Detection of *Mycobacterium tuberculosis* in Clinical Samples from Patients with Tuberculosis or Other Pulmonary Diseases by Polymerase Chain Reaction

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Polymerase chain reaction (PCR) using primers targeting the IS6110 repetitive sequence was employed to detect *Mycobacterium tuberculosis* in 228 samples from patients with tuberculosis or other pulmonary diseases and controls, and the results were compared with culture and clinical findings. None of culture negative samples from 17 healthy controls were PCR positive. Of 109 active tuberculosis patients under chemotherapy, 88 (80.7%) were PCR positive and were significantly higher than 63 (57.8%) positive by culture. Fifty-nine (93.7%) of 63 culture positive and 29 (63.0%) of 46 culture negative specimens contained *M. tuberculosis* detectable by PCR. In 41 specimens from inactive tuberculosis patients who visited to the chest clinic because of chest problems, 16 (39.0%) also gave PCR positive results. In addition, 14 (46.7%) of 30 specimens submitted for *M. tuberculosis* culture from patients with pulmonary diseases were PCR positive. Presumptive diagnosis of these PCR positive patients was bronchitis, pneumonia, bronchial asthma, etc. Therefore, this study suggests that PCR is sensitive and specific in detecting *M. tuberculosis* in clinical specimens. However, the interpretation of the PCR results in specimens from patients with pulmonary diseases should be done cautiously in areas with a high prevalence of tuberculosis.

**Key Words:** Polymerase chain reaction, *Mycobacterium tuberculosis*, diagnosis, tuberculosis

Laboratory diagnosis of tuberculosis has been made by culture and identification of *Mycobacterium tuberculosis* in clinical specimens from patients with illness compatible with the disease. Despite simplicity and rapidity, the smear examination of acid-fast bacilli showed low sensitivity and specificity; on the other hand, although it is highly sensitive and specific, a culture of *M. tuberculosis* requires a long term incubation (Crawford et al. 1989). Even with the combination of radiometric culture system and DNA probes, it took 10~20 days to confirm *M. tuberculosis* in clinical specimens (Ellner et al. 1988).

Recent development of polymerase chain reaction (PCR) (Saiki et al. 1988), however, has brought tremendous interests for the sensitive, specific and rapid diagnosis of tuberculosis. In the literature, there have been various target sequences for PCR and DNA probes specific for *M. tuberculosis* (Hance et al. 1989; Eisenach et al. 1990; Pao et al. 1990; Patel et al. 1990; Hermans et al. 1990; Plikaytis et al. 1990; Sjobring et al. 1990; Thierry et al. 1990; Cousins et al. 1992; Portillo et al. 1991). Most of the reports showed that PCR with or without DNA probes seemed very sensitive, so that even one to
ten organisms are detectable by the method. In addition, PCR provides virtually 100% specificity in detecting the organisms belonging to *M. tuberculosis* complex. Particularly, the repetitive sequences such as IS6110 (Eisenach et al. 1990; Thierry et al. 1990) or IS986 (Hermans et al. 1990a & 1990b), have been widely used because of its multiple copies in the *M. tuberculosis* chromosomal DNA and of its presence only mycobacteria of *M. tuberculosis* complex.

When PCR was used for the detection of *M. tuberculosis* in clinical specimens, it gave the sensitivity of 97~100%, regardless of target DNAs; however, specificity of PCR varied widely depending on the studies and ranged from 71% to 100% when the culture method was used as a gold standard (Pao et al. 1990; Thierry et al. 1990; Cousins et al. 1992; Eisenach et al. 1991; Brisson-Noel et al. 1991). Specificity of PCR in the diagnosis of tuberculosis seemed influenced by the target DNAs, the presence of other mycobacterial species in the specimens, the efficiency of culture methods, the previous history of tuberculosis among controls, etc. In this study, therefore, we attempted to evaluate the PCR tool for the diagnosis of tuberculosis using primers based on the IS 6110 repetitive sequence (Eisenach et al. 1990) in sputum samples from active pulmonary tuberculosis, patients with other pulmonary diseases, and controls in the areas with high prevalence of tuberculosis.

**MATERIALS AND METHODS**

**Specimens**

Sputum specimens were obtained from 109 patients with active pulmonary tuberculosis presented at the Severance Hospital of the Yonsei University Medical Center. The diagnosis of tuberculosis was based on a culture of *M. tuberculosis* in 98 (90%) patients and chest X-ray evidences along with clinical findings in 11 (10%) patients. Clinical samples were also obtained from 41 inactive tuberculosis patients visited to the clinic with chest complaints, 30 patients visited to the chest clinic with pulmonary disorders other than tuberculosis, and 23 patients with illness other than pulmonary diseases. Sputum samples from 17 medical students who had no evidence of tuberculosis by chest X-ray, no history of tuberculosis, and no history of family contacts were included as controls.

**Culture of *M. tuberculosis***

For digestion and decontamination, sputum samples were mixed vigorously with 3~4 volumes of 2% NaOH solution containing acetylcysteine and sodium citrate. After centrifugation, the sediment was resuspended in 1~2 mL of residual solution. About four-fifths of the suspension was used for inoculation into two egg-based Ogawa media (Chadwick 1982) including one with antibiotics and for smear examination. The rest of the suspension was then frozen at -20°C until used for PCR analysis. The growth of mycobacteria in Ogawa media with and without antibiotics was examined once a week for 8 weeks. The identification was based on the colony morphology, pigmentation, and growth rate, followed by biochemical tests.

**DNA Preparation for PCR**

From the residual suspension above, a 200 μl portion was transferred to a 2 mL screw capped conical tube and microfuged for 10 min. After decanting the soup, a 100 μl volume of 0.1 mm zirconium beads (Biospec Products, Bartlesville, Okla.) in Tris-EDTA (pH 7.4) was added, and the residual soup was removed after the beads settled. To the tube, 100 μl of Tris-EDTA-NaCl (pH 8.0) and 50 μl of phenol: chloroform: isoamylalcