and after illumination of the gel under U-V light results were

documented in gel documentation system (BIORAD, Italy).

2.4. Statistical Analysis

All the data were analyzed using Statistical Package for Social Science (SPSS) 11.5 software for windows. To test the significance of difference between different groups ANOVA was used for normally distributed variables, whereas the Kruskal-Wallis test was used for variables having non-normal distribution. P value < 0.05 was taken as minimum level of significance. As the rate of positivity by AFB smear and culture is very low sensitivity and specificity of ADA assay was calculated considering 53 TB cases as disease positive and 28 control cases as disease negative.

2.5. Ethical Consideration

Ethical clearance from the Institutional Ethical Review Committee was taken and informed written consent was sought from the patients before commencing the study.

3. Results

3.1. Results of AFB Smear, Culture, PCR and ADA Assay in the Study Population

Out of 53 suspected TB samples highest 42 (79.2%) was positive by ADA. Only 10 (18.7%) samples were positive by AFB smear and culture. IS6110 PCR was found positive in 18 (33.9%) cases (Table 1). AFB smear, culture and PCR all together improved the sensitivity to 19 (35.8%). These 19 cases were considered as confirmed TB cases, while rest of the suspected cases was regarded as probable TB cases. All the 28 samples from non TB control were found negative by AFB smear, culture or PCR.

Table 1. AFB smear, culture, PCR and ADA positivity in suspected TB and non TB cases.

Specimen	No of cases	Positive by			
		Smear or culture n (%)	PCR n (%)	Smear, culture or PCR n (%)	ADA n (%)
CSF	27	5 (18.5)	9 (33.3)	9 (33.3)	23 (85.1)
Peritoneal fluid	10	2 (20.0)	2 (20.0)	2 (20.0)	7 (70.0)
Pleural fluid	16	3 (18.8)	7 (43.7)	8 (50.0)	12 (75.0)
Total	53	10 (18.7)	18 (33.9)	19 (35.8)	42 (79.2)

NB: All the non-TB samples were subjected to AFB smear, culture and PCR where none of the specimen was found positive.

3.2. Comparative ADA Positivity among Confirmed, Probable and Non TB Cases

While considering ADA positivity in confirmed and probable TB cases (Table 2), there was 100% positivity in all types of samples. About 67.6% probable cases were found positive for ADA, where pleural fluid showed the highest

recovery rate of 70%. ADA was higher than the cut-off value in 2 CSFs and 2 pleural fluids, together comprises 14.3% of the non TB cases. As the control cases were selected on the basis of detailed diagnostic evidences, these 14.3% cases indicated false positivity by ADA.

Table 2. Comparison of ADA positivity among confirmed, probable and non TB cases.

Cases	ADA positivity in			
	CSF	Peritoneal fluid	Pleural fluid	Total
Confirmed TB cases	9 (100%) (n=9)	2 (100%) (n=2)	8 (100%) (n=8)	19 (100%) (n=19)
Probable TB cases	12 (66.7%) (n=18)	5 (62.5%) (n=8)	6 (75%) (n=8)	23 (67.6%) (n=34)
Non TB cases	2 (20%) (n=10)	0 (0%) (n=8)	2 (20%) (n=10)	4 (14.3%) (n=28)

3.3. Mean ADA Value of CSF, Peritoneal Fluid and Pleural Fluid in the Study Population

Mean ADA value in confirmed TB cases were 17.9 ± 2.7 , 96.3 ± 24.1 and 71.9 ± 32.7 respectively in CSF, peritoneal fluid and pleural fluid. These values were significantly higher than 5.3 ± 4.1 , 11.1 ± 9.6 , 20.6 ± 16.9 values of non TB cases. The values for the same samples in probable TB cases were

16.8 ± 5.5 , 54.5 ± 28.6 and 52.5 ± 31.0 which were also significantly higher than those of the non TB cases (Table 3). In each of the comparison p value was found ≤ 0.05 .

The sensitivity and specificity of ADA was found 79% and 86% respectively when the cut off value used ≥ 10 IU/L in CSF and ≥ 40 IU/L in pleural or peritoneal fluid.

Table 3. Mean ADA values of CSF, peritoneal fluid and pleural fluid in confirmed, probable and non TB cases.

