Detection of *Mycobacterium tuberculosis* in Sputum Samples by Poly-merase Chain Reaction Using a Simplified Procedure

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A repetitive sequence of *Mycobacterium tuberculosis* DNA was amplified by polymerase chain reaction (PCR), from sputum samples, for the diagnosis of pulmonary tuberculosis. The method of heating the sample in a boiling water bath to break down the bacterial cell wall and to release the DNA was compared with that of enzymatic lysis of bacteria and then phenol-chloroform extraction of DNA. Heating the sample was the better method with a sensitivity of approximately 10 microorganisms. A total of 78 sputum specimens prepared by heating were examined by PCR, and the results were compared with the results of acid-fast stained smears, cultures, and clinical data. *M. tuberculosis* was detected by PCR in all smear- and culture-positive and smear-negative, culture-positive cases. Additionally, PCR was capable of detecting four of nine cases which were smear and culture negative but clinically suspected of tuberculosis. DNA amplification by PCR is a sensitive and specific method for the diagnosis of tuberculosis, and with this simplified DNA isolation procedure it can be used in routine clinical practice.

Although a presumptive diagnosis of pulmonary tuberculosis can be made on the basis of patient histories and clinical and radiological findings, the definitive bacteriological diagnosis of tuberculosis continues to depend on the microscopic examination of acid-fast stained sputum smears and then cultural confirmation. Direct microscopy by Ziehl-Neelsen staining to identify acid-fast bacilli (AFB) is the most rapid method, but it lacks sufficient sensitivity and specificity. On the other hand it takes 4 to 8 weeks to culture pathogenic mycobacteria because of their slowly growing nature. Recently there has been great progress in developing rapid, sensitive, and specific tests for the diagnosis of tuberculosis (3).

Amplification of specific nucleic acid sequences by using polymerase chain reaction (PCR) has become a powerful tool for the rapid and specific detection of many infectious agents (15). Many primers which amplify specifically the DNA of *Mycobacterium tuberculosis* have been designed and successfully used for identification of this microorganism from culture (5, 10, 13, 16) and also from clinical samples (1, 2, 4, 6–8, 12, 14). It has been stated that the technology is too complex for application in developing countries, but it has potential for simplification (3); the necessity of further efforts to establish the sensitivity of the test and to develop routine and simple procedures for use in clinical practice is emphasized (2). In previous studies the isolation of *M. tuberculosis* DNA, by enzymatic lysis and phenol-chloroform extraction, was usually performed before amplification. In this study we used heat as the only agent to break down the bacteria and to release DNA for amplification by PCR, which simplified the overall procedure, making the method potentially useful for routine clinical practice.

**MATERIALS AND METHODS**

**Primers.** The primers used for the amplification were originally designed by Eisenach et al. from sequences which are repeated several (10 to 16) times in the chromosome of *M. tuberculosis* (6). The sequences of the primers (synthesized by MedProbe A.S., Oslo, Norway), which amplify a 123-bp fragment of the repetitive sequence (6) were 5′ to 3′ CTGCGAGCGCTAGGGC CGTTCGG.

**Comparison of DNA isolation methods.** Two different DNA isolation methods for preparation of template DNA were compared. A suspension of a clinical isolate of *M. tuberculosis* having ~10^9 microorganisms per ml was prepared in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), by adjusting its turbidity with McFarland standards. A total 10 ml of this suspension (~10^9 organisms) was put into two tubes. One of these was placed on boiling water for 10 min and then used directly for amplification without any further processing. The other tube was centrifuged, and the pellet was resuspended in TE containing lysozyme (10 mg/ml) and incubated on ice for 30 min. Proteinase K and sodium dodecyl sulfate (SDS) were then added to final concentrations of 10 mg/ml and 2%, respectively, and the incubation was continued at 37°C for 3 h. DNA was extracted by phenol-chloroform and precipitated by ethanol. The pellet was resuspended in 10 ml of TE buffer and used in PCR.

