Polymerase Chain Reaction of Pleural Biopsy Is a Rapid and Sensitive Method for the Diagnosis of Tuberculous Pleural Effusion*

Nadia A. Hasaneen, MD, PhD; Maysaa E. Zaki, MD, PhD; Hala M. Shalaby, MD; and Ahmad S. El-Morsi, MD, PhD

Background: Tuberculous pleural effusion occurs in 30% of patients with tuberculosis (TB). Rapid diagnosis of a tuberculous pleural effusion would greatly facilitate the management of many patients. Polymerase chain reaction (PCR) has been used to detect Mycobacterium tuberculosis in pleural fluid with highly variable sensitivity.

Objective: To improve our laboratory diagnosis of tuberculous pleural effusion.

Methods: We applied PCR to detect DNA specific for M tuberculosis in 33 of the studied pleural biopsy specimens using an IS986-based primer that was specific for mycobacterium complex, and compared it to the results of pleural fluid and biopsy cultures performed on either Lowenstein-Jensen (LJ) medium or BACTEC 12B liquid medium (Becton Dickinson Microbiology Systems; Cockeysville, MD), Ziehl-Neelsen (ZN) staining, and histopathology in 45 patients with pleural effusion.

Results: Of the 45 patients with pleural effusion who were studied, 26 patients received diagnoses of tuberculous pleural effusion that had been confirmed by either culture and or histopathology, 10 patients received diagnoses of exudative effusion due to causes other than TB, and 9 patients received diagnoses of transudative effusion. Histopathology of the pleural biopsy specimen had a sensitivity of 53.8%. The sensitivity of the ZN staining of pleural fluid and biopsy specimens was 0.0% and 3.8%, respectively. The sensitivity of the culture on both BACTEC 12B liquid medium and LJ medium was higher in pleural biopsy specimens (92.3%) than in pleural fluid specimens (15.4%; p > 0.001). The improvements of the BACTEC culture system improved and shortened the detection time of M tuberculosis in pleural biopsy specimens. PCR of pleural biopsy specimens had 90% sensitivity and 100% specificity. The positive predictive value and the negative predictive value for pleural biopsy specimen cultures were 100% and 90.5% vs 100% and 86.7% for pleural biopsy specimen PCRs.

Conclusion: The overall accuracy of PCR of pleural biopsy was similar to the results of pleural biopsy culture, however, PCR of the pleural biopsy was much faster in reaching diagnosis. PCR of pleural biopsy is a useful method when used in combination with the BACTEC culture system and histopathologic examination of pleural biopsy to reach a rapid diagnosis of tuberculous pleural effusion.

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Key words: BACTEC system culture; pleural biopsy; pleural effusion; polymerase chain reaction; tuberculosis pleural effusion

Abbreviations: AFB = acid-fast bacilli; bp = base pair; LJ = Lowenstein-Jensen; NAP = P-nitro-acetylamin0 α-hydroxy-proionophenone; NPV = negative predictive value; PCR = polymerase chain reaction; PPV = positive predictive value; TB = tuberculosis; ZN = Ziehl-Neelsen