Samples	ADA value in IU/L (Mean \pm SD)		
	CSF	Peritoneal fluid	Pleural fluid
Confirmed TB group	17.9 ± 2.7	96.3 ± 24.1	71.9 ± 32.7
Probable TB group	16.8 ± 5.5	54.5 ± 28.6	52.5 ± 31.0
Non TB group	5.3 ± 4.1	11.1 ± 9.6	20.6 ± 16.9

4. Discussion

As Bangladesh is TB-endemic country a considerable number of TB patients are presented with tuberculous pleuritis, peritonitis or meningitis. Due to lack of appropriate laboratory methods, the diagnosis of these cases largely depends on clinical suspicion and non-microbiological parameters. Consequently, the cases having atypical clinical presentation remain undiagnosed and even untreated. In this cross sectional study, out of 53 suspected TB samples only 18.7% revealed smear and culture positivity. The poor sensitivity of AFB smear and culture has widely been reported in many other studies [6-9].

The present study demonstrated 33.9% PCR positivity among suspected TB cases which was higher than that of the bacteriological methods. But this rate of PCR positivity is inferior comparable to different other studies done with body fluids [7,12,22]. PCR positivity largely depends on sample volume, processing, stringent operation and expertise. As a result several authors reported varying degree of sensitivity of this test. Another inconvenience of PCR in body fluids is presence of inhibitors, which negatively influence the result. This problem can be overcome by a multistep process of removing PCR inhibitors and thus improve the recovery of purified *M. tuberculosis* DNA [22]. Most importantly in this study PCR was performed targeting IS6110 gene sequence which is recently been reported to be absent in genome of some isolates of *M. tuberculosis* in south-east India, Tanzania, Malaysia, Oman and Denmark [23,24]. So, IS6110 PCR result sometimes found negative in TB cases. Use of combination of primers targeting different site of mycobacterial genome or using nested PCR may improve the detection of *M. tuberculosis*.

In 53 suspected TB cases adenosine deaminase assay demonstrated highest positivity among all the methods applied in this study. In about 85% CSF ADA value exceeded the diagnostic cut-off for tuberculosis. For peritoneal and pleural fluid raised ADA value was found in 70% and 75% cases, respectively. In a considerable number of suspected TB cases (11 out of 53) ADA activity was found negative. In this study, besides bacteriological confirmation, cases enrolled on the basis of clinical, radiological or cytological ($\geq 50\%$ lymphocytes) evidences. So, there may be some overlapping features with other clinical conditions that influenced the results. Nevertheless, in this study bacteriologically confirmed all the 19 cases (100%) showed a distinct higher value of ADA which signifies the precision of this test. Moreover, among bacteriologically negative 34 probable TB cases 67.6% cases was found positive with highest detection rate in pleural fluid (75%). This finding is in concordance with the study of Kashyap *et al.* who found 96% sensitivity in culture positive CSF and 78% in culture negatives [16]. Ocana *et al.* and Martinez- Vazquez *et al* found 100% positivity of ADA assay in culture positive pleural and peritoneal fluids respectively [17,18]. In addition, the mean ADA value of confirmed TB and probable TB cases was found significantly higher than that of non TB cases ($P < 0.05$) which indicates the strong relationship between presences of TB infection and ADA activity.

As discussed earlier, the sensitivity of AFB smear and culture is very low in diagnosing extrapulmonary TB. Thus it is not reliable to judge any other tests considering these methods as gold standard. Therefore, in the present study a suspected TB case was regarded as disease positive and against this variable sensitivity of ADA was found 79.2%. Out of 28 cases in the non TB control group two CSFs and two

pleural fluids revealed ADA positivity, consequently specificity of the test found 85.7%. Of note, in few other conditions other than TB like pyogenic meningitis and empyema where fluids contain high cell counts can depict high total ADA value. It makes dilemma in differentiating non tubercular conditions from tubercular origin. ADA value detected in most of the assays is the total ADA which includes both ADA-1 and ADA-2 isoenzymes. In tubercular disease particularly elevated isoform is ADA2 which gives rise to the elevation of total ADA also [9,13]. So, analysis of specific ADA2 isoenzyme may help to correctly classify these cases. Furthermore, the sensitivity and specificity of ADA largely depends upon the setting of cut-off values [7,13,16,19]. Thus specificity can be improved by elevating the cut-off value and overlapping values between suspected TB and non TB cases can be avoided.

5. Conclusion

In conclusion, adenosine deaminase level in body fluids is a good indicator of extrapulmonary TB especially where clinical suspicion is high. It can be performed with minimum time, cost and equipments compared to PCR. However, culture is still the gold standard and superior to ADA in determining drug resistance prior initiation of treatment. So, for achieving best clinical outcome ADA values should be carefully correlated with the clinical and other biochemical parameters and cases should be confirmed bacteriologically where facility is available. Meanwhile empirical treatment can be employed in patients having high ADA value as it efficiently differentiates TB from non-TB cases.

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