**PCR procedure.** A reaction mixture of 100 ml containing the primers (20 pmol each), 1× Taq polymerase buffer (25 mM Tris HCl [pH 9.5], 50 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol, bovine serum albumin [1 mg/ml], activated calf thymus DNA [300 µg/ml], 0.2 mM [each] deoxynucleoside triphosphates [dNTP], Taq polymerase [Amersham International; 2.5 U]), and the sample was prepared. The DNA was denatured for 5 min at 94°C; 35 amplification cycles were performed with an automated thermal cycler (DNA Thermal Cycler; Perkin-Elmer Cetus). Each cycle consisted of denaturation at 94°C for 1 min, annealing of
primers at 68°C for 2 min, and primer extension at 72°C for 2 min. The presence of the 123-bp amplification product was analyzed by electrophoresis of 10 μl of the amplified mixture on a 2% agarose gel. The DNA was stained by ethidium bromide and photographed on a UV transilluminator.

**Determination of sensitivity.** To determine the sensitivity of detection of *M. tuberculosis*, 10-fold serial dilutions of the same clinical isolate mentioned above were prepared, and DNA was prepared by just boiling the organisms. Tubes containing $-10^8$ to $-10^2$ organisms per ml were placed on boiling water for 10 min, and 10 μl from each tube ($-10^8$ to $-1$ organism) was used for amplification.

**Patients and clinical specimens.** Samples were obtained from 78 patients who were admitted to Atatürk Chest Diseases and Chest Surgery Center, Ankara, Turkey, with or without suspected tuberculosis. The clinical diagnosis of tuberculosis was established by patient histories, clinical and radiological findings, and response to antituberculosis drug therapy. In Turkey, the prevalence of being seropositive for human immunodeficiency virus (HIV) is very low. According to the statistics of the Ministry of Health, a total of 129 patients seropositive for HIV and 67 cases of AIDS were reported by 29 February 1992 (9). In a survey, 262 patients with tuberculosis were tested for HIV, and all of them were found to be negative (18). In another survey done at the same hospital from where the samples were collected for this study, 3 of 57 patients with tuberculosis were found out to be HIV seropositive (11). Therefore, the patients included in this study were not likely to be HIV positive.

Sputum samples were treated with the standard protocol of n-acetylcysteine-NaOH and concentrated by centrifugation (1,500 × g). From the sediments smears were made, stained by Ziehl-Neelsen, examined for AFB, and inoculated on Loewenstein-Jensen medium. The remaining of the sediments were stored at −20°C until used for PCR amplification.

**Specimen processing and DNA amplification.** The sediments of sputum samples were washed at least three times by centrifugation (12,000 × g) and resuspension in TE buffer. This was critical to rid the sample of proteins and salts produced by decontamination with NaOH. The final sediment was resuspended in 50 μl of TE buffer, and the tube was placed in a boiling water bath for 10 minutes. Of this, 10 μl was used for PCR. The amplification was performed by the above-described procedure. *M. tuberculosis* from culture and human epithelial DNA were used as positive and negative controls.

## RESULTS

The amplification of the 123-bp fragment of *M. tuberculosis* DNA by PCR, after preparation of the template DNA by two different methods, is shown in Fig. 1. A stronger band was produced by PCR amplification when bacterial DNA was obtained by boiling compared with lysis by lysozyme and SDS and then proteinase K digestion and phenol-chloroform extraction. The experiment was repeated twice with the same results (data not shown). The sensitivity of PCR for the detection of *M. tuberculosis* with the boiling method was determined by making serial 10-fold dilutions of the bacterial suspension (Fig. 2). Less than 10 microorganisms can be detected.

