Combined Use of the Polymerase Chain Reaction and Detection of Adenosine Deaminase Activity on Pleural Fluid Improves the Rate of Diagnosis of Pleural Tuberculosis*

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**Study objectives:** Evaluation of the combined use of polymerase chain reaction (PCR) and adenosine deaminase (ADA) activity on the diagnosis of pleural tuberculosis (pTB) in a region of high prevalence of tuberculosis.

**Patients:** PCR and determination of ADA activity were performed on the pleural fluid of every patient presenting with pleural effusion suspected to be associated with tuberculosis. The case definition of pTB involved parameters including the combination of clinical and radiologic findings; biochemical, microbiologic, and cytologic examination of the pleural fluid; and the histopathologic findings of pleural fragments obtained by biopsy. The diagnosis of pTB was confirmed in any patient presenting with positive culture findings of *Mycobacterium tuberculosis*, either on the pleural fluid or other biological material, or the presence of histopathologic findings suggestive of pTB on pleural biopsy, and also, in the absence of negative laboratory results, those patients with clinical improvement after empirical treatment.

**Results:** We studied 45 patients with pleural effusion. Of these, 16 patients met the diagnosis of pTB by our broad case definition. PCR findings were positive in six patients. The reaction was also positive in a patient whose diagnosis of tuberculosis could not be confirmed. ADA activity was considered positive in 11 patients with pTB. The combined use of PCR and ADA activity confirmed pTB in 14 patients; however, when analyzed in combination with the conventional methods, diagnosis of pTB was achieved in all 16 patients.

**Conclusion:** Our results show that, even in a highly endemic area, neither PCR nor ADA activity should be relied on as a single test that substitutes for the diagnostic methods already available, but rather they should be used as an extra tool in the diagnosis of pTB. The combined analysis of PCR and ADA activity, however, is a very useful diagnostic approach to achieve a more rapid and precise diagnosis in the cases of pTB.

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**Key words:** adenosine deaminase activity; diagnosis; *Mycobacterium tuberculosis*; pleural fluid; polymerase chain reaction; tuberculosis pleuritis

**Abbreviations:** ADA = adenosine deaminase; AFB = acid-fast bacilli; PCR = polymerase chain reaction; pTB = pleural tuberculosis

Tuberculosis is the single most frequent cause of death by an infectious agent worldwide.\(^1\) Among the extrapulmonary presentations, pleural tuberculosis (pTB) is second only to tuberculous lymphadenitis.\(^2\) The difficulty in determining the cause of a pleural effusion, which is shown by the “unknown etiology” rates of up to 20% in some published case series, is largely due to the great variety of diseases that can bring about this condition. The etiology of pleural effusions depends on the geographic region,
patient age, and advances in the diagnosis and treatment of the underlying cause.\textsuperscript{3} In the case of tuberculosis, the prevalence rates of a specific region is of particular importance, and the rapid and precise detection of \textit{Mycobacterium tuberculosis} may have a strong impact on the medical care of infected people, thus leading to an improvement of the treatment and control of the disease.\textsuperscript{4}

pTB diagnosis is usually accomplished through the analysis of clinical and radiologic findings; biochemical, microbiological, and cytologic examination of the pleural fluid; and the histopathology of pleural fragments obtained by biopsy. The laboratory diagnosis of tuberculosis is based on the Ziehl-Neelsen staining for acid-fast bacilli (AFB) and on the growth of the causative organism, \textit{M tuberculosis}. Ziehl-Neelsen staining is rapid and inexpensive but lacks sensitivity. The culture, although sensitive, takes a long period for positive results, and very often clinical and therapeutic decisions need to be made before the laboratory diagnosis becomes available.\textsuperscript{5} Even though several approaches are available for the diagnosis of tuberculosis, a method leading to early diagnosis is still warranted.