Tuberculosis (TB) is a major health problem especially in developing countries. The most effective approach for controlling the disease is accurate diagnosis and treatment of active TB cases.1 Tuberculous pleural effusion occurs in up to 30% of patients with TB2 and constitutes the major portion of the extrapulmonary TB morbidity.3 Diagnosis of
tuberculous pleural effusion depends on the demonstration of the presence of tubercle bacilli in the sputum, pleural fluid, or pleural biopsy specimen, or the demonstration of granuloma in the pleura by histopathologic examination. In many cases, the findings of the histopathologic examination of the pleural biopsy specimen may be negative or nonspecific. Microbiological methods for diagnosing tuberculous pleural effusion include Ziehl-Neelsen (ZN) acid-fast bacilli (AFB) stain and laboratory culture of the causative organism Mycobacterium tuberculosis. The detection of mycobacterium in pleural fluid by microscopy has low sensitivity and cannot be used to distinguish between the various members of the mycobacterium genus. On the other hand, a laboratory culture of M. tuberculosis is sensitive, but it takes up to 8 weeks to yield a positive result. The radiometric BACTEC 460 TB system (Becton Dickinson Diagnostic Instrument System; Sparks, MD) has been an important improvement in culture methods. The use of this method has reduced the average detection time of both smear-positive and smear-negative samples by nearly 50%. The BACTEC 460 TB system also has been adopted to differentiate M. tuberculosis from other mycobacteria as well as to perform antimicrobial susceptibility testing, particularly for M. tuberculosis. Diagnostic assays based on nucleic acid amplification methods such as polymerase chain reaction (PCR) dramatically decrease the time required to identify an organism in clinical specimens. This method has been tested extensively for the detection of M. tuberculosis in sputum specimens, and pericardial fluid and biopsy specimens. PCR also has been used to detect M. tuberculosis in pleural fluid samples, with highly variable sensitivity (11 to 81%) in different studies. Comparatively little work has focused on the utility of PCR and the BACTEC 460 TB system in detecting M. tuberculosis in pleural biopsy specimens.

In this study, we examined whether PCR of pleural biopsy specimens would be superior to that of pleural fluid in detecting M. tuberculosis. We applied PCR to detect DNA (IS986) specific for M. tuberculosis complex in pleural biopsy specimens and compared the results to those of pleural fluid and biopsy cultures using the BACTEC system, ZN examination, and histopathology.

Materials and Methods

The present study comprised 45 patients with pleural effusion, who had been admitted to the Thoracic Medicine Department, Mansoura University, Egypt, between February and December 2000.

Study Design

The study was performed prospectively in a blinded manner in which the clinical diagnosis was not available to the laboratory personnel. Informed consent was received from each patient involved in the study. This study was approved by Human Subject Research Committee. Each patient underwent the following procedures:

1. Mantoux tuberculin skin test, in which an induration of >10 mm was considered to be positive;
2. Radiologic examination including the following:
   a. Chest radiographs in the posteroanterior and lateral views;
   b. Ultrasound of the pleural space to identify the appropriate site for thoracentesis; and
   c. CT scan to determine the presence of any parenchymal lesion underlying the effusion;
3. Laboratory investigation including CBC, blood sugar test, liver function, and kidney function;
4. Sputum examination, including Gram and ZN staining, and culture; and
5. Diagnostic thoracentesis and pleural biopsy using the Abram needle.

Methods

Processing of Pleural Fluid and Biopsy Samples: One hundred milliliters of the pleural fluid was aspirated and divided into two parts, one for cytology examination and the other for microbiology studies. Pleural fluid samples sent to the Microbiology Department were subjected to decontamination and then were concentrated by centrifugation for 20 min at 10,000g at 4°C. Then the sediments were resuspended in sterile normal saline solution, and used for ZN smear and culture. The pleural biopsy was performed using Abram needles, and 4 to 5 punches of parietal pleura were divided equally into two parts. One part of the specimen was fixed in formalin for histopathologic examination, while the other part was put into sterile normal saline solution, then homogenized and used for ZN smear, culture, and PCR studies.

Laboratory Methods

ZN Smear of Pleural Fluid and Biopsy: Smears of pleural fluid sediments or pleural biopsy specimens were stained by ZN stain according to the method of Jenkins. The slide then was examined under an oil immersion lens for the presence of AFB.

Loewenstein-Jensen Culture of Pleural Fluid and Biopsy Specimen: Ready to use bottles of Loewenstein-Jensen (LJ) medium (Hispan Lab; Medicopharma Co; Madrid, Spain) were inoculated with 0.1 mL concentrated pleural fluid or biopsy specimen and were kept in a CO2 incubator for 8 weeks. Bottles were inspected twice a week for visible colonies, and suspicious growth was subjected to ZN staining. Negative culture was discarded after 8 weeks. Resulting growth was left in light for 2 h and was examined for yellow pigments to identify photochromogen species.