A total of 78 sputum samples were tested by PCR with the boiling method. (some are shown on Fig. 3.) The comparison of amplification results with smears, cultures, and clinical data is summarized in Table 1. A total of 57 of these were from patients with diagnosis of tuberculosis and 21 were from patients with nontuberculous pulmonary disease, on the basis of clinical and radiological findings. All of the 26 samples which were smear and culture positive were also positive with PCR; 3 samples which were negative on the smear for AFB but culture positive were PCR positive. There were 9 patients who were smear and culture negative but for whom tuberculosis was strongly suspected clinically. Four of these were PCR positive. There were 19 patients with pulmonary tuberculosis who were receiving antituberculosis therapy. Of these, 12 were smear and/or culture positive before therapy but were both smear and culture negative at the time the sample was taken for PCR. The duration of therapy ranged between 2 weeks and 1 year. Two of these were positive with PCR. One of these cases had taken therapy for 2 weeks and the other had therapy for 3 weeks. PCR-negative patients had taken at least 4 weeks of therapy. Seven patients with low clinical suspicion of tuberculosis but for whom smears and cultures were repeatedly negative were negative by PCR. Finally, of the 21 patients with nontuberculous pulmonary disease with sputum smear and culture negative for AFB, 1 was positive with PCR.

![Fig. 1. Comparison of the 123-bp amplification products by PCR from *M. tuberculosis* DNA isolated by two different methods from the same number ($-10^8$) of bacteria. A total of 10 μl from each tube after amplification was run on a 2% agarose gel. Lane M, *Hae*III-digested phage φX174 DNA as molecular size markers; lane 1, DNA prepared by enzymatic lysis followed by phenol-chloroform extraction; lanes 2 and 4, no DNA; lane 3, template DNA prepared by heating the bacteria on boiling water.](http://jcm.asm.org/)

![Fig. 2. Detection of *M. tuberculosis* by PCR with template DNA prepared by heating the bacteria on boiling water. Amplification products from serial 10-fold dilutions of *M. tuberculosis*. A total of 10 μl from each tube after amplification was run on a 2% agarose gel. Lane M, *Hae*III-digested phage φX174 DNA as molecular size markers; lanes 1 to 7, DNA from reaction tubes containing $-10^8$, $-10^5$, $-10^4$, $-10^3$, $-10^2$, and −1 bacterium, respectively; lane 8, human epithelial DNA; lane 9, no DNA.](http://jcm.asm.org/)
Unfortunately there is no real "gold standard" to compare and to determine the sensitivity and specificity of each method. Table 2 shows the sensitivity, specificity, and predictive values of PCR compared with AFB smear and culture, assuming clinical diagnosis as the gold standard.

**DISCUSSION**

DNA amplification by PCR is a rapid and sensitive method for detection of *M. tuberculosis* in sputum samples. Preparing samples by boiling simplifies the method so that it can be used in routine clinical practice. The repetitive nature of the target sequence amplified by PCR probably contributes to the high sensitivity. Fewer than 10 microorganisms could be detected. Precise estimation of the sensitivity is difficult. Since the number of organisms used in PCR to determine the sensitivity of the assay was adjusted depending on the turbidity of the suspension, the actual number of organisms per sample may have been higher, because of possible clumping of organisms, and thus the sensitivity may have been overestimated. All culture-positive samples, including three that were smear negative, were positive with PCR. Of the nine samples which were both smear and culture negative, obtained from patients clinically suspected of tuberculosis, four were positive with PCR, suggesting that the assay is probably more sensitive than the culture by detecting nonviable and/or fewer viable organisms.

One false-positive result from the sputum sample of a nontuberculous pulmonary disease patient may be due to cross-contamination with target sequences because of an error in handling the samples. (Although the PCR procedure was performed in a laboratory located in another building, the processing of the sputum samples for smear, culture, and PCR was performed in the busy laboratory of the chest diseases hospital, where about 50 AFB-positive smears are identified each day.) The clinical diagnosis was accepted as the gold standard of this research, but since there was no way to confirm this in a smear- and culture-negative case, the possibility of false diagnosis must also be considered.