New approaches for the rapid detection of mycobacterial growth have been developed with the aim to reduce the time needed for diagnosis. Radiometric and nonradiometric systems have the advantage of detecting the presence of the bacilli in a much shorter time than the traditional Löwenstein-Jensen culture,\textsuperscript{6} contributing to the institution of specific treatment for tuberculosis. Other methods, such as measurement of adenosine deaminase (ADA) activity levels, have proven to be sensitive and specific for pTB in special circumstances, such as in regions with a high prevalence of tuberculosis,\textsuperscript{2} even though several other studies have not found that to be true in clinical practice.\textsuperscript{7} For these reasons, these methods have not been widely used for the diagnosis of tuberculosis.

The polymerase chain reaction (PCR) is a new strategy used for the tuberculosis diagnosis, and two kits have been approved by the US Food and Drug Administration for use on clinical samples; however, their cost is prohibitive for developing countries where tuberculosis remains an important public health problem. “Homemade PCR” might be a solution for these countries, but this test has not been approved for general use. PCR has been used to detect mycobacterial DNA in pleural fluid, with sensitivities ranging from 20 to 80\% and specificities of 78 to 100\%, depending on the area of the genome that is amplified and the technique used for DNA extraction.\textsuperscript{8} The high biological sensitivity and specificity of PCR for \textit{M tuberculosis} suggest that this method, when used in combination with measurement of ADA activity levels, could improve the efficiency of the laboratory diagnosis of pTB.\textsuperscript{2} Our results confirm that when used alone, PCR and ADA activity do not yield very good results, but when used in combination, they can be very useful as additional diagnostic methods for the achievement of a more rapid and precise diagnosis of pTB.

**Materials and Methods**

**Patients**

We analyzed 58 samples of 45 patients with pleural effusion suspected to be related to pTB that were attended at the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto. Their pleural effusions were analyzed in order to evaluate the characteristics and to determine the etiology. In order to be included in this study, patients had to present with clinical manifestations suggestive of tuberculosis, i.e., the presence of productive cough, low-grade fever, night sweats, weight loss, and chest pain, especially if these symptoms lasted \(\geq 4\) weeks. If the patients presented with less than two of these symptoms, and especially if the clinical manifestations were of \(< 4\) weeks in duration, they were excluded from the study. The study was conducted from March 2000 to July 2001, and was approved by the Ethics Committee of the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto.

**Case Definition of Tuberculosis**

Cases of pTB were defined as those patients with clinical manifestations suggestive of tuberculosis presenting with one of the following: a positive Ziehl-Neelsen result, a positive culture finding of the pleural fluid or other biological material, and/or histopathologic findings suggestive of tuberculosis on pleural biopsy. Patients with clinical and radiologic findings that lacked microbiological/histopathologic confirmation but responded positively to empirical therapy were also considered to be tuberculosis cases. We defined a positive response to therapy as the improvement of clinical and radiologic findings after 2 months of the therapy.

**DNA Extraction**

The DNA extraction method used in this study employed the Qiagen DNA kit (Qiagen; Santa Monica, CA). Samples were processed according to the instructions of the manufacturer. Briefly, 200 \(\mu\)L of the pleural fluid were added to an Eppendorf tube with 25 \(\mu\)L of proteinase K (20 \(\mu\)g/mL) and 200 \(\mu\)L of the lysis buffer, and incubated at 56°C for 10 min. After incubation, 210 \(\mu\)L of 100\% ethanol were added to the mix and centrifuged at 6,000g for 1 min. The mix was transferred to a silica column, centrifuged at 6,000g for 1 min, and the collection tube containing the filtrate was discarded. Five hundred microliters of the wash buffer were added to the column and centrifuged at 6,000g for 3 min, and again the collection tube containing the filtrate was discarded. The column was then placed in a centrifuge tube, and the DNA was eluted with 200 \(\mu\)L of the elution buffer after incubation at room temperature for 1 min. This was followed by centrifugation at 6,000g for another minute.

