

# Original Article

## Laboratorial validation of an automated assay for the determination of adenosine deaminase activity in pleural fluid and cerebrospinal fluid\*

Validação laboratorial de um método automatizado de dosagem da atividade de adenosina desaminase em líquido pleural e em líquido cefalorraquidiano

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### Abstract

**Objective:** The incidence of tuberculosis worldwide has emphasized the need for better assays designed to diagnose the disease, principally the extrapulmonary form. The objective of the present study was to validate the performance of an automated method for the determination of adenosine deaminase (ADA) activity in pleural fluid (PF) and cerebrospinal fluid (CSF), comparing it with a conventional method (the modified Giusti method). **Methods:** In total, 134 samples were selected from among those tested in our laboratory: 94 PF samples and 40 CSF samples. The ADA activity was determined using the two methods. Inter- and intra-assay precision was determined, linear regression analysis was performed, simple concordance tests were conducted, and the means of the differences were calculated. **Results:** The correlation coefficients for PF and CSF samples were, respectively, 0.96 and 0.95. Inter-assay precision was determined using 21 replicates at 3 different activity levels: low, medium and high. The percentage coefficient of variation (%CV) was, respectively, 5.9, 8.1 and 5.8 for PF samples, compared with 21.9, 18.6 and 13.8 for CSF samples. Intra-assay precision in %CV was 1.3 and 11.7, respectively, for PF and CSF samples. The concordance between the methods in PF and CRF samples was, respectively, 96.8% and 100%, considering the reference values for the diagnosis of TB to be 40 U/L (conventional) and 30 U/L (automated) in PF samples, versus 9 U/L (for both methods) in CSF samples. **Conclusions:** The results validate the use of the automated method of determining ADA activity in PF and CSF samples as an alternative to the conventional method.

**Keywords:** Adenosine deaminase; Tuberculosis/diagnosis; Pleural effusion; Cerebrospinal fluid.

### Resumo

**Objetivo:** A incidência global de tuberculose reforça a necessidade de melhores ensaios para o diagnóstico desta doença, principalmente a tuberculose extrapulmonar. O objetivo do trabalho foi validar o desempenho de um método automatizado para a determinação da atividade de adenosina desaminase (ADA) no líquido pleural (LP) e no líquido cefalorraquidiano (LCR), comparando-o com um método convencional (Giusti modificado). **Métodos:** Selecionaram-se 134 amostras da rotina laboratorial: 94 de LP e 40 de LCR. Foram realizadas as determinações da atividade de ADA através dos dois métodos. Calculou-se a precisão inter- e intra-ensaios, análise de regressão linear, testes de concordância simples e médias das diferenças. **Resultados:** Os coeficientes de correlação para as amostras de LP e LCR foram, respectivamente, 0,96 e 0,95. A precisão interensaio foi determinada pela média de 21 amostras replicadas em ensaios diferentes para 3 níveis de atividade: baixa, média e alta. Os coeficientes de variação em porcentagem (%CV) foram, respectivamente, 5,9, 8,1 e 5,8 para amostras de LP; e 21,9, 18,6 e 13,8 para amostras de LCR, respectivamente. A precisão intra-ensaio em %CV foi, respectivamente, 1,3 e 11,7% para amostras de LP e LCR. A concordância entre os dois métodos em amostras de LP e LCR foi, respectivamente, 96,8% e 100%, considerando-se como valores de referência para o diagnóstico de TB 40 U/L (convencional) e 30 U/L (automatizado) em amostras de LP, e 9 U/L em amostras de LCR para os dois métodos. **Conclusões:** Os resultados validaram o método automatizado de determinação da atividade de ADA para o uso em amostras de LP e LCR como alternativa ao método convencional.

