Clinical Evaluation of Polymerase Chain Reaction DNA Amplification Method for the Diagnosis of Pulmonary Tuberculosis in Patients with Negative Acid-fast Bacilli Smear

SATOSHI MITARAI, KAZUNORI OISHI, MASASHI FUKASAWA*, HIROSHI YAMASHITA, TSUYOSHI NAGATAKE and KEIZO MATSUMOTO

Department of Internal Medicine and *Preventive Medicine, Institute of Tropical Medicine, Nagasaki University, Nagasaki 852


We evaluated the sensitivity and specificity of polymerase chain reaction (PCR) for the detection of Mycobacterium tuberculosis among 109 patients who were suspected to have active pulmonary tuberculosis (PTB) and showed negative acid-fast bacilli (AFB) smears in a total of 393 samples of sputum (169), gastric aspirate (134), and urine (90) which fulfilled different criteria for the positivity of PCR. The patients were subsequently divided into one group of active PTB composed of 15 patients with definite PTB and 43 patients with highly suspected PTB, and another group of 51 non-active PTB patients. The PCR assay using samples of sputum and gastric aspirate proved to be specific for active PTB. The PCR method for diagnosis of active PTB using sputum samples was sensitive (97.8%) but lacked specificity (27.0%) when regarded as PCR positive when at least one positive reaction was obtained among all samples examined. However, the PCR of gastric aspirate demonstrated a sensitivity of 63.4% and a specificity of 76.7%. Our data supports that the PCR method for detecting active PTB in AFB smear negative patients using gastric aspirate shows markedly improved sensitivity over the conventional method (25.9%), although it still lacks specificity. PCR assay for M. tuberculosis using multiple samples of gastric aspirate in conjunction with careful clinical observations for the presence of active infection is essential for the diagnosis of active PTB among patients with negative AFB smear.

polymerase chain reaction; Mycobacterium tuberculosis; negative acid-fast bacilli smear; diagnosis

Tuberculosis (TB) is still a serious disease world wide and is becoming even more important with the increasing number of individuals who have the acquired

Received February 15, 1995; revision accepted for publication June 13, 1995.
Address for reprints: Satoshi Mitarai, M.D., Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University, 1–12–4 Sakamoto-machi, Nagasaki 852, Japan.

immunodeficiency syndrome (AIDS) (Chaison et al. 1987). The conventional method for diagnosing TB using clinical samples by the acid-fast bacilli (AFB) smear and culture for *Mycobacterium tuberculosis* (*M. tuberculosis*) is a time consuming process which has low sensitivity and specificity (Bates 1979). In clinical practice, a presumptive diagnosis of pulmonary tuberculosis (PTB) can be made on the basis of typical clinical and roentgenographic findings for patients strongly suspected to have PTB. But they frequently show negative results on the AFB smear. Recently, the polymerase chain reaction (PCR) DNA amplification method for *M. tuberculosis* has made it possible to identify DNA sequence unique to the *M. tuberculosis* complex of organisms (Boddinghaus et al. 1990; De Wit et al. 1990; Hermans et al. 1990; Brisson-Noel et al. 1991). The technique is much more sensitive than other conventional mycobacterium detecting methods and shows almost 100% bacteriological specificity (Boddinghaus et al. 1990; Hermans et al. 1990; Pao et al. 1990; Wilson et al. 1993). The PCR method provides rapid and almost equal information compared to conventional methods, but still now few data are available regarding the usefulness of the PCR method in the diagnosis of PTB patients showing negative conventional result. This study was designed to evaluate the sensitivity and specificity of the PCR method for the diagnosis of active PTB in patients showing clinical findings suggesting tuberculosis, but negative AFB smear in the first clinical samples.

**MATERIALS AND METHODS**

*Subjects*

One hundred nine individuals suspected to have active PTB by clinical findings which included chest roentgenogram and who had received antituberculosis drugs for at least three months were included in this study. The conventional examinations of sputum AFB smears from all individuals were negative when they were enrolled. Thirteen of 109 proved to be *M. tuberculosis* positive by sputum culture (Table 1) and two cases were diagnosed as having active PTB by lung biopsy. These 15 patients composed the definite PTB group. Forty-three patients were subsequently diagnosed as active PTB by the improvement of clinical symptoms and typical chest roentgenographic findings after administration of the antituberculosis drugs; rifampicin, ethambutol and isoniazide for three months or more (highly suspected PTB group). Thus, the group of active PTB