Pleural Fluid and Biopsy Culture Using Radiometric BACTEC 460 System: The BACTEC 460 system and BACTEC 12B vials (Becton Dickinson Microbiology Systems; Cockeysville, MD) were used for radiometric culturing of M. tuberculosis. BACTEC 12B vials were prepared by the addition of the antimicrobial agent PANTA, which contains polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin. One half milliliter concentrated pleural fluid or biopsy specimen was inoculated into each BACTEC 12B vial, was kept at 37°C, and then was tested.
three times a week, with vials showing a growth index of ≥ 10 being considered positive and tested daily.

**BACTEC Differentiation Test:** When the inoculated BACTEC 12 B vials showed a growth index of 50 to 100, a Gram stain and subculture were performed to make sure that the culture was not contaminated, then the culture was homogenized with a tuberculin syringe, and 1 mL was transferred to the vial containing P-nitro-acetylamino-α-hydroxy-proionophenone (NAP). The NAP vial and the control vial (ie, the original culture) were tested daily with the BACTEC instrument for the next 2 to 6 days. A decreased or unchanging GI in the NAP vials indicated the presence of a mycobacterium other than TB.

**PCR of Pleural Biopsy Specimen:** PCR was used for the detection of DNA (IS986) specific for the mycobacterium complex on 33 of the studied pleural biopsy samples. The size of the amplification product was 123 base pairs (bp). A PCR reaction was performed using an M tuberculosis kit (code H.02; Expertteam; Venice, Italy) according to the manufacturer’s instructions.

**DNA Extraction:** Pleural biopsy specimens (2 mm in diameter, and 2 to 3 punches per patient) were homogenized in normal saline solution using a sterile wide-gauge needle for each patient sample to avoid cross-contamination. Pleural biopsy homogenates were centrifuged at 5,000 g for 10 min, and the pellet was resuspended in 162 μL lysis buffer. Eighteen microliters each lysozyme and mutanolysin were added to the pellet, which then was incubated at 37°C for 1 h. Then, after 20 μL proteinase K and 200 μL buffer alkaline lyses were added to the pellet and incubated at 56°C for 30 min, and for a further 30 min at 95°C. Later, DNA was extracted from the pellet with mini spin columns (QIAamp; Qiagen; Valencia, CA). To monitor for cross-contamination, one water-containing negative control tube was used per five sample tubes.

**PCR:** A typical PCR reaction mixture contained 2.5 μL 10 × buffer, 2.5 μL deoxynucleoside triphosphates, and 1 μL each primer BMT002 (CCTGCGAGCGTAGGCGTCGG) and BMT003 (CTCGTCCAGGCCCCGCTTCCG), to which 0.3 μL Taq DNA polymerase, 7.7 μL distilled water, 10 μL DNA solution extracted with QIAamp columns from each pleural biopsy sample, and 25 μL mineral oil were added. Each set of the PCR reaction contained a positive control containing DNA extracted from TB bacilli that had been isolated in our laboratory and two negative controls, one containing the same amount of DNA extracted from the lymphocytes of healthy individuals and the other containing the same amount of distilled water. The thermal cycle was programmed for 5 min at 94°C for 30 cycles, denaturation at 94°C for 2 min, annealing at 68°C for 2 min, and extension at 72°C for 2 min. The amplified products were detected by gel electrophoresis using 2% agarose gel with ethidium bromide, and a 123-bp amplified band was visualized on an ultraviolet transilluminator.

### Statistical Analysis

The results of clinical evaluation and diagnostic tests were analyzed using computer software (SPSS, version 10; SPSS; Chicago, IL). The results of ZN examination, culture, histopathology, and PCR were compared to the final diagnosis of TB with individual patients used as the unit of analysis. The data were presented as percentages and compared using the χ² test, with a p value of < 0.05 considered to be significant. Culture for AFB and histopathologic examination for caseating granuloma were used as the “gold standard” tests.

### Results

The study included 45 patients (27 men and 18 women) with a mean (± SD) age of 46 ± 2.30 years. None of our patients were tested for HIV, as HIV infection is uncommon in our setting. The patients were categorized into the following three groups.