**Descritores:** Adenosina desaminase; Tuberculose/diagnóstico; Derrame pleural; Líquido cefalorraquidiano.

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## Introduction

The incidence of tuberculosis (TB) has increased worldwide; it is estimated that there are 100,000 new cases annually, and 80% of those cases are concentrated in 22 countries, one of which is Brazil.<sup>(1,2)</sup> In Western countries, there has been an increase in the number of cases of pulmonary and extrapulmonary TB, especially due to the prevalence of HIV infection.<sup>(3)</sup>

Clinical laboratories perform a series of tests that contribute to the diagnosis of TB. Direct testing for mycobacteria using Ziehl-Neelsen staining is considered a priority. Ziehl-Neelsen staining is extremely important, since treatment can be initiated immediately if the result is positive. In addition, it is an inexpensive test that is rapid and easily performed. Culture on Löwenstein-Jensen, or similar, medium is performed in parallel. However, sputum smear microscopy has a major problem, that of its low sensitivity.<sup>(2,4)</sup> Culture, although more sensitive, is limited by the time required to obtain results, which has an extremely negative effect on treatment.<sup>(3)</sup> In the case of pleural fluid (PF), the yield obtained by sputum smear microscopy is near zero, whereas the yield obtained by culture ranges from 10% to 35%.<sup>(5)</sup>

These factors make diagnosis even more difficult. Automated or semi-automated methods of mycobacterial growth in liquid media allow earlier diagnosis, since detection occurs between one and three weeks, compared with the three to eight weeks required when using solid media. Among such methods is the rapid radiometric system for detection of mycobacteria in clinical specimens (BACTEC 460 TB R; Becton Dickinson, Sparks, MD, USA), which is well established (international validation) and has been approved by the World Health Organization.<sup>(6)</sup> However, studies indicate that, in addition the problem created by the discharge of radioactive material, the rate of false-positive culture results, due to contamination between specimens during the machine reading, ranges from 1.4% to 4%.<sup>(6)</sup>

In Brazil, this system is falling into disuse in referral laboratories, being replaced by faster, nonradiometric systems, after assessment of their cost-effectiveness in different situations.<sup>(7)</sup>

Biopsy facilitates the differential diagnosis between granuloma with caseous necrosis and other

nonspecific granulomatous lesions without necrosis, especially in cases in which the microbiological study was not sufficient to establish the etiological diagnosis.<sup>(5,6)</sup>

Currently, serologic testing for antibodies using enzyme immunoassays or radioimmunoassays is not as definitive as is the isolation of the etiologic agent itself, since these assays usually have low specificity.<sup>(5,7)</sup>

The intradermal test, which is based on a delayed hypersensitivity reaction to purified protein derivative, can be used only as a screening test, since a positive result indicates only exposure to the bacillus or to the vaccine strain. In Brazil, mass vaccination with the bacillus Calmette-Guérin vaccine is a limiting factor for the use of this test.<sup>(8)</sup> It should be noted that immunocompromised patients present negative reactions even in the presence of a positive culture or active infection.<sup>(2,9)</sup>

The tuberculostearic acid present in *Mycobacterium tuberculosis* can be detected by gas chromatography and mass spectrometry. Despite being more sensitive, these techniques are less specific, as well as being more complex and more costly.<sup>(1)</sup>

If, in the diagnosis of pulmonary TB, we still have difficulty in finding methods that can meet the needs of clinical practice, in the case of extrapulmonary TB, the situation is even worse. Conventional methods, which are useful for the diagnosis of pulmonary TB, perform poorly when PF or any other cavitary fluid is used in cases of extrapulmonary TB.<sup>(1,7)</sup>

The recognition of these difficulties in the diagnosis of TB have led to the search for other methods that could optimize the approach to patients with pleural effusion who are suspected of having one or more of the several extrapulmonary forms of this disease, such as meningeal, renal, pleural, osseous and lymph node TB. Chief among the new techniques are polymerase chain reaction,

**Table 1** - Intra-assay precision determined by the mean, standard deviation and coefficient of variation of the repeated determinations.

