

## Pleural fluid adenosine deaminase detection for the diagnosis of pleural tuberculosis\*

MORRYS CASAGRANDE KAISEMANN, AFRÂNIO LINEU KRITSKI,  
MARIA DE FÁTIMA C PEREIRA, ANETE TRAJMAN

**Background:** The diagnosis of pleural tuberculosis continues to be a challenge due to the low sensitivity of traditional diagnostic methods. Histopathological examination of pleural tissue is the most accurate method, with a sensitivity of up to 80%. Determination of adenosine deaminase levels is a recently introduced method, although its usefulness in the diagnosis of pleural tuberculosis in Brazil has yet to be well elucidated.

**Objective:** To verify the sensitivity and specificity of an experimental method of measuring adenosine deaminase activity in pleural fluid in a sample of pleural effusion patients evaluated between August 1998 and November 2002 in Rio de Janeiro.

**Results:** Of 137 cases, 111 pleural fluid samples were available. Of those, 83 were from pleural tuberculosis patients. Among the 67 pleural tuberculosis patients tested, 10 (14.9%) presented human immunodeficiency virus. The adenosine deaminase cutoff value of 35U/L was determined through analysis of a receiver operator characteristic curve. The sensitivity, specificity, positive likelihood ratio and negative likelihood ratio were 92.8%, 93.3%, 25.8 and 13.9, respectively. Mean adenosine deaminase in the pleural tuberculosis group was  $84.7 \pm 43.1$  U/L, versus  $15.9 \pm 11.1$  U/L in the group with other diseases. There was no significant difference in adenosine deaminase activity between patients with and without human immunodeficiency virus co-infection.

**Conclusion:** Determination of adenosine deaminase levels in pleural fluid is a sensitive and specific method for the diagnosis of pleural tuberculosis and its use can preclude the need for pleural biopsy in the initial workup of pleural effusion patients. An adenosine deaminase cutoff value of 35U/L is recommended.

*J Bras Pneumol 2004; 30(6)*

**Key Words:** Pleural fluid adenosine deaminase detection for the diagnosis of pleural tuberculosis

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\* Study carried out at and sponsored by Gama Filho University. Jointly sponsored by FAPERJ E-26/170.647/2001.  
Correspondence to: Anete Trajman. Departamento de Medicina. Universidade Gama Filho. Rua Manoel Vitorino 553, Piedade, CEP 20748-900 – Rio de Janeiro, RJ.  
Submitted: 4 March 2004. Accepted, after review: 5 September 2004.

## INTRODUCTION

Tuberculosis (TB) is the leading cause of death from infectious diseases worldwide. Brazil belongs to the group of 22 countries that account for 80% of all cases, and it is estimated that there are approximately 100,000 new cases per year in the country<sup>(1,2)</sup>. Pleural TB is one of the most common extrapulmonary manifestations of the disease and may represent up to 10% of all cases<sup>(3,4)</sup>.

The diagnosis of pleural TB continues to be a challenge in clinical practice. Traditional diagnostic methods are very useful for the diagnosis of pulmonary TB but have a low yield when applied to pleural fluid. Direct analysis of pleural fluid for detection of acid-fast bacilli (AFB) by the Ziehl-Neelsen or similar method is positive in less than 50% of cases, and the culture on Löwenstein-Jensen medium does not surpass a 40% positivity rate<sup>(5)</sup>. In Brazil, detection of granulomas by histopathological examination of samples obtained through biopsy of the parietal pleura is considered to be the best method for the diagnosis of pleural TB<sup>(5)</sup>, with a positivity of approximately 80%<sup>(6)</sup>. However, pleural biopsy increases the risk of complications from thoracentesis, principally in children and immunodepressed patients. In addition, it increases the cost of patient care since it presupposes the presence of a physician who is trained to perform the procedure and an appropriate facility for its performance, as well as a pathological anatomy laboratory and an experienced pathologist who can interpret the findings.

Approximately 20% of all cases of pleural effusion go undiagnosed. In such cases, treatment for TB is adopted based solely on clinical criteria. In countries with high incidence of TB, such as Brazil, the clinical profile of pleural TB has a high positive predictive value. Nevertheless, in countries where the prevalence of TB is lower, there is higher risk of incorrect long-term use of potentially toxic pharmaceuticals<sup>(7)</sup>.