**Group 1: Tuberculous Pleural Effusion:** This group included 26 patients (17 men and 9 women) with a mean age of 43.15 ± 2.8 years. Diagnosis of pleural TB was confirmed by culture and/or histopathology of pleural biopsy specimens, supported by laboratory, radiographic, and clinical data. Eight of 26 patients (30.8%) in this group presented with left-sided pleural effusion, 16 of 26 patients (61.5%) presented with right-sided pleural effusion, and 2 of 26 patients (7.7%) presented with bilateral pleural effusion. There was no underlying parenchymal infiltration, cavitation, or lymphadenopathy.

**Group 2: Exudative Pleural Effusion:** This group included 10 patients (six men and four women) with a mean age of 47.3 ± 5.96 years. Within this group, malignant pleural effusion was diagnosed in seven patients (small cell carcinoma, two patients; lymphoma, one patient; mucoepidermoid, one patient; adenocarcinoma, two patients; and undifferentiated carcinoma, one patient), systemic lupus erythematosus (SLE) was diagnosed in two patients, and parapneumonic effusion was diagnosed in one patient. Five of 10 patients (50%) presented with left-sided pleural effusion, 2 of 10 patients (20%) presented with right-sided pleural effusion, and 3 of 10 patients (30%) presented with bilateral effusion. Five of 10 patients (50%) had underlying parenchymal infiltrates or lymphadenopathy but no cavitation.

**Group 3: Transudative Pleural Effusion:** This group included nine patients (four men and five women) with a mean age of 52.78 ± 5.37 years. Bilharzial liver cirrhosis was diagnosed in five patients, and congestive heart failure was diagnosed in four patients. Eight of nine patients (88.9%) presented with right-sided pleural effusion, and one of nine patients (11.1%) presented with left-sided pleural effusion. There was no underlying parenchymal infiltration, lymphadenopathy, or cavitation.

**Tuberculin Skin Test**

Tuberculin skin test results were positive in 19 of 26 patients (73.1%) in the tuberculous pleural effusion group and were negative in all other patients having exudative and transudative pleural effusion.
ZN Staining of Sputum, Pleural Fluid, and Biopsy Specimen

ZN staining results of sputum and pleural fluid were negative in all three groups. ZN staining results of pleural biopsy specimens were positive in only 1 of 26 patients (3.8%) in the tuberculous pleural effusion group, and were negative in both the exudative and transudative pleural effusion groups (Tables 1 and 2).

Culture of Sputum, Pleural Fluid, and Biopsy Specimens on Either LJ Medium or BACTEC System

Sputum culture on LJ medium showed no mycobacterial growth. A culture of pleural fluid on both LJ medium and BACTEC 12B medium showed the growth of TB bacilli in 4 of 26 patients (15.4%) in the tuberculous pleural effusion group. A pleural biopsy specimen culture using BACTEC culture system showed a higher detection rate of *M. tuberculosis* (24 of 26 patients; 92.3%) than did a culture on LJ medium (22 of 26 patients; 84.6%) in the tuberculous pleural effusion group. Pleural fluid and biopsy specimen cultures showed no mycobacterium growth in nontuberculous pleural effusions. A pleural biopsy culture was negative in two cases of tuberculous effusion, in which the diagnosis was confirmed by histopathology showing caseating granuloma (Tables 1 and 2). The mean detection time of *M. tuberculosis* was 13 days for BACTEC 12B medium vs 28 days for LJ medium.

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<th>Patients With Tuberculous Pleural Effusion</th>
<th>Patients Without Tuberculous Pleural Effusion</th>
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<tr>
<td><strong>Method</strong></td>
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<tr>
<td>ZN examination‡</td>
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<tr>
<td>LJ culture†</td>
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<tr>
<td>Pleural biopsy</td>
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<td>BACTEC culture†</td>
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<td>PCR of pleural biopsy</td>
<td>18/20 (90)</td>
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*Values given as No. of specimens positive for TB/No. of specimens tested (%). ‡p < 0.05 for pleural fluid specimen vs pleural biopsy specimen.

