Characterization Molecular Biochemical Method for Diagnostic of Tuberculosis in the Pleural Samples

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Abstract

Objectives: To compare the diagnostic efficiency of adenosine deaminase, isoenzyme adenosine deaminase-2 and concentration of interferon-γ in patients with tuberculous pleural effusion.
Materials and Methods: The prospective study was done on 114 patients who were divided into 3 groups: tuberculous, non-tuberculous infectious pleurisy, and malignant effusion. The adenosine deaminase, adenosine deaminase-2 and interferon-γ were analyzed by receiver operating characteristic curves.
Results: There was increase of all three markers in tuberculous pleural effusion but not in nontuberculous effusion. The cut-off values for adenosine deaminase, adenosine deaminase-2 and interferon-γ were 40, 26 U/l and 299 pg/ml respectively. Adenosine deaminase, adenosine deaminase-2 activities were significantly higher in tuberculosis effusion than in malignant pleural effusion (more than 5 times) and in non-tuberculous infectious pleurisy (more than 4 times). The median of interferon-γ concentration in pleural fluid of tuberculosis patients was 1514.2 pg/ml (931.2-2187.5pg/ml) which was 10 times more than the median values of other groups of patients. There was no significant difference between patients with malignant effusion and those with non-tuberculous pleural effusion.
Conclusions: All three markers had higher diagnostic yield for tuberculous effusion.

Key words: Adenosine deaminase, isoenzyme, interferon γ, tuberculous pleural effusion.
1 Introduction

Worldwide, tuberculosis (TB) is the single most frequent cause of death by an infectious agent and also is a major cause of pleural effusion. The diagnosis of tuberculous pleuritis should be considered in any patient with an exudative pleural effusion. However, it is sometimes difficult to establish the diagnosis using only conventional methods. A reliable clinical marker permitting the rapid and accurate diagnosis of tuberculous pleuritis is greatly needed [1, 2]. A variety of biological markers have been proposed to facilitate the diagnosis of tuberculous pleuritis, including increased pleural fluid concentrations of adenosine deaminase (ADA), interferon (INF)-γ, interleukin (IL)-12p40, IL-18, immunosuppressive acidic protein (IAP), and soluble IL-2 receptors (sIL-2Rs). The diagnosis of pleurisy itself is not very difficult but carrying out proper treatment at the right time requires clear definition of its etiology. Differential diagnosis of tuberculosis pleural effusion (TP) usually includes invasive procedures like pleural biopsy and thoracoscopy. These manipulations require expertise and can sometimes worsen the patient condition. High cost and time lag between diagnostic procedures also contribute to the efficacy of pleural biopsy and bacteriological test application. These methods, however, are considered to be a “gold standard” of diagnostics. Relatively low sensitivity of other commonly used diagnostic methods pose difficulties in TP diagnostics. Acid-fast bacteria could be seen only in 20-30% cases of pleural fluids and 30-45% specimens of pleural biopsy. Even use of polymerase chain reaction for mycobacteria has sensitivity of less than 89% [2, 3, 4].

Pleural fluid contains sensitive biochemical markers which can be used to facilitate the differential diagnostics of TP. In response to an antigenic stimulation by M. tuberculosis a cell-mediated immune reaction starts leading to production in of interferon-γ (IFN-γ) by T-lymphocytes. The ability of IFN-γ to intensify the phagocyte activity of macrophages that is directed against mycobacteria causes its hyper production in case of TP [1, 5, 6]. Adenosine deaminase (ADA) is an enzyme, whose chief role is the proliferation and differentiation of lymphocytes, especially T-lymphocytes and is called as a marker of cell-mediated immunity, which encompasses the delayed hypersensitivity reaction. There are several isoforms of ADA, but the prominent ones are ADA1 and ADA2. The ADA1 isoenzyme is found in all the cells with highest concentration in lymphocytes and monocytes, whereas ADA2 is found only in monocytes/macrophages which release it when stimulated by the presence of live microorganisms. ADA2 is the more efficient marker of tuberculous pleural effusion [1, 3, 6].