Recognition of the difficulty in diagnosing pleural TB led to a search for methods that would optimize the workup of pleural effusion patients with suspected TB. Of note among these new techniques are those, such as polymerase chain reaction, that identify *Mycobacterium tuberculosis* DNA by amplifying known segments of its genome, as well as those that detect chemical markers, such

as interferon- $\gamma$  and adenosine deaminase (ADA), that are produced during the inflammatory process triggered by the *M. tuberculosis*.

The generic designation ADA is given to a group of enzymes of different molecular weights that have similar chemical functions in the purine metabolism, catalyzing the conversion of adenosine and deoxyadenosine into inosine and deoxyinosine<sup>(8)</sup>. These ADAs are present in almost all invertebrates, and, in humans, are found principally in the lymphocytes, being directed related to lymphocyte activation. As a result, in diseases presenting greater lymphocyte participation, elevated levels of ADA are usually detectable.

Several authors have demonstrated ADA sensitivity and specificity for the diagnosis of pleural TB<sup>(9-13)</sup>. However, in other diseases that cause pleural effusion with a predominance of lymphocytes, such as systemic lupus erythematosus and lymphoma, high levels of this enzyme may also be seen<sup>(14-18)</sup>. The specificity of the examination increases when ADA-2 fraction is determined<sup>(19)</sup>. This isoenzyme is only produced by macrophages. However, when comparing the results of the tests for ADA-2 to those for total ADA, no significant difference is observed. Determination of ADA-2 increases the cost of the test without offering any significant gain in sensitivity in relation to determination of total ADA<sup>(20)</sup>.

Although it is a very sensitive method for the diagnosis of pleural TB, a universal ADA cutoff value for pleural fluid, which may be adopted as reference, has yet to be established since results vary widely from country to country and even among different health facilities within a given region<sup>(21)</sup>. Therefore, the establishment of an ADA cutoff value for the diagnostic test of pleural TB should be regionalized due to different prevalences of TB and other diseases yet to be elucidated among pleural effusion patients. In Brazil, the Brazilian Society of Pulmonology and Phthisiology recommends a value of 40 units per liter (U/L)<sup>(22)</sup>. Another difficulty is standardizing the method. Despite the fact that the colorimetric method described by Giusti<sup>(23)</sup> has been available for at least two decades, that other methods for determination of ADA levels in pleural fluid have been described, and that determination of ADA levels in pleural

fluid is recommended by the Brazilian Society of Pulmonology and Phthisiology, there is no reliable industrial kit available<sup>(24)</sup>. The favorable results that have been observed so far refer to ADA determination through the Giusti technique in reference or research laboratories (experimental or "in-house" ADA testing).

The objective of the present study was to validate the usefulness of determining ADA levels in pleural fluid in the diagnosis of pleural TB at the *Hospital Universitário Clementino Fraga Filho* (HUCFF, Clementino Fraga Filho University Hospital) of the *Universidade Federal do Rio de Janeiro* (UFRJ, Federal University of Rio de Janeiro). The method adopted and standardized in the HUCFF biochemistry laboratory was used in a group of patients who were evaluated prospectively at the *Hospital Geral da Santa Casa da Misericórdia do Rio de Janeiro* (Santa Casa da Misericórdia General Hospital of Rio de Janeiro – hereafter referred to as "Santa Casa General Hospital").

## METHODS

Between August 1998 and November 2002, pleural effusion in-patients at the Santa Casa General Hospital underwent thoracentesis and Cope needle biopsies of the parietal pleura. The tests were carried out by one of the three trained pulmonologists of the Seventh Ward team, in compliance with the investigation protocol. The pleural fluid was analyzed in terms of glucose, protein, albumin, amylase, lactate dehydrogenase, cholesterol and pH levels, Gram stain, culture for the detection of nonspecific germs, general and specific cytometry, and cytopathologic examination (data not available). Stain for AFB and culture on Löwenstein-Jensen medium were also performed. On average, four pleural samples were obtained: three were sent for histopathological examination and one for culture on Löwenstein-Jensen medium. An aliquot of the pleural fluid was set aside for ADA determination. On the day of thoracentesis, a blood sample was collected in order to measure protein, glucose, lactate dehydrogenase, amylase and cholesterol levels (data not available). Samples of spontaneous or induced sputum were collected for detection of AFB and culture on Löwenstein-Jensen medium.