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Number of patients (n=109)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active PTB</td>
<td>58</td>
</tr>
<tr>
<td>Definite PTB</td>
<td>15/58</td>
</tr>
<tr>
<td>Highly suspected PTB</td>
<td>43/58</td>
</tr>
<tr>
<td>Non-active PTB</td>
<td>51</td>
</tr>
</tbody>
</table>
POE Clinical Evaluation for the Diagnosis of Pulmonary Tuberculosis

consisted of the definite and the highly suspected PTB patients. The chest roentgenographic findings of the active PTB group according to the criteria of the American Thoracic Society were composed of 30 minimal, 20 moderately advanced and 8 far advanced. No clinical response to the antituberculosis treatment was observed in the other 51 patients. Among these, 13 had prior PTB, 9 were found to have pathologically confirmed lung cancer alone, 3 had lung cancer and prior PTB, and 4 were bacteriologically proven to have atypical mycobacterial infection; the other 22 had inflammatory lung diseases. These 51 patients including prior tuberculosis and other pulmonary diseases belonged to the non-active PTB group in contrast to the active PTB group.

Clinical samples

A total of 109 patients who were seen in the out- or in-patient clinics of the Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University Hospital or other affiliated hospitals in Nagasaki, were suspected to have active PTB. Sixty-three were male and 46 were female, and their mean age was 63 years. A total of 393 samples (169 sputum samples, 134 samples of gastric aspirate and 90 samples of urine) were obtained from those patients and examined. These samples were stored at -20°C until and after DNA extraction.

Sensitivity and specificity of PCR

In this study, sensitivity refers to the detection rate of active PTB by PCR in the active PTB group. Specificity refers to the detection rate of non-active PTB patients by PCR in the non-active group. In the present study, the sensitivity of conventional methods using TB smear and culture was only 25.9% (15/58), while the specificity was 100% (51/51).

Treatment of clinical samples

All samples were cultured. Sputum was mixed well with a four fold volume of 0.1 N NaOH to reduce its viscosity. The samples of gastric aspirate and urine were mixed with an equal volume of 20% polyethylene glycol in 2.5 M NaCl and left for 15 min at room temperature (De Wit et al. 1990), and were then centrifuged for 10 min at 1,700×g. The pellet was suspended in 5 ml of buffer (pH 8.0) which contained 50 mM Tris-HCl, 5 mM EDTA and 50 mM NaCl (TNE buffer) and centrifuged once again under the same conditions. Finally, the pellet was suspended in 5 ml of TNE buffer with a final concentration of 1% sodium dodecyl sulfate and 100 µg/ml of proteinase K (Wako Pure Chemical Industries Ltd., Osaka) and incubated at 42°C for 2 hr with gentle shaking. The digested specimen was extracted with an equal volume of phenol followed by phenol/chloroform/isoamylalcohol and chloroform. The last aqueous phase was precipitated with two volumes of ice cold pure ethanol. Two hr later, the DNA fraction thus extracted was centrifuged for 10 min at 2,500×g at 4°C. The pellet
was dissolved in 300 μl of distilled water.

**DNA amplification**

Several primer sets for detection of mycobacteria have already been reported (Boddinghaus et al. 1990; De Wit et al. 1990; Pao et al. 1990; Shankar et al. 1990; Eisenach et al. 1991; Manjunath et al. 1991; Kaneko et al. 1992; Uematsu et al. 1992; Waker et al. 1992). In a preliminary experiment with 65 kDa, 19 kDa, dna J and MPB64 coding gene detecting primer pairs, the MPB64 detecting primer pair was found to be the most sensitive and thus was used in subsequent experiments. The DNA target for amplification was a 240-base pair sequence of the MPB64 coding gene in the *M. tuberculosis* complex genome (Yamaguchi et al. 1989). The primers used for the PCR process have the following sequences as reported by Shankar et al.: Forward Primer: 5’ TCCGCTGCCAGTCGTCTTCC 3’, Reverse Primer: 5’ GTCCTCGCGAGTCTAGGCCA 3’. The reaction mixture contained 10 μl of each sample, 200 μM each of dNTP, 50 pmole each of primer, 1 unit of *Tth* DNA polymerase (TOYOBO Co. Ltd., Osaka) and a reaction buffer (Tris-HCl: 10 mM, KCl: 80 mM, MgCl₂: 1.5 mM, sodium cholate: 0.1%, sodium cholate: 0.1%, Triton X-100: 0.1%, BSA: 500 μg/ml). Amplification was carried out for 40 cycles by a thermalcycler TSR-300 (IWAKI Co., Chiba). The PCR condition was 1 min at 94°C, 2 min at 63°C and 2 min at 72°C. The PCR product was analyzed by electrophoresis with 2% agarose gel and stained with ethidium bromide (Kolk et al. 1992; Uematsu et al. 1992).