The ADA activity and IFN-γ concentration are valuable biochemical markers with high sensitivity and specificity for TB diagnosis but their diagnostic usefulness also depends on the local prevalence of TB and population’s ethnicity. Some workers showed that the lower ADA levels among Asians might compromise its usefulness in TB detection in these populations. A meta-analysis of studies conducted in Europe had significantly better diagnostic performance than those from other regions. Studies from Japan and Singapore demonstrated better sensitivities and specificities than those from Thailand and India.

The present study was done to see the diagnostic usefulness of the ADA, ADA2 and IFN-γ tests in pleural fluid due to various etiologies in Belarussian and Iranian population.

2 Materials and Methods

Sample collection: This prospective study was done on 114 adult patients who were admitted to the Belarusian Republican Center of Pulmonology and Tuberculosis and Mycobacteriology department of Masoud Laboratory from September 2009 to December 2010. All pleural effusions were clinically divided into 3 groups:

Group 1: with TB,
Group 2: non-tuberculosis effusion (pneumonia, postoperative pleurisy, empyema),
Group 3: malignant effusions (adenocarcinoma, epidermoid carcinoma, small-cell carcinoma, metastatic carcinoma, lymphoma and pleural mesothelioma).

Pleural fluid specimens were obtained by thoracocentesis where about 30 ml of pleural fluid was obtained and analyzed for cell count, cytology, acid-resistant stain, protein concentration and lactate dehydrogenase activity. The rest of collected fluid was centrifuged for 10-15 min at 1500 rpm; supernatant was separated and stored at -20°C for ADA and IFN-γ analysis. Pleural biopsy was also done and tissue was sent for histopathology and microbiology.

The diagnosis of tuberculosis was based on investigation listed above, presence of *M. tuberculosis* in pleural fluid or pleural biopsy specimens, detection of granulomas in pleural tissue with positive acid-resistance stain or display of negative acid-resistant stain granulomas in pleural tissue along with response to antituberculous therapy.

Malignant effusions were diagnosed on positive pleural fluid cytology or presence of malignant cells in pleural biopsy specimens.

2.1. Measurement of ADA and ADA$_2$ activity:
The ADA activity was determined by Giusti and Galanti method. This technique is based on Bertholet reaction of colored indophenol complex production assisted by ammonia liberated from adenosine and spectrophotometric estimation of its concentration. The specimen was incubated with the substrate for 1 h in 37°C water bath. The results were expressed as International Activity Units (U) and one unit of ADA activity was taken an enzyme quantity which was required to liberate 1 mmol of ammonia per minute.

To distinguish between ADA$_1$ and ADA$_2$ forms, 200 µmol/L erythro-9-(2-hydroxy-3-nonyl)-adenosine hydrochlorite (ENHA) (Sigma, USA) was added to the reaction solution, because ENHA is a potent inhibitor of only ADA$_1$ isoenzyme. The same technique was repeated to find the ADA$_2$ value, and ADA$_1$ activity was then calculated by subtracting the ADA$_2$ activity from total ADA activity.

2.2. Measurement of IFN-γ concentration:
Interferon-γ concentration was determined using enzyme-linked immunosorbent assay (Vector-best, Russia). Measurable concentration range was 0-2000 pg/ml, sensitivity of analysis was 20 pg/ml.

**Statistical Analysis:** Statistical analysis was performed using statistical programs “Statistica 6.0” and SPSS. The results were expressed as median and interquartile range (median: 25th percentile – 75th percentile). The differences between groups were analyzed by non-parametric Mann-Whitney U-test. P<0.05 was accepted as significant. The selection of cut-off points was based on the standard Receiver Operator Characteristic (ROC) analysis. The diagnostic value of the parameters studied was assessed in terms of sensitivity, specificity, positive predictive value and diagnostic accuracy according to the cut-off values.
3 Results

The age of the patients ranged from 20-75 years and their characteristics are shown in Table 1.