A diagnosis of pleural TB was made in two situations. Situation one was when the patient

fulfilled at least one of the following criteria: detection of AFB in pleural fluid or in spontaneous/induced sputum; positive culture for *M. tuberculosis* in pleural fluid or biopsy sample; and detection of a granuloma in the pleural biopsy sample (confirmed diagnosis). Situation two was when the patient presented a clinical profile that included evening fever and night sweats for at least three weeks, in combination with exudative pleural effusion (3 g/dL of protein in the pleural fluid or a ratio of protein in pleural fluid/protein in serum of more than 0.5) with lymphocyte predominance (over 50% of mononuclear cells)<sup>(25)</sup>, and was satisfactorily responsive to treatment, although all the other exams were negative (presumptive clinical diagnosis). Patients were evaluated as to the presence of human immunodeficiency virus (HIV) antibodies by the ELISA method using two antigens in the same serum sample. The study was approved by the Ethics Committee of the Santa Casa General Hospital. All patients gave written informed consent prior to thoracentesis.

Determination of ADA levels was carried out in the UFRJ/HUCFF Clinical Pathology Laboratory using the Giusti method<sup>(26)</sup>. After centrifugation of the sample, 25 µL of the pleural fluid supernatant were placed into a test tube and 500 µL of an adenosine solution were added. The mixture was heated at 37°C for 60 minutes, and the reaction was then interrupted by the addition of a phenol-nitroprusside solution and a hypochlorite solution. The resulting solution was subsequently heated at 37°C for 30 minutes. The reading of the amount of ammonia liberated by ADA action was performed with the aid of a spectrophotometer at a wavelength of 620 nm. Each series of tests was carried out under a reaction control and a negative control for each sample. In addition, the reading was taken by technicians who were blinded as to the origin of the pleural fluid samples (from which group of patients). The readings were converted to U/L in order to make the statistical calculations. The ADA sensitivity and specificity were determined by using the final diagnosis of pleural TB (confirmed or not) as the gold standard.

The results were stored using the SPSS 10.0 program (SPSS Inc., Chicago, IL, USA), and a receiver operator characteristic (ROC) curve was used for determining the ideal cutoff value for the test. Mean ADA values in the different groups were

compared using the Student's *t*-test. The McNemar test was used to compare concordance among the tests. A *p* value < 0.05 was considered significant.

## RESULTS

Between August 1998 and November 2002, 137 consecutive pleural effusion in-patients were evaluated. Among those 137, pleural fluid samples were available for determination of ADA levels in 115 cases. In 2 cases, it was not possible to make a diagnosis, and the patients were excluded from the analysis. The 2 cases of empyema in which ADA levels were determined were also excluded. The remaining 111 cases comprised 91 men (82%) and 20 women, and the age mean was 44 years (range, 15 to 85). A final diagnosis of pleural TB was made in 83 patients (74.7%) and was confirmed by at least one of the laboratorial methods described above in 65 of the 83 (78.3%), whereas the diagnosis was made clinically in the 18 remaining cases.

Serology for HIV was performed in 83 patients and was positive in 10 (14.9%) of the 67 pleural TB patients tested. None of the patients tested who were diagnosed with other diseases presented HIV. Table 1 shows the sensitivity of the diagnostic examinations. The best sensitivity (77.5%) was obtained through histopathological examination of pleural samples.

Of the 28 remaining cases, 10 were diagnosed as metastatic lung cancer, 5 as congestive heart failure, 3 as lymphoma, 2 as parapneumonic effusions, 2 as liver cirrhosis, 2 as chronic renal failure, 1 as systemic lupus erythematosus, 1 as

traumatic hemothorax, 1 as metastatic breast cancer and 1 as metastatic ovarian cancer.

Through analysis of a ROC curve, the ideal ADA cutoff value determined to be 35 U/L (Figure 1). Based on this cutoff value, ADA sensitivity and specificity were 92.8% and 96.4%, respectively. The area under the curve was 0.977.

The positive likelihood ratio and the negative likelihood ratio were 25.8 and 13.4, respectively. Taking into account the cutoff value of 40 U/L recommended by the Brazilian Society of Pulmonology and Phthisiology<sup>(27)</sup>, the sensitivity of the method was 84.3% (specificity was unaffected), with a positive likelihood ratio and negative likelihood ratio of 23.4 and 6.1, respectively. Figure 2 shows the distribution of ADA levels within the groups.