PCR Detection of TB Bacilli in Pleural Biopsy Specimen

PCR was performed in only 33 of the studied pleural biopsy specimens (tuberculous pleural effusion, 20 patients; exudative effusion, 7 patients; and transudative effusion, 6 patients). The PCR result was positive in 18 of 20 patients in the tuberculous pleural effusion group, and in none of the patients in the transudate and exudate pleural effusion groups (Fig 1). PCR had a false-negative result in two patients with tuberculous effusion in whom the diagnosis of TB was confirmed by histopathology showing caseating granuloma and by the response of these patients to antituberculous drugs. Based on the results of pleural biopsy specimen culture, the PCR of the pleural biopsy specimen had 100% sensitivity, but, based on the results of combined culture and histopathology, it had 90% sensitivity, 100% specificity, 93.3% accuracy, 100% positive predictive value (PPV), and 86.6% negative predictive value (NPV) [Table 2].

Histopathology of Pleural Biopsy Specimen

Histopathologic examinations of the pleural biopsy specimens showed a caseating granuloma with central necrosis in 14 of 26 patients (53.8%) with tuberculous pleural effusions. In the exudative pleural effusion group, histopathology was diagnostic of malignant effusion in seven patients and showed nonspecific pleurisy in three patients (systemic lupus erythematosus was diagnosed in two patients, and parapneumonic effusion was diagnosed in one patient). The diagnosis of systemic lupus erythematosus was based on a positive result of testing for anti–double-strand DNA antibodies and LE cells, in addition to other clinical criteria. The diagnosis of parapneumonic effusion was confirmed by Gram staining and culture of sputum showing Gram-positive cocci (Streptococcal pneumonia) and also by the response of the effusion to antibiotic therapy. In the transudative effusion, histopathology revealed normal pleura (Tables 1, 2).

Discussion

Rapid diagnosis and treatment of tuberculous pleural effusion is crucial to reduce morbidity and mortality from untreated TB. Because the differential diagnosis of exudative pleural effusion is broad, the rapid diagnosis of tuberculous pleural effusion would greatly facilitate the management of these patients. At present, the most reliable method for the diagnosis of tuberculous pleural effusion is the identification of *M. tuberculosis* in the pleural speci-
The diagnosis of tuberculous pleural effusion depends on the demonstration of tubercle bacilli in the sputum, pleural fluid, or pleural biopsy specimens, or the demonstration of granuloma in pleural biopsy specimens, with high sensitivity of the latter up to 80% and low sensitivity of the former. Histopathology alone cannot reliably establish the diagnosis because it cannot distinguish disease caused by *M tuberculosis* from that caused by mycobacteria other than TB or other granuloma.

In this study, our goal was to improve our diagnosis of tuberculous pleural effusion and to determine the most sensitive, specific, and rapid diagnostic methods. We used PCR to detect DNA (IS986) specific for *M tuberculosis* complex on pleural biopsy specimens and compared it to the results of pleural biopsy cultures using the BACTEC 460 system and LJ medium.

Our study showed that the results of sputum ZN staining and culture for AFB on LJ medium were negative in all cases. A sputum study that was negative for AFB in our study was explained by the absence of any underlying pulmonary parenchymal disease, as determined by radiologic studies, and the sputum induction method that has been reported to improve the yield of sputum culture for mycobacterium was not used. Our result was in agreement with that of the previous study showing low sensitivity of the sputum examination in pleural effusion patients with normal chest radiograph findings.

ZN staining had a low detection rate of *M tuberculosis* in pleural fluid (0%) and pleural biopsy specimens (3.8%). A culture of the pleural fluid on both LJ medium and BACTEC 12B medium showed a lower sensitivity (15.4%) in comparison with the 92.3% seen in cultures of pleural biopsy specimens. The lower detection rate in pleural fluid samples studied with ZN staining and culture when compared to that of pleural biopsy ZN staining and culture can be explained by the presence of viable bacilli in the pleural tissue causing infection, in which the pleural fluid is the manifestation of a hypersensitivity reaction to the mycobacterium.