Table 1: Patient characteristics

<table>
<thead>
<tr>
<th>Groups</th>
<th>Nos</th>
<th>Men</th>
<th>Women</th>
<th>Age interval (in years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuberculous pleurisy</td>
<td>45</td>
<td>28</td>
<td>17</td>
<td>20–75</td>
</tr>
<tr>
<td>Non-tuberculous infection</td>
<td>30</td>
<td>17</td>
<td>13</td>
<td>24–75</td>
</tr>
<tr>
<td>Malignant effusion</td>
<td>28</td>
<td>15</td>
<td>13</td>
<td>29–73</td>
</tr>
</tbody>
</table>

Table 2 shows the ADA and ADA$_2$ activities in pleural fluid in each group. The values were significantly higher in patients with tuberculous effusion than in patients with malignant pleural effusion (more than 5 times) and patients with non-tuberculous infections pleurisy (more than 4 times). The ADA$_1$/ADA$_2$ ratio in tuberculous group was lowest; it made up 64% of corresponding median for “non-tuberculous pleurisy” group and 68% of corresponding median in patients with malignant effusion.

Table 2: ADA activity in pleural fluid of patients with pleurisy

<table>
<thead>
<tr>
<th>Determined indices</th>
<th>Examined groups</th>
<th>Tuberculous pleurisy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-tuberculous infection</td>
<td></td>
</tr>
<tr>
<td>ADA U/l</td>
<td>18.7 (12.6–21.7)</td>
<td>15.5 (13.1–21.1)</td>
</tr>
<tr>
<td>ADA$_2$ U/l</td>
<td>11.4 (7.9–16.8)</td>
<td>12.1 (9.6–16.5)</td>
</tr>
</tbody>
</table>

* p<0.05 in comparison with “non-tuberculous infection” group;
* * p<0.05 in comparison with “malignant” group.

IFN-γ concentration in pleural fluid of tuberculous patients was 1514.2 pg/ml (931.2–2187.5) which is considerably higher (> 10 times) than the median values of other groups. There was no significant difference between patients with malignant effusion and non-tuberculous effusion (Figure 1).

Figure 1: IFN-γ concentration in pleural fluid of patients with pleurisy
To see the optimal cut-off value we built characteristic curves (ROC-curves) which are generally accepted for diagnostic efficacy estimation (Figure 2, 3). Sensitivity and specificity were plotted for various cut-off levels for pleural fluid IFN-γ and ADA to construct the ROC curve. The area under the ROC curve (AUC) was estimated to compare the diagnostic utility of IFN-γ levels with ADA for the diagnostic utility of IFN-γ levels with ADA for the diagnosis of tuberculous pleurisy. Both IFN-γ and ADA had high AUC values, although IFN-γ and ADA2 were marginally superior to ADA (IFN-γ: 0.996; 95% confidence interval [CI]: 0.988 -1.004; ADA2: 0.996; 95% CI: 0.987-1.005 and ADA: 0.987; 95% CI: 0.967-1.007). The best cut-off was taken at the point where the curve sharply angulated. It was found to be 299.5 pg/ml for IFN-γ whereas it was 39.8 U/l for ADA and 26.2 U/l for ADA2.

For the best cut-off of IFN-γ the sensitivity and the negative predictive value for diagnosis of TP were 100%, whereas specificity and positive predictive value were 92.5% and 93.1% respectively (Table 3).

Table 3: Descriptive profile of diagnostic utility of ADA, ADA2 and IFN-γ estimation for tuberculous pleurisy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ADA U/l</th>
<th>ADA2 U/l</th>
<th>IFN-γ pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut-off value</td>
<td>38</td>
<td>25</td>
<td>288</td>
</tr>
<tr>
<td>True-positive</td>
<td>38</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>False-positive</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>False-negative</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>True-negative</td>
<td>28</td>
<td>28</td>
<td>33</td>
</tr>
<tr>
<td>Sensitivity %</td>
<td>92.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Specificity %</td>
<td>95.7</td>
<td>95.7</td>
<td>92.5</td>
</tr>
<tr>
<td>Positive predictive value %</td>
<td>96.3</td>
<td>96.3</td>
<td>93.1</td>
</tr>
<tr>
<td>Negative predictive value %</td>
<td>89.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Diagnostic efficacy %</td>
<td>93.5</td>
<td>97.6</td>
<td>95.3</td>
</tr>
</tbody>
</table>

☐ Positive predictive value (probability of fact that patient really has tuberculous pleurisy in case if test result exceeds cut-off value).
☐ Negative predictive value (probability of fact that patient has non-tuberculous pleurisy in case if test result doesn’t exceed cut-off value).