**PCR**

PCR was performed in a centrifuge tube containing 5 \(\mu\)L (approximately 220 ng) of total DNA, 0.1 mM of deoxynucleoside
triphosphates, 1 U of Taq DNA polymerase, and 50 pmol of each primer TB1 (5'-CTGGCCAGCTAGGCGTCGG-3') and TB2 (5'-CTCGTCACGGCTTCCG-3'). PCR was conducted according to the following protocol: 94°C for 1 min, followed by 65°C for 2 min and 72°C for 1 min. These cycles were repeated 35 times with a final step where the temperature was maintained at 72°C for 10 min, and the sample was then stored at 4°C. The specific amplicon for the *M. tuberculosis* has 123 base pairs, and it was detected on a 3% agarose gel electrophoresis, stained with ethidium bromide, and visualized by the UVP Vision Works software (UVP; Cambridge, UK). As positive and negative controls, extracted DNA from a reference strain H37Ra (American Type culture Collection; Manassas, VA) and phosphate-buffered saline solution, respectively, were used on the reactions.

**Histopathologic and Microbiologic Tests**

Ziehl-Neelsen staining and Löwenstein-Jensen culture were performed according to our hospital protocol. Histopathologic examination of pleural biopsies was conducted in a blinded fashion, without any interference by the authors.

**ADA**

ADA activity was determined in 20 μL of pleural fluid using the colorimetric method described by Giusti. A positive result was defined as a value > 40.0 U/L, which is based on previous studies of pleural fluid samples of patients with proven tuberculosis.

**RESULTS**

**Microbiologic Tests**

Ziehl-Neelsen staining results of the pleural fluid were negative in all 16 patients with pTB; however, AFB was detected in three patients (lymph node, sputum, and BAL samples). The culture finding was positive for *M. tuberculosis* in six patients, and five patients fulfilled the case definition of pTB (Table 1). The reaction was positive in a patient’s sample where the diagnosis of tuberculosis could not be confirmed due to patient’s death. If we considered this patient as a non-pTB patient, the PCR sensitivity was 31.3%, with a specificity of 96.6% (Table 2).

**Histopathologic Examination**

Plural biopsy was performed in 27 of the 45 patients. Of the 16 patients with pTB, plural biopsy was performed in 9 patients. Histopathologic findings suggestive of tuberculosis were found in five patients (Table 1). Sensitivity of pleural biopsy was 55.6%, and the specificity was 100%. This high specificity can be explained by the fact that we considered the suggestive results (granulomatous pleurisy) as indicative of tuberculosis, even though the presence of *M. tuberculosis* on Ziehl-Neelsen staining was never detected on the biopsy specimens.

**PCR**

PCR findings were positive for *M. tuberculosis* in six patients, and five patients fulfilled the case definition of pTB (Table 1). The reaction was positive in a patient’s sample where the diagnosis of tuberculosis could not be confirmed due to patient’s death. If we considered this patient as a non-pTB patient, the PCR sensitivity was 31.3%, with a specificity of 96.6% (Table 2).

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**Table 1—Results of 16 Patients With Tuberculosis Diagnosis by the Broad Case Definition**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Therapy</th>
<th>Culture</th>
<th>Zielh-Neelsen</th>
<th>ADA</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T</td>
<td>Negative</td>
<td>Positive</td>
<td>ND</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>T</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Positive</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>ND</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Positive</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>T</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Positive</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>T</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>Suggestive</td>
</tr>
<tr>
<td>11</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>ND</td>
<td>Suggestive</td>
</tr>
<tr>
<td>14</td>
<td>T</td>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>Suggestive</td>
</tr>
<tr>
<td>15</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
<td>ND</td>
</tr>
<tr>
<td>16</td>
<td>T</td>
<td>Negative</td>
<td>ND</td>
<td>Negative</td>
<td>ND</td>
</tr>
</tbody>
</table>

*PF = pleural fluid; OT = other biological materials (lymph node, sputum, BAL); ND = not done; T = tuberculosis therapy.*
ADA

The mean value of ADA activity levels in all 45 patients was 43.6 U/L. Among the patients with the diagnosis of tuberculosis, the mean ADA activity level was 53 U/L, while in the group of patients where the diagnosis of tuberculosis was ruled out, it was 37 U/L. Considering 40 U/L as a cutoff value, the test result was considered positive in 11 patients with tuberculosis; however, in the samples in which the tuberculosis diagnosis was ruled out, 8 patients presented activity levels > 40 U/L, which lowers the sensitivity (68.8%) and specificity (72.4%) of the test (Table 2).