Mean ADA in the group of pleural TB patients was  $84.7 \pm 43.1$  U/L, versus  $15.9 \pm 11.1$  U/L in the group with other diseases ( $p < 10^{-4}$ ). Mean ADA in the group of pleural TB patients without HIV co-infection was  $86.2 \pm 49.2$  U/L, whereas in the group of co-infected patients, the mean was  $75.6 \pm 17.9$  U/L ( $p = 0.45$ ).

The sensitivity of ADA determination in the group without HIV co-infection was 89.3%, versus 100% in the group with co-infection ( $p = 0.36$ ). In comparison with the sensitivity of the histopathological examination, ADA determination was significantly more sensitive (77.5% vs. 92.8%; McNemar:  $p = 0.0007$ ). In 15 of the 18 patients whose histopathological examination was inconclusive (83.3%), as well as in all of the patients with positive culture results, ADA

TABLE 1  
Sensitivity of the different tests in the diagnosis of 83 pleural tuberculosis patients in Rio de Janeiro from 1998 to 2002

Method	Number of exams performed		Positive (%)
	In a total of 111 patients (%)	In the 83 pleural TB patients (%)	
Sputum AFB	69 (62.2)	54 (64.2)	1 (1.9)
Sputum culture	30 (27)	24 (28.9)	4 (16.7)
Liquid AFB	99 (89.2)	74 (89.2)	1 (1.4)
Liquid culture	63 (56.8)	49 (59)	3 (6.1)
Sample culture	51 (45.9)	38 (45.8)	14 (36.8)
Histopathology	104 (93.7)	80 (96.4)	62 (77.5)
Clinical examination	111 (100)	83 (100)	76 (91.6)

TB: tuberculosis; AFB: acid-fast bacilli

determination was positive. Determination of ADA levels was consistent with the final diagnosis of pleural TB (McNemar:  $p = 0.22$ ).

The single false-positive result occurred in a patient who had chronic renal failure and persistent

pleural effusion. There were two cases with false-negative results among the patients with confirmed diagnosis of pleural TB: one with a granuloma in the pleural biopsy and one with a positive result for AFB in the pleural fluid. The remaining false-negative cases all occurred in patients with clinically diagnosed pleural TB.

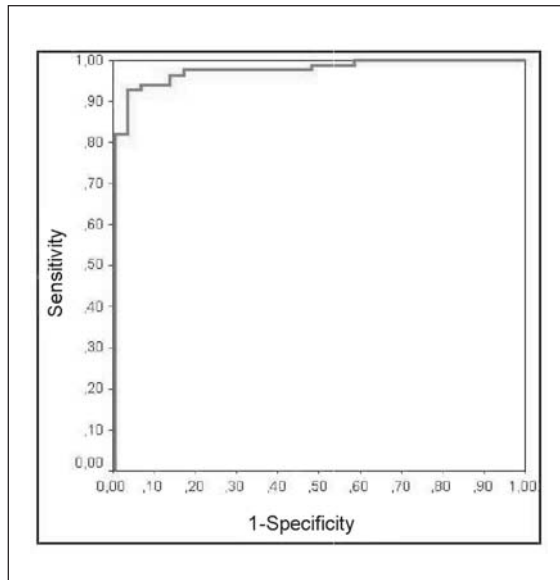


Figure 1 - ROC curve for determination of ADA levels in the pleural fluid of 111 patients with nonpyogenic pleural effusion evaluated in Rio de Janeiro from 1998 to 2002.

ROC: receiver operator characteristic; ADA: adenosine deaminase

## DISCUSSION

Since 1997, the Brazilian Society of Pulmonology and Phthisiology has recommended routine determination of ADA levels in pleural fluid and has recommended a cutoff value of 40 U/L for the diagnosis of TB<sup>(27)</sup>. However, determination of ADA levels is not performed in the majority of the public health facilities. In the present study, a cutoff value of 35 U/L elevated the sensitivity of the test without compromising its specificity.

For making a final diagnosis of pleural TB, the results obtained by determining ADA levels were similar to those of the sum of all the diagnostic criteria. Determination of ADA levels was more sensitive than histopathological examination of pleural tissue, which is the best test available for the diagnosis of pleural TB, and vastly superior to bacteriological tests. These results are similar to those of studies carried out in regions where there is high prevalence of TB. Those studies showed sensitivity to be between 68.8% and 100%, and specificity to be between 72.4% and 95%<sup>(26-29)</sup>.