Sample type	Mean, U/L	SD, U/L	CV, %
Pleural fluid	16.3	0.2	1.3
Cerebrospinal fluid	0.6	0.07	11.7

CV: coefficient of variation; CV = 1.3 ± 0.2% for pleural fluid; and CV = 11.6 ± 0.07% for cerebrospinal fluid.

which identifies *M. tuberculosis* DNA through the amplification of known genome sequences, and the techniques that detect biochemical markers, such as interferon gamma and adenosine deaminase (ADA), produced during the inflammatory process triggered by *M. tuberculosis*.<sup>(4,10)</sup>

This abbreviation—ADA—is a designation given to a group of enzymes with different molecular weights that have similar chemical functions in the metabolism of purines, catalyzing the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively.<sup>(9)</sup> In nearly all vertebrates, ADA is present. In humans, ADA is mainly found in T lymphocytes and is directly related to the activation of these cells. Activation of T lymphocytes can occur in various clinical situations in which increased ADA levels are found. The determination of ADA levels is particularly useful in cases of meningitis and tuberculous pleuritis.<sup>(3,10-12)</sup> Therefore, the determination of ADA levels is extremely relevant for the laboratory testing-based diagnosis.<sup>(12)</sup>

Some researchers have reported that serum ADA activity is increased in patients with acute hepatitis or liver cirrhosis, as well as in those with other conditions.<sup>(10)</sup> Other authors have carried out studies relating ADA activity levels to diseases such as rheumatoid arthritis, lymphoproliferative disorders, systemic lupus erythematosus, psoriasis and eclampsia.<sup>(13-15)</sup> However, due to its lack of specificity, the determination of serum ADA activity has been more often used for the follow-up treatment of TB than for diagnosis. In the literature, there are innumerable studies involving the determination of ADA levels in cases of suspected TB, especially the extrapulmonary form, studies in which this group of enzymes is considered an excellent biochemical marker of TB.<sup>(7,9,10)</sup>

The determination of ADA levels has been performed using the method proposed by Giusti in 1974,<sup>(16,17)</sup> and which has undergone certain modifications over time. The modified Giusti method includes the Berthelot reaction in order to obtain better results.<sup>(3,10,16)</sup> Despite being considered efficient, this method still has a series of limitations: it requires reagent preparation; it is performed manually; readings are taken using a spectrophotometer; and all steps are performed in-house, that is, by each laboratory individually—therefore, there is a lack of standardization that does not allow this assay to be

**Table 2** - Inter-assay precision at three different activity levels (low, medium and high) using pleural fluid and cerebrospinal fluid samples.

Level	Mean, U/L	SD, U/L	CV, %
Low <sup>a</sup>	5.9	0.4	5.9
Medium <sup>a</sup>	19.4	1.6	8.1
High <sup>a</sup>	60.1	3.5	5.8
Low <sup>b</sup>	0.8	0.2	21.9
Medium <sup>b</sup>	5.4	1.0	18.6
High <sup>b</sup>	7.2	1.0	13.8

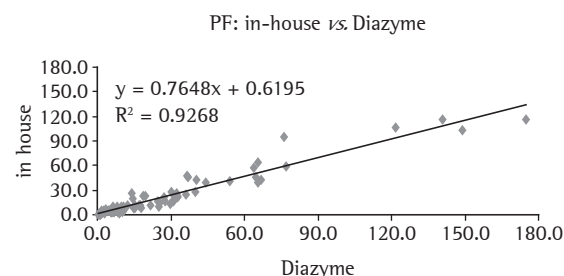
CV: coefficient of variation; <sup>a</sup>Pleural fluid samples; and <sup>b</sup>cerebrospinal fluid samples.

used as a parameter that can meet all the criteria of a good diagnostic test.<sup>(18)</sup>

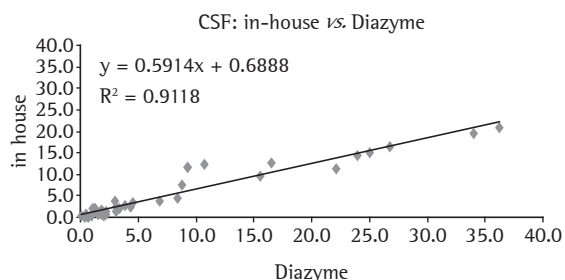
The objective of the present study was to test an automated method (commercial kit) for the determination of serum ADA activity in PF and cerebrospinal fluid (CSF) samples, as well as to standardize it for the first time, comparing its results with those obtained using the modified Giusti method, which is considered a reference test for our biochemical study.<sup>(16)</sup>

## Methods

A total of 134 samples (94 PF samples and 40 CSF samples) were selected from among those tested in the Central Laboratory of the *Hospital São Paulo/Universidade Federal de São Paulo* (HSP/UNIFESP, São Paulo Hospital/Federal University of São Paulo) and in the Laboratory of the Association for the Incentive Funding of Psychopharmacology/Laboratory Medicine. In the samples selected, ADA activity levels were previously determined, in-house, using a conventional method—the modified Giusti method.