The preparation of mycobacterial DNA by just boiling rather than enzymatic lysis followed by phenol-chloroform extraction was better for amplification with PCR. This might be due to the loss of some of the DNA during purification steps in the latter method. Thus, the procedure is simplified with the elimination of DNA purification, and the time required and the cost of the assay were reduced by half. Sritharan and Barker compared eight different procedures, including different enzymatic and detergent lysis methods, to release PCR-amplifiable DNA from *M. tuberculosis* bacilli (17). Of methods which worked, they selected a similar boiling method, because of its simplicity, to examine its efficacy for detecting *M. tuberculosis* in clinical samples. As in our method, they used Tris-EDTA buffer to suspend and boil the samples, but they also included 1% Triton X-100, and the duration of boiling was 30 instead of 10 min. They determined that with this method, fewer than 10 microorganisms can be detected by PCR.

All the smear-positive samples were positive with PCR. When the incidence of seropositivity for HIV is very low and *Mycobacterium avium* subsp. *intracellulare* complex (MAC) is hence a rare possibility, as it was in the patients included in this study, PCR positivity only confirms that the AFB seen on the smear is *M. tuberculosis*. Since detection of AFB by smear is an inexpensive and rapid method from the point of beginning antituberculosis therapy, PCR positivity is not very beneficial for the clinician in these cases. However, in HIV-positive patients it is very important to differentiate *M. tuberculosis* from MAC in choosing the right therapeutic regimen. In these cases the detection of *M. tuberculosis* by PCR might be very useful, even when the smear is positive, especially if it can be used together with a PCR procedure that can detect MAC. Since there was no smear-positive and PCR-negative patient identified in this

### TABLE 1. Correlation of PCR findings with smear and culture results and clinical data

<table>
<thead>
<tr>
<th>Patient status</th>
<th>No. of patients</th>
<th>No. positive by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear and culture positive</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Smear negative, culture positive</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Smear and culture negative, with high clinical suspicion; not on treatment at time of study</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>Smear and culture positive previously, became negative by therapy at the time sample was taken for PCR</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>On treatment for tuberculosis, repeatedly smear and culture negative, with low clinical suspicion</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Smear and culture negative; nontuberculous pulmonary disease</td>
<td>21</td>
<td>1</td>
</tr>
</tbody>
</table>

* Of the four patients who were smear and culture negative, PCR positive, and with high clinical suspicion, two had pleural fluid with adjacent parenchymal lesion and were diagnosed to have tuberculosis by pleural biopsy; the other two had moderate fibronodular infiltration without cavitation. All of these patients had symptoms for more than 4 weeks and responded to antituberculosis therapy, showing clinical and radiological improvement.

### TABLE 2. Sensitivity, specificity, and predictive values of various methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear</td>
<td>68</td>
<td>100</td>
<td>100 70</td>
</tr>
<tr>
<td>Culture</td>
<td>76</td>
<td>100</td>
<td>100 76</td>
</tr>
<tr>
<td>PCR</td>
<td>87</td>
<td>96</td>
<td>97 84</td>
</tr>
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</table>

* Clinical diagnosis with high suspicion is considered the gold standard. The total cases of tuberculosis was 38 (26 were smear and culture positive; 3 were smear negative, culture positive; and 9 were smear and culture negative but with clinical diagnosis of tuberculosis with high suspicion). True negatives numbered 28 (21 patients with nontuberculous pulmonary disease and 7 who were always smear, culture, and PCR negative, with low clinical suspicion).
study, the utility of this method in HIV-positive cases remains to be determined.

The detection of M. tuberculosis with PCR in smear-negative and culture-positive patients provides the bacteriological data 4 to 8 weeks earlier. For smear- and culture-negative specimens, PCR is the only currently available method that can provide bacteriological diagnosis. Since detection of AFB by smear is an inexpensive and rapid method, for HIV-negative patients clinically suspected of tuberculosis, it might be rational to use PCR for smear-negative specimens.

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REFERENCES