Pleural biopsy specimens cultured on BACTEC 12B medium had high sensitivity and short detection time when compared to pleural biopsy specimen cultures on LJ medium. The detection time of mycobacterium was shortened from a mean of 25 days using LJ medium to a mean of 13 days when BACTEC 12B medium was used.

Histopathologic examination of the pleural biopsy specimen had a sensitivity of 53.8% and was a rapid method for reaching the diagnosis of tuberculous pleural effusion, however, its sensitivity was low in comparison to that of pleural biopsy culture (92.3%). These results are in agreement with the results reported by Katiyar et al that showed that the culture of pleural biopsy was more sensitive in diagnosing tuberculous pleural effusion when compared to the pathologic examination of pleural biopsy specimens. In contrast, other studies have found that the histopathology of pleural biopsy specimens was more sensitive than the pleural biopsy specimen culture in the diagnosis of tuberculous pleural effusion. The difference in detection rates in the histopathologic examinations in these studies could be attributed to the fact of repeated sampling needed for histopathologic examinations to detect caseating lesions, while for cultures one sample seems to be sufficient. In addition to the need for multiple sampling, histopathology alone cannot distinguish between a disease caused by *M tuberculosis* from other causes of granuloma.

In our study, a PCR of a pleural biopsy specimen
had a 90% sensitivity, a 100% specificity, a 100% PPV, a 86.6% NPV, and 93.9% accuracy, which are values that approach those of pleural biopsy specimen culture using both BACTEC B12 medium and LJ medium. The results of pleural biopsy specimen PCR and culture were negative in only two patients with tuberculous pleural effusion, in which the diagnosis was established by histopathology showing caseating granuloma and by the positive response to antituberculous drugs. False-negative results of pleural biopsy specimen PCR and culture can be explained by the presence of a low number of TB bacilli in these lesions where immunologic reaction with extensive caseation was predominant. The sensitivity of nucleic amplification techniques depends on the number of mycobacteria, their homogenous distribution in the sample, the presence of the amplification inhibitor in the sample,29 and type of the primer.30 In our study, we select the primer pair amplifying 123 bp, which is specific for members of the M tuberculosis complex.31 Also, this primer pair has been reported32 to be the most sensitive primer of three different primer pairs in detecting mycobacterium DNA. Applying PCR to a fresh pleural biopsy specimen in this study showed a higher sensitivity when compared to PCR detecting mycobacterium in a paraffin-embedded pleural biopsy specimen by avoiding DNA inhibitors in paraffin-embedded tissues.29 In our study, the sensitivity of a PCR of a pleural biopsy specimen to detect M tuberculosis was higher when compared to that of pleural fluid PCR in earlier studies, which have demonstrated a sensitivity ranging from 11 to 81%.16–18,32,33 Only one study, by Takagi et al34 addressed the utility of a PCR of a pleural biopsy specimen to detect M tuberculosis with similar sensitivity and specificity to our study. However, the study by Takagi et al34 used histopathology alone as a "gold standard" because they had no BACTEC culture studies of pleural biopsy specimens. The negative finding of the PCR of pleural biopsy specimens in two cases implies the importance of combining PCR of pleural biopsy specimens with microbiological and histopathologic examination.

In this study, we showed that a PCR of the pleural biopsy specimen has similar sensitivity and specificity to that of a culture of the pleural biopsy specimen, however, the potential advantage of PCR is that it may give faster results. Although PCR provides a rapid result, it has some limitations. It cannot replace conventional culture techniques and must be used in conjunction with culture to ensure optimal isolation rates for test sensitivity and specificity. Also, multiple negative, positive, and inhibition controls must be performed with each assay to ensure quality and reliability.35 In conclusion, a PCR of pleural biopsy specimens is a useful tool when used in combination with the BACTEC culture system and histopathologic examination of pleural biopsy specimens to reach a rapid diagnosis of tuberculous pleural effusion.

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