**Bacteriological sensitivity and specificity of the primer set**

The primer pair detecting MPB64 coding gene is very popular and specificity for typical and atypical mycobacteria has been reported (Shankar et al. 1990; Manjunath et al. 1991; Shankar et al. 1991; Uematsu et al. 1992). Bacteriological specificity of PCR for detection of *Mycobacterium tuberculosis* complex was tested using commonly isolated non-mycobacterial DNA templates from the respiratory system; these included *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, α-streptococcus, *Candida albicans* and human peripheral blood cells. The PCR product was seen only when *Mycobacterium tuberculosis* DNA template was used. Bacteriological sensitivity was determined by using a 10-fold dilution of 240-base pair-fragment-integrated control plasmid. The limit of detection by this system was theoretically 10 organisms (Fig. 1).

**Statistical methods**

The significance of differences in the positive rate of the PCR was analyzed by chi-square test and Fisher’s exact probability test. Data were considered statistically significant if *p* value was less than 0.05.
RESULTS

Positive rate of PCR

The positive rate of PCR for sputum samples (94.5%) was much higher than that of gastric aspirate (47.7%) or urine (32.8%) among patients with active PTB (Table 2). The positive rate of PCR for sputum samples was also higher (74.6%) than that of gastric aspirate (19.6%) or urine (12.5%) among those with non-active PTB. The positive rate of PCR for samples of sputum and gastric aspirate, but not for those of urine, was significantly higher in patients with active PTB than in patients with non-active PTB ($p < 0.01$ for sputum, $p < 0.01$ for gastric aspirate, $p > 0.05$ for urine). This means that the PCR examination for PTB with samples of sputum and gastric aspirate, but not with those of urine, is

<p>| Table 2. PCR positivity in the group of active and non-active pulmonary tuberculosis |
|----------------------------------|---------|---------|</p>
<table>
<thead>
<tr>
<th>Active PTB</th>
<th>Non-active PTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>103/109 (94.5%)$^a$</td>
</tr>
<tr>
<td>Gastric aspirate</td>
<td>42/88 (47.7%)$^a$</td>
</tr>
<tr>
<td>Urine</td>
<td>22/67 (32.8%)$^b$</td>
</tr>
</tbody>
</table>

$^a$chi-square, $p$ value $< 0.01$

$^b$Fisher's exact method, $p$ value $> 0.05$
specific for active PTB.

**Sensitivity and specificity of PCR for detection of active PTB**

We first evaluated the sensitivity and the specificity of PCR in 109 patients, and interpreted the results of PCR as positive when at least one of all specimens from different sites was positive (Table 3). Eighty-five patients were PCR positive according to the above criteria. In 55 patients with positive PCR in active PTB, there was no patients whose PCR was positive only in the urine sample (Table 2). However, 30 of these patients belonged to the non-active PTB group. Among them, 8 had had prior PTB, 8 had primary lung cancer, 2 had prior PTB and primary lung cancer, and 12 had other lung diseases. Twenty-four cases were PCR negative based on similar criteria; 3 of these patients had active PTB. The gastric aspirate samples from 2 patients and a sputum sample from one case were PCR negative. The sensitivity and specificity of PCR, when PCR was interpreted as positive, when at least one of all specimens from different sites was positive, was 94.8% and 41.2%, respectively. The positive and negative predictive value of PCR was 64.7% and 87.5%, respectively. Then, to improve those four factors (sensitivity, specificity, positive predictive value and negative predictive value), the result of PCR was interpreted as positive when at least one

<table>
<thead>
<tr>
<th>PCR</th>
<th>Active PTB (n=58)</th>
<th>Non-active PTB (n=51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive PCR</td>
<td>55</td>
<td>30</td>
</tr>
<tr>
<td>Negative PCR</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Sensitivity for diagnosis of active PTB</td>
<td>55/58 (94.8%)</td>
<td></td>
</tr>
<tr>
<td>Specificity for diagnosis of active PTB</td>
<td>21/51 (41.2%)</td>
<td></td>
</tr>
</tbody>
</table>

*The result of PCR was interpreted as positive when at least one of the specimens tested was PCR-positive.