<table>
<thead>
<tr>
<th>Patients</th>
<th>PCR Positive</th>
<th>ADA Positive</th>
<th>PCR Positive or ADA Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confirmed pTB, No.</td>
<td>5</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td>Sensitivity, %</td>
<td>31.3</td>
<td>68.8</td>
<td>87.5</td>
</tr>
<tr>
<td>Specificity, %</td>
<td>90.6</td>
<td>72.4</td>
<td>72.4</td>
</tr>
<tr>
<td>Positive predictive value, %</td>
<td>83.3</td>
<td>57.9</td>
<td>63.6</td>
</tr>
<tr>
<td>Negative predictive value, %</td>
<td>71.8</td>
<td>80.8</td>
<td>91.3</td>
</tr>
</tbody>
</table>

**Table 2—Sensitivity, Specificity, and Predictive Values of the Diagnostic Methods Used in the Study**

**Discussion**

Over the last decades, tuberculosis has been one of the main causes of death in the world, responsible for almost 3 million deaths annually. The use of PCR in the routine diagnosis of extrapulmonary tuberculosis requires further evaluation. This evaluation should consider all the peculiarities of this disease in a given region and compare it with the diagnostic methods available in the same region.

In this study, of the 16 patients who met the criteria for the diagnosis of pTB using the broad case definition, PCR findings were positive in 5 patients. The microscopic examination of the pleural fluid was negative in all of the samples, confirming its low diagnostic yield. The small number of positive results in the culture can be due to the difficulties of culturing *M tuberculosis*, since this technique is only capable of detecting *M tuberculosis* in samples containing 50 to 1,000 bacilli per milliliter. The low sensitivity of the culture in the pleural fluid was also demonstrated in a study done by Berger and Mejia, who verified that in the pleural fluid cultures, it is only possible to detect the presence of the *M tuberculosis* in 20 to 30% of infected patients; however, biopsy culture results were positive in 50 to 80% of the patients with the disease. Even though evaluation of pleural biopsies is considered a good approach for the diagnosis of pTB, in our study 27 patients underwent pleural biopsy during their diagnostic evaluation but none of them either grew or showed the presence of the *M tuberculosis*; however, the characteristic histopathologic findings (granulomas) were present in five biopsy samples, which helped in making the correct diagnosis.

ADA is an enzyme involved in purine catabolism. It is found in the majority of the cells, but particularly in lymphocytes, where its concentration is inversely related to the degree of differentiation. High levels of ADA have been found in patients with tuberculous pleurisy. It may take an important role on the diagnosis of pTB because the measurement of ADA activity levels is simple, quick, and inexpensive. In a study done by Reechaipichitkul et al, the pTB diagnosis by ADA activity levels in the pleural fluid had a sensitivity of 80% and specificity of 80.5%. They conclude that ADA levels are a useful test for diagnosing tuberculous pleural effusions.

In our study, ADA activity measurements also yielded good results in the diagnosis of pTB; however, ADA activity values may be increased due to other clinical entities. We detected an ADA activity value of 117 U/L in a patient with pleural lymphoma, a well-known condition associated with high concentrations of this enzyme in the pleural fluid. Other authors have also reported high levels of ADA in patients with other causes of pleural effusions (mainly lymphomas, adenocarcinomas, systemic lupus erythematosus, and pneumonia).

The ADA cutoff value indicative of tuberculosis is subject to debate, since the literature presents a great variation of these values, ranging from 30 to 50 U/L; higher cutoff values (70 U/L) have been described by Bañales et al. It is difficult to define a universal cutoff value for ADA activity. This test has to be validated for each region, and eventually for every service where the test is to be used. We had previously standardized this test in our laboratory and concluded that the best cutoff value was 40U/L.