Two recent studies, one of which was conducted in Brazil, reported low sensitivity for ADA determination<sup>(29,30)</sup>. However, both studies described samples of pleural TB cases in which only a fraction of the patients were definitively diagnosed through culture or histopathological examination. It is possible that some of the patients evaluated in these studies were not TB cases, since the values described in both articles are well below the range usually reported in the literature<sup>(31)</sup>. On the other hand, in a study of 416 adult pleural effusion patients carried out in the city of São Paulo, Fiuza de Melo et al. evaluated four different tests used in the diagnosis of pleural TB. In a multivariate analysis that also included age, determination of protein levels in pleural fluid and the total lymphocyte counts, ADA determination showed an impressive adjusted odds ratio of 186. The authors used ROC curve analysis to determine a cutoff value of 30 U/L, lower than that

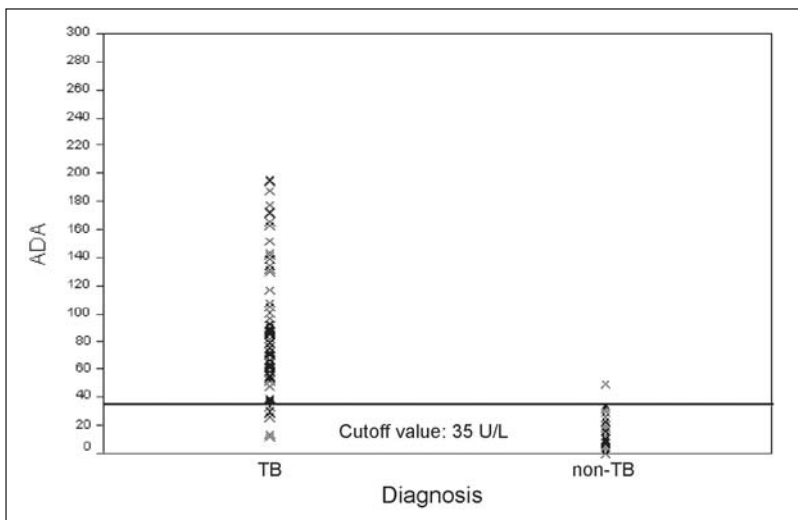


Figure 2 - Distribution of ADA levels by group in 111 patients with nonpyogenic pleural effusion evaluated in Rio de Janeiro from 1998 to 2002.

ADA: adenosine deaminase; TB: tuberculosis

recommended by the Brazilian Society of Pulmonology and Phthisiology and that determined for our sample, and concluded that, using this cutoff value, determination of ADA levels was the most useful test<sup>(26)</sup>. Since it may vary among samples and by region, an ideal cutoff value has yet to be established.

In our study, the specificity of ADA determination was high, with only one false-positive test: a patient on hemodialysis with chronic renal failure and persistent exudative right pleural effusion. In this case, none of the tests performed on the samples collected were conclusive. In the USA, Jarratt and Sahn<sup>(32)</sup> studied 100 patients on hemodialysis and found an incidence of pleural effusion of 21%, of which a significant part was exudates. In most cases, the effusion found was of cardiac origin, and, in the remaining cases, the most commonly found diagnoses were uremic pleurisy, atelectasis and pneumonia. No cases of pleural TB were found. In contrast, in a retrospective study conducted in Taiwan and involving 62 patients with chronic renal failure and TB, 15 of whom presented pleural effusion, 8 (12.9%) were diagnosed with pleural TB<sup>(33)</sup>. In the chronic renal failure case included in the present study, the patient was diagnosed with uremic pleural effusion, and TB was not detected in the two-month follow-up after thoracentesis.

In cases of nonspecific pleural empyema, an elevated level of ADA activity is a common finding, and the test is therefore considered of little use in the differential diagnosis of pleural effusion<sup>(34)</sup>. Since the clinical profile and the characteristics of the pleural fluid in empyema cases are quite distinct, their exclusion from the analysis does not affect the quality of the test or the results obtained in this study. Empyema caused by *M. tuberculosis* occurs only rarely<sup>(35)</sup>. In addition, co-infection of the pleural space by *M. tuberculosis* and nonspecific germs is also seen in practice, although this clinical condition had not been reported in the literature. In a recent study, conducted in South Africa, the clinical status of nine HIV-positive patients with pulmonary TB and community acquired pneumonia was evaluated and no pleural effusion was reported<sup>(36)</sup>.