For the best cut-off of ADA2 both the sensitivity and the negative predictive value for diagnosis of TP were 100%, whereas specificity and positive predictive value were 95.7% and 96.3% respectively. The
latter two characteristics were almost equal to those for the best cut-off of ADA (95.7 and 96.6%, respectively). On the other hand, the choice of the cut-off for ADA resulted in a lower sensitivity (92.0%) and negative predictive value (89.6%). Therefore, the diagnostic efficiency of using ADA test was slightly lower (92.5%) than for ADA2 (97.6%) and IFN-γ (95.3%).

Difference in the sensitivity between the ADA and two other markers resulted because of three extra false-negatives with ADA (table 3). All patients with low IFN-γ levels as well as ADA2 activity did not have tuberculosis (TB) (there were no false-negatives). Among the patients with low ADA, 3 patients out of 32 (9.3%) turned out to have TB. All of the remaining 29 patients with low ADA activity did not have TB. Only 1 out of 41 and 44 patients (2.4%, 2.2%) had ADA and ADA2 activities respectively above the threshold value, but that patient did not have TB, and only 2 out of 41 patients (4.8%) had IFN-γ levels above the threshold value, but these patients did not have TB. For 43 patients with proven TB, ADA missed diagnosis in 3 patients (6.9%), more than what were missed by IFN-γ and ADA2 estimations. That is, for every 100 patients with TB, IFN-γ and ADA2 estimations results in the detection of 6.9 patients those are missed by ADA estimation.

Both the measurement of IFN-γ and ADA, when combined with the determination of ADA2, yielded high sensitivity (100%) (table 4). The combination of two markers where both these tests are positive made increased diagnostic efficacy of all methods except ADA2. The PPVs and NPVs of all combined methods were almost equally high (97.6, 97.7 and 100%, respectively).

Table 4: Utility of the combination of pleural fluid ADA, ADA2 and IFN-γ in the diagnosis of tuberculous pleurisy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ADA+ADA2+IFN-γ</th>
<th>ADA+ADA2</th>
<th>ADA+IFN-γ</th>
<th>ADA2+IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with confirmed TP</td>
<td>39</td>
<td>39</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>Sensitivity %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Specificity %</td>
<td>95.7</td>
<td>95.7</td>
<td>95.7</td>
<td>95.7</td>
</tr>
<tr>
<td>Positive predictive value%</td>
<td>96.3</td>
<td>96.3</td>
<td>96.3</td>
<td>96.3</td>
</tr>
<tr>
<td>Negative predictive value%</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Diagnostic efficacy %</td>
<td>97.6</td>
<td>97.6</td>
<td>97.3</td>
<td>97.6</td>
</tr>
</tbody>
</table>

5 Discussion

Previous reports have suggested that these biological markers are useful for the diagnosis of tuberculous pleuritis. However, which of these six markers is most useful for the diagnosis of tuberculous pleuritis has not been determined. To determine the marker with the greatest diagnostic significance, we performed receiver operating characteristic (ROC) analysis on these six markers (1, 6).

Making a differential diagnosis between tuberculous and nontuberculous pleural effusions is a critical clinical problem, and conventional methods of diagnosis are often inadequate. The direct examination of pleural fluid by Ziehl-Neelsen staining has low sensitivity. Culture is gold standard but is dependent on expertise and requires several weeks to grow *M. tuberculosis*. The sensitivity of pleural biopsy is higher than that of thoracocentesis both by culture and histology. However, a biopsy requires greater expertise and is more invasive, and the examination of a biopsy specimen is subject to sampling error(2, 4, 5).

Pleural levels of a number of biological markers have been proposed as aids in the diagnosis of tuberculous pleuritis, including those of ADA, INF-γ, IL-12p40, IL-18, IAP, and sIL-2R, the levels of which are all significantly higher in tuberculous pleural effusions than in nontuberculous pleural effusions. However, the sensitivities of these markers have never been compared directly.