**Figure 1** - Correlation (linear regression) between the conventional (in-house) and the automated (Diazyme) methods for pleural fluid (PF) samples. The correlation coefficient (r) was 0.96.



**Figure 2** – Correlation (by linear regression) between the conventional (in-house) and the automated (Diazyme) methods for cerebrospinal fluid (CSF) samples. The correlation coefficient ( $r$ ) was 0.95.

Repeated determinations of ADA activity levels in those same samples were performed simultaneously using the modified Giusti method and the Diazyme commercial kit (Diazyme Laboratories, San Diego, CA, USA) with automation (ADVIA 1650 analyzer; Bayer Diagnostics, Tarrytown, NY, USA).

In order to perform the modified Giusti method,<sup>(16)</sup> the samples were centrifuged, and 25  $\mu$ L of the supernatant were transferred into test tubes, to which 500  $\mu$ L of an adenosine solution were added. The mixture was heated at 37°C for 60 min, and the reaction was interrupted by the addition of a phenol and nitroprusside solution and a hypochlorite solution. The resulting solution was then reheated at 37°C for 30 min. The quantity of ammonia released by the ADA activity was determined using spectrophotometry at 620 nm. In order to perform the automated Diazyme method, 11  $\mu$ L of PF sample and 11  $\mu$ L of commercial control material were used at two activity levels (high and low). For CSF samples, quantities of 22  $\mu$ L were

used. The results obtained using the two methods are expressed in U/L.

Inter- and intra-assay precision tests were performed for the automated method. Intra-assay precision was determined using 21 replicates of PF and CSF. Inter-assay precision was determined using 21 replicates at 3 different ADA activity levels in a pool of PF and CSF. Mean, standard deviation and coefficient of variation (CV) were calculated for the two types of precision (Tables 1 and 2).

The results obtained using the conventional method and those obtained using the automated method were submitted to the correlation test (Figures 1 and 2) and the simple concordance test in order to determine the equivalence between them,<sup>(17-19)</sup> considering the reference values for the diagnosis of TB in PF samples to be 40 U/L (modified Giusti method) and 30 U/L (automated method), versus 9 U/L (for both methods) in CSF samples (Table 3).

During the study, the stability of the automated method reagents was evaluated for one year. The sample volume needed when assays are performed using the automated method was also evaluated, as were execution time and the time needed for obtaining results.

## Results

Intra-assay precision for each type of biological fluid, which was determined using 21 assay replicates of PF and CSF, is shown in Table 1.

Inter-assay precision, which was determined using 21 replicates at 3 different ADA activity levels (low, medium and high) in PF and CSF samples, is shown in Table 2. Mean, standard deviation and CV were calculated.

**Table 3** – Simple concordance test of the results obtained using the automated and the conventional methods in pleural fluid and cerebrospinal fluid samples.

Automated method: pleural fluid	Conventional method: $\geq 40$ U/L	Conventional method: $< 40$ U/L	Total
$\geq 30$ U/L	14	2	16
$< 30$ U/L	1	77	78
Total	15	79	94*
Automated method: cerebrospinal fluid	Conventional method: $\geq 9$ U/L	Conventional method: $< 9$ U/L	Total
$\geq 9$ U/L	11	0	11
$< 9$ U/L	0	29	29
Total	11	29	40**

\*96.8% concordance; and \*\*100% concordance.

For PF samples, the %CV values obtained at the 3 different ADA activity levels (inter-assay precision) did not exceed 10%, whereas, for CSF samples, the mean values ranged from 13% to 20%.

Figures 1 and 2 show the correlation between the two methods for PF and CSF samples at different activity levels.

Table 3 presents the results of the simple concordance test. Considering the cut-off values of 40 U/L (conventional method) and 30 U/L (automated method) for the detection of pleural TB in PF samples, the concordance was 96.8%. For the detection of meningeal TB in CSF samples, the cut-off value was 9 U/L (for both methods), and the concordance was 100%.