Despite the small number of samples of the present study, we did not observe the significant difference in ADA levels between pleural TB patients

with and without HIV co-infection that has been described in the literature<sup>(37)</sup>. On the contrary, in the group of co-infected patients, the sensitivity of ADA determination was 100%, and the mean optical readings were similar in both groups. A larger number of patients would be needed for more definitive conclusions, but determination of ADA levels in pleural fluid for the diagnosis of pleural TB seems to be also useful in this group of patients.

The McNemar test demonstrated that, from a statistical viewpoint, ADA determination was more sensitive for the diagnosis of pleural TB than was histopathological examination ( $p < 0.0007$ ). Of the 18 pleural TB patients whose histopathological examinations were negative for granulomas, determination of ADA levels in pleural fluid identified 15 (83.3%). Routine use of ADA determination would have significantly reduced the number of cases that were treated on the basis on clinical data alone.

The present study has its limitations. Traditional diagnostic methods (sputum smear microscopy and culture) and ADA determination was not used in all patients, which may have impaired the analysis of the data due to selection bias. In addition, determination of ADA levels was not performed under routine conditions, that is, with no knowledge of the final diagnosis. However, the clinical samples were never selected for the tests, but used according to the availability of the material. Priority was always given to routine laboratorial examinations (biochemistry, cytometry, cytology and histopathology). Therefore, the volume of pleural fluid collected from some patients was, unintentionally, insufficient to perform the cultures and measure ADA activity.

Some patients, three with pleural TB, did not undergo pleural biopsy, and, as a result, no histopathological examination of pleural tissue was performed. This could impair the analysis of the results, since the latter is the best method available for the diagnosis of the disease. In all cases in which pleural biopsy was not performed, there was some clinical impediment, such as the severity of the case or a limited volume of pleural fluid. Pleural needle biopsy performed with little or no pleural fluid may double the risk of pneumothorax or other complications during thoracentesis (from less than 5% to approximately 11%)<sup>(38-40)</sup>.

The number of patients in the group with other pleural diseases (control group) was small. However, the formation of the patient groups reproduced typical conditions in everyday clinical practice. There was no case selection or patient recruitment. Patients were voluntarily referred to the Seventh Ward (from other health facilities in Rio de Janeiro) or sought treatment there spontaneously.

The number of women investigated in this study was relatively small, and the analysis of the capacity of ADA determination to confirm pleural TB diagnoses may therefore have been impaired. Some diseases, such as systemic lupus erythematosus, that present lymphocytic pleural effusion similar to that produced by pleural TB may have been underrepresented since they are more common in the female gender. Although there are no statistical data regarding the prevalence of lupus in Brazil, a recently published study of the prevalence of pleural TB among the pleural effusion cases treated in the city of Niterói (also in the state of Rio de Janeiro) reported a prevalence of systemic lupus erythematosus of 4%. As in our study, those authors found pleural TB to be the most diagnosed pleural disease, followed by neoplasia and transudates<sup>(41)</sup>.

As would be expected, the number of reported cases of TB (102.7/100,000 inhabitants, Rio de Janeiro, 2001) was higher than that of other diseases presenting pleural effusion. For example, in 1998, the prevalence of lung cancer was estimated at 39.2/100,000 inhabitants in São Paulo, and 54.9/100,000 inhabitants in the city of Porto Alegre, in the state of Rio Grande do Sul<sup>(43)</sup>. In view of these considerations, this sample may be considered representative of the population studied.

The selection bias may have occurred due to the fact that the sample studied was not randomly selected from the population. Nevertheless, diseases presenting pleural effusion lead patients to seek medical attention in hospitals since they usually cannot be treated in outpatient clinics. There is no reason to believe that the Santa Casa General Hospital would receive more pleural effusion patients than would other hospitals with the same patient care characteristics. As a result, the sample studied can be considered representative of the group of patients that seek treatment in public hospitals for the diagnosis and treatment of pleural effusion in the city of Rio de Janeiro.

In conclusion, determination of ADA levels in pleural fluid in the present study was a useful tool for the etiologic diagnosis of nonpyogenic exudative pleural effusion, even in patients with HIV co-infection. A cutoff value of 35 U/L is recommended. In Brazil, the routine use of ADA determination in pleural fluid for the diagnosis of pleural TB may significantly preclude the need for pleural biopsy in the initial workup of pleural effusion patients. The development and validation of an industrial diagnostic kit for different health facilities and regions of the country would facilitate the use of this test in the *Sistema Único de Saúde* (Unified Health System), thereby having a significant impact on TB control programs in Brazil.

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