Present research demonstrates the results of the first investigation conducted in Iran which is devoted to the utility of pleural fluid ADA activity and IFN-γ concentration in differential diagnosis of tubercular
pleural effusions. The growth of total ADA activity in pleural fluid is caused mainly by ADA$_3$ isoenzyme form. The same phenomenon was also observed by other researchers. In contrast, 2 studies have shown that ADA levels are of limited value. However, one was performed in an area of low tuberculous prevalence, and the other included poorly defined population group. Carrying out total ADA activity definition test in our study proved to be characterized by higher sensitivity and specificity values (93.0% and 96.7%) in comparison with results from other reports. Determination of ADA$_2$ activity in pleural fluid was found to be even more sensitive (100%) regarding TP.

Refers to the literature, cut-off values of ADA activity in pleural fluid of patients with tuberculous effusion from European and Asian countries varied from 41 - 70 U/L while test sensitivity varied from 79 - 100%. Cut-off values of IFN-γ had even greater variations (from 12 - 240 pg/ml). The differences found in the diagnostic capacity of IFN-γ and ADA might be due to the assay used enzyme-linked immunosorbent assay (RIA), or to the range of diseases included in the studies. In addition, different cut-off levels were used, perhaps because of interlaboratory variability, different prevalence of tuberculosis in population and peculiarities of population itself. Every laboratory should determine the most suitable cut-off point (2-5).

Our data are similar to reports of other investigators, who researched IFN-γ levels in pleural fluid and demonstrated high IFN-γ concentrations in tuberculosis. Patients with malignant pleural effusions, nonspecific pleural effusion, parapneumonic effusions, and pleural transudates had low levels of IFN-γ. In our study, the increased IFN-γ concentration in pleural fluid of patients with tuberculosis has extremely high sensitivity and rather high specificity – 100 and 92.5%, respectively. IFN-γ, a cytokine associated with Th1 type of cell-mediated immune response was found to be highly associated with a tuberculous etiology of pleural effusion. The clear separation of 95% confidence intervals for values of this cytokine in the pleural fluid of patients with confirmed tuberculosis from those with nontuberculous pleural effusions, and the high sensitivity, specificity and predictive values observed for IFN-γ levels in this case series substantiate its diagnostic potential.

Thus, all three markers of TP demonstrated their usefulness in Iranian population. However none of these have an absolute specificity because they did not reach 100%.

The question arises of which marker should be preferred for practical use. Although ADA and ADA$_2$ activity, IFN-γ measurement have been evaluated individually in the diagnosis of tuberculosis, the results of using all three methods in the same patients have not been available previously in Iran, and their combined use has not been explored.

Our data show that together, measurement of IFN-γ levels and determination of ADA and ADA$_2$ activity can be utilized to preferentially optimize sensitivity in either or combination, or specificity (in case of IFN-γ) in a combination requiring both methods used to be positive. On this respect it is necessary to note that among examined patients there were three patients with confirmed diagnosis of tuberculosis whose ADA activity was lower than determined cut-off value and there were one patient with fibrinous-exudative non-tuberculous pleurisy who had ADA and ADA$_2$ activity higher than determined cut-off values. IFN-γ concentration in these cases was found to be correspondingly higher and lower than cut-off value. In other words, use of IFN-γ could supply the results of ADA test, improving to 100% its negative predictive value. However there was one patient with right-side non-tuberculous exudative pleurisy and one patient with left-side multilacunar non-tuberculous empyema whose IFN-γ concentrations proved to be higher than cut-off value.

Investigations display that total ADA activity determination in pleural fluid has not only clinical but also economical efficacy as this method is easy to execute, it doesn’t require expensive equipment and reagents. The result can be received within 2 hours. This method should be recommended first of all for the application in medical practice. The definition of ADA$_2$ activity and IFN-γ concentration in pleural fluid also has high diagnostic value. Use of these methods improves the diagnostic efficacy of ADA test in TP.

In the present investigation, alternative diagnostic methods were evaluated individually and in combination within the context of the case mix encountered in clinical practice. The understanding of the strengths and
weaknesses of each diagnostic method, used individually or in combination, and the consideration of the results together with the symptoms and signs associated with a determined etiology of pleural effusion propitiates the rational and optimal election of methods in different diagnostic scenarios.

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References