<table>
<thead>
<tr>
<th>Sputum (n=83)</th>
<th>Gastric aspirate (n=71)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Active PTB</td>
</tr>
<tr>
<td>Positive PCR</td>
<td>45</td>
</tr>
<tr>
<td>Negative PCR</td>
<td>1</td>
</tr>
<tr>
<td>Sensitivity for diagnosis of active PTB</td>
<td>45/46 (97.8%)</td>
</tr>
<tr>
<td>Specificity for diagnosis of active PTB</td>
<td>10/37 (27.0%)</td>
</tr>
</tbody>
</table>

*The result of PCR was interpreted as positive when at least one of the samples from different sources was PCR-positive.
of multiple clinical samples from a single site was positive (Table 4). Evaluation of 83 and 71 cases for samples of sputum and gastric aspirate, respectively, showed PCR sensitivity of 97.8% for sputum and 63.4% for gastric aspirate. However, the sputum samples from 27 cases in non-active PTB were PCR positive. These included 7 cases of primary lung cancer or prior PTB, 2 cases associated with primary lung cancer and prior PTB, 3 cases of atypical mycobacterial infection and 8 cases of other pulmonary diseases. Thus, the specificity for sputum sample was low (27.0%). In contrast, PCR specificity for gastric aspirate was relatively high (76.7%). The positive and negative predictive value of sputum was 62.5% and 90.9%, while that of gastric aspirate was 78.8% and 60.5%, respectively. Finally, when one PCR result was simultaneously positive in sputum and gastric aspirate, it was interpreted as PCR positive (Table 5); the sensitivity and specificity was 62.5% and 76.2%, respectively. PCR positivity even when sputum and gastric aspirate were included in the evaluation improved neither the sensitivity nor specificity. These values were similar to those when at least one of several samples of gastric aspirate was PCR positive. The values of sensitivity and specificity of the PCR in patients with active PTB (definite and highly suspected) were comparable to those in highly suspected PTB.

**Discussion**

Previous studies demonstrated high sensitivity and specificity of PCR in the diagnosis of active TB infection (Boddinghaus et al. 1990; Hermans et al. 1990; Eisenach et al. 1991; Altamirano et al. 1992; Wilson et al. 1993). Altamirano et al. (1992) employed only sputum samples which were culture positive for *M. tuberculosis*, but did not describe the AFB smear results in their study. They showed 98% sensitivity and 100% specificity by PCR in the diagnosis of TB infection. In addition, almost all patients with positive sputum culture for TB who were studied by Eisenach et al. (1991) showed positive AFB smear. On the other hand, Schluger et al. (1994), however, recently reported that the sensitivity and specificity of PCR for the diagnosis of active TB was 100% and 45% among

---

**Table 5. Sensitivity and specificity for diagnosis of active PTB by PCR**

<table>
<thead>
<tr>
<th>PCR</th>
<th>Sputum + gastric aspirate</th>
<th></th>
<th></th>
</tr>
</thead>
</table>
|                    | Active PTB 
                    | Non-active PTB 
(n = 32) | (n = 21) |
| Positive PCR       | 20                        | 5        |
| Negative PCR       | 12                        | 16       |

| Sensitivity for diagnosis of active PTB | 20/32 (62.5%) |
| Specificity for diagnosis of active PTB | 16/21 (76.2%) |

*The result of PCR was interpreted as positive when at least one of the specimens of sputum and gastric aspirates was PCR-positive.*
65 patients with or without TB infection, respectively. Twenty-three definitely active TB patients with negative AFB sputum smears were involved in their study. Their findings showed that positive PCR results were obtained from those with a history of TB or contact with tuberculosis patients, as also described by Waker et al. (1992). The latter group concluded that the role of PCR would be limited to well-defined clinical situations, such as HIV-positive patients with intrathoracic adenopathy. Yoon et al. (1992) similarly reported that 39% of sputum samples from patients with prior PTB were PCR positive. The pathologic evidence for the persistence of mycobacteria in patients with residual PTB might support these data (Kanai 1987). Additionally, Kocagoz and his coworkers evaluated smear and culture negative but clinically suspected TB cases by PCR using a simplified procedure (Kocagoz et al. 1993). Their PCR assay detected only 4 of 9 cases.