The PCR protocol used in this study was based on the amplification of a portion of the *M tuberculosis* genome located in the *IS6110* insertion sequence. Thierry and collaborators described that this sequence is repeated approximately 20 times in the genome of the mycobacteria, and it is specific for the *M tuberculosis* complex. In our study, the sensitivity of PCR was 31.3% and specificity was 96.6%. These data are in agreement with those obtained by De Wit et al, Folgueira et al, Pao et al, and Shankar et al, in that the PCR sensitivity ranged from 13 to 100% and specificity ranged from 88 to 100%. Pierre et al and Soini et al report low sensitivities of 63% and specificity of 55.9%, respectively, for their PCR in sputum samples, a specimen that contains a much higher concentration of *M tuberculosis* than pleural fluid.
fluid. Our results suggest a low PCR sensitivity compared to ADA, but PCR specificity was much better than any other technique used in this study; however, PCR results vary significantly according to the material studied, as well as with the extraction method used.

The fact that the pleural fluid is paucibacillary can partially explain the small number of positive results observed in this study. Studies have shown that PCR also has a low sensitivity when using samples sharing the same characteristics as pleural fluids (cerebrospinal fluid, ascitic fluid, etc).

In a study done by Shankar et al the sensitivity in cerebrospinal fluid ranged from 43 to 75%, while in a series described by Pierre et al using gastric aspirate, the sensitivity was between 60% and 100% and the specificity around 100%. In more recent study done by Nagesh et al using pleural fluid, PCR has a sensitivity of 70% and specificity of 100%.

In this study, PCR findings were positive in a sample in which the tuberculosis diagnosis could not be confirmed. The patient had been admitted to the hospital with a diagnosis of neoplastic involvement of the pleura; however, this patient had a history of tuberculosis 20 years previously, and we cannot exclude the possibility of reactivation of the disease, perhaps due to immunodeficiency associated with this malignancy. A possibility of cross contamination must be considered due to sensitivity of PCR; however, in our experiments, we have always used assay conditions designed to prevent any contamination. Furthermore, all samples were tested twice and the results were reproducible. Another explanation is the detection of a contaminant M tuberculosis by PCR due to its ability of detecting low copy numbers of DNA.

Finally, even with a broad definition of a tuberculosis case, this patient may represent an exception in our case definition since we did not have enough time to make the diagnosis due to the death of the patient. Furthermore, patient 9 (Table 1) had a diagnosis of chronic myeloid leukemia and presented with pneumonia in the right lower lobe and evidence of hepatic involvement. The etiologic investigation presented negative results in the pleural fluid for all of the techniques used here; however, this patient was considered to have tuberculosis because he had a positive Ziehl-Neelsen staining in BAL. This patient was started on antituberculosis chemotherapy but died 18 days after beginning treatment. The cause of death was cardiac tamponade, a clinical situation where tuberculosis could not be ruled out, but that could also be the result of the tumor infiltration or adverse effects of antituberculosis chemotherapy. However, the BAL sample resulting in positive AFB findings was not evaluated by PCR, a situation that could also result in the detection of M tuberculosis.

Another possible explanation for the low PCR sensitivity is the presence of Taq polymerase inhibitors on pleural fluid. This may have occurred in our study, since 13 samples of 10 patients with pTB were hemorrhagic, and 4 samples of 3 patients were purulent. Of the hemorrhagic samples, two patients had the tuberculosis diagnosis according to our case definition. Of the purulent samples, the culture finding was positive in three of them, all from patients considered as tuberculosis cases. All of these samples were negative by PCR, strongly suggesting the presence of an inhibitor of the reaction, since it is known that purulent material contains nucleases that degrades DNA, and hemorrhagic samples are associated with low amplification rates. Substances, such as heparin, were found to inhibit the activity of the both murine leukemia virus reverse transcriptase and Taq DNA polymerase. Another substance that can inhibit PCR is hemoglobin, due to the binding of heme and/or porphyrin to Taq DNA polymerase.

Our results show that, even in a highly endemic region, neither PCR nor ADA activity should be relied on as a test that will substitute for diagnostic methods already available, but as an extra tool for the diagnosis of pTB. Furthermore, the combined analysis of PCR and ADA activity is a useful diagnostic approach to achieve a more rapid and precise diagnosis in cases of pTB (Table 2).

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REFERENCES

6 Grange JM. The rapid diagnosis of paucibacillary tuberculosis. Tuber Lung Dis 1989; 70:1–4

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