## Discussion

It is of great relevance to evaluate the efficiency of alternative tests for the diagnosis of TB, especially that of the determination of ADA activity, which can be used in countries where TB prevalence is high, as announced by the Pan American Health Organization. In addition, the determination of ADA activity can, in many cases, favor the diagnostic confirmation, replacing biopsy, laparoscopy and other tests that definitively confirm the diagnosis but are more sophisticated, expensive and, in many health care facilities, unavailable.

Pulmonology societies, including the *Sociedade Brasileira de Pneumologia e Tisiologia* (SBPT, Brazilian Thoracic Association),<sup>(20)</sup> recommend the determination of ADA activity in organic fluids, especially in PF and CSF.<sup>(21)</sup> Therefore, the standardization of reliable automated methods is needed in order to define the diagnosis more rapidly and favor TB treatment.

However, the practicality and rapidity of the automated method proposed in the present study should be accompanied by a proper and useful validation so that the method can be used on a large scale.<sup>(22)</sup> There is considerable laboratory and clinical interest in standardizing an automated method that can replace the use of in-house methods, such as the modified Giusti (Berthelot) method, which, despite being considered definitely appropriate and having been available for at least two decades, is influenced by several interfering factors related to the execution of the technique, which decrease precision and accu-

racy, consequently weakening interlaboratory and intralaboratory correlations. In addition, the preparation of the specific, highly toxic reagents for use in the test requires trained, qualified professionals.

The organic fluid (PF and CSF) samples were selected at random from among those collected from patients with various diseases (among which were infectious, immunologic and neoplastic diseases) treated as inpatients at the HSP/UNIFESP. The present study did not focus on clinical practice, since the authors were more interested in validating the laboratory method of ADA level determination, and, therefore, the samples selected had a wide range of ADA activity levels (high, medium and low).

The values found for the two types of organic fluid confirmed the strong correlation between the two methods (Figures 1 and 2).

The importance of the determination of ADA levels in organic fluids has been well established,<sup>(11,23,24)</sup> although the ideal cut-off level for this test, in the diagnosis of TB, remains controversial.

In the present study, we considered the cut-off level for the conventional method in PF samples to be 40 U/L, in accordance with recommendation of the SBPT,<sup>(8,12,19)</sup> although various studies have associated the clinical aspect with different ADA cut-off points.<sup>(21)</sup> For the automated method in PF samples, we suggested a cut-off value of 30 U/L, after the mean percentage of the differences between the results obtained by the two methods had been calculated, and a value approximately 30% lower was found for the automated method. With these cut-off levels, the concordance between the methods was found to be 96.8% (simple concordance test), which is in accordance with the findings of other authors.<sup>(23)</sup>

In the literature, the cut-off value recommended for the diagnosis of TB in CSF samples is 9 U/L. We considered this value for the two methods, since, using this value, concordance was found to be 100% (simple concordance test), although the mean percentage of the differences between the results obtained by the two methods was approximately 35%. It is likely that this value will be analyzed in detail and reevaluated in future studies, selecting samples of patients with a definite diagnosis.

The results of the inter- and intra-assay precision tests were satisfactory, with %CV values appropriate for PF samples. However, for CSF samples, %CV values were higher because ADA activity levels are lower in CSF than in other cavitory fluids. In addition, CSF is much poorer in protein elements, as well as being known to present more unstable enzymatic and immunologic differences. In the present study, the %CV values for low ADA activity in PF samples were higher than those found for medium and high ADA activity in CSF samples.

Other aspects analyzed during the use of the automated method include reagent stability, which remained unaltered at 4°C for 6 months, the rapidity of obtaining results—approximately 10 min—and the (smaller) sample volume.

Based on the results obtained in the present study, the automated method, which was performed as described above, proved to be appropriate to replace the conventional (in-house) method without affecting the interpretation of the final result.

After the automated method is implemented in laboratory practice, it will be possible to perform clinical studies analyzing its sensitivity and specificity<sup>(22,25)</sup> for the diagnosis of TB and of other diseases that stimulate increased ADA activity, as well as to have standardized methods, which will allow better comparison of interlaboratory results.

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