Amano et al. (1993) and Shankar et al. (1991) have demonstrated the high sensitivity and specificity of the PCR method using primers amplifying a 240-base pair sequence in the MPB64-coding gene to diagnose PTB and extrapulmonary TB, including tuberculous meningitis. In the present study, we evaluated the sensitivity and specificity of the PCR technique employing the same primers in patients who were suspected to have active PTB but had AFB-smear-negative samples of sputum or gastric aspirate according to several different criteria for PCR positivity. The active PTB group comprised definite and highly suspected PTB individuals who responded to antituberculosis chemotherapy. In cases of highly suspected PTB, the clinical diagnosis was accepted since there was no alternative to confirm the presence of active PTB in patients with negative AFB smear and negative culture for *M. tuberculosis*. But 40 patients of the 43 highly suspected PTB group were basically positive by PCR and only 3 were negative. These 3 patients were in doubt for diseases other than tuberculosis responding to rifampicin, although one of them was finally diagnosed conventionally. One of the others was for several years an inpatient and no endemy of mycoplasmal or chlamydial infection was observed in the area. Therefore, it is possible that actually only one patient might have had chlamydial or mycoplasmal infection.

When we had at least one positive PCR result among all the clinical specimens tested, we found a high sensitivity of 94.8% and a low specificity of 41.2% (Table 3). The sensitivity was obviously influenced by the high positive rate of PCR in sputum samples among patients with active PTB (Table 2), while the low specificity was due at least to one positive PCR result in 30 patients with non-active PTB. Most of these patients were those with prior PTB or primary lung cancers. Previous reports showed false positive PCR in patients with cancer and malignant pleural effusion (Brisson-Noel et al. 1991; De Wit et al. 1992). Our positive PCR patient among those with primary lung cancer may have been due to latent or new tuberculous infection around the tumor.

Pierre et al. (1993) evaluated the PCR method for the diagnosis of PTB in
children employing samples of gastric aspirate. All samples were negative for AFB smear and *M. tuberculosis* culture. These researchers demonstrated that testing multiple samples from the same individual increased the sensitivity and specificity of the PCR method because not all samples contained detectable numbers of mycobacteria and because of the risk of sporadic false positive results. Similarly, we found it useful to examine gastric aspirate in diagnosing active PTB. The sensitivity (63.4%) and specificity (76.7%) were shown to be relatively high when at least one of the gastric aspirates was interpreted as PCR-positive. The specificity was low (27.0%) by similar criteria for PCR positivity with sputum samples (Table 4). Although the values of sensitivity and specificity for PCR positivity in samples of gastric aspirates were still lower than those in the literature because the samples we examined were from patients with negative AFB smear, the sensitivity (63.4%) of PCR by the criteria using gastric aspirate was much higher than that by conventional methods (25.9%). Furthermore, up to three PCR examinations with gastric aspirate improved the sensitivity, although it remained at the same level when repeated more than three times (data not shown). Thus, up to three PCR examinations with gastric aspirate are recommended when active PTB is strongly suspected from the clinical symptoms and data. In addition, provided PCR-data were interpreted as positive when at least one sample of both sputum and gastric aspirate was simultaneously PCR positive, the sensitivity (62.5%) and specificity (76.2%) were comparable to those of which at least one of multiple samples of gastric aspirate was PCR positive. Thus, the combination of PCR examination for TB using samples of sputum and gastric aspirate did not improve the sensitivity or specificity in the diagnosis of PTB in patients with negative AFB smear, because the PCR sensitivity of the sputum sample limited that of gastric aspirate. Nor was there any significant difference between the values of sensitivity and specificity among patients with active and highly suspected PTB. This confirms the validity of the diagnosis of highly suspected PTB based on the clinical efficacy of antituberculosis chemotherapy and improved findings of chest roentgenograms.

In summary, the PCR method for detecting active PTB in AFB smear negative patients using samples of gastric aspirate showed greater sensitivity than the conventional method, but still lacked specificity. The PCR method should be utilized not only from bacteriological but also clinical view points in a rational approach to a definite diagnosis of tuberculosis. Finally, the PCR assay for *M. tuberculosis* using a sputum sample should be recommended to screen a patient, because it has a high sensitivity and high negative predictive value. Three samples of gastric aspirate combined with careful clinical observations can be a helpful diagnostic tool in diagnosing active PTB among patients with negative AFB smear and culture.
Acknowledgment

We are grateful to Ms. Naoko Tanaka for her excellent laboratory assistance and all the staff members of the Department of Internal Medicine, Institute of Tropical Medicine, Nagasaki University for their clinical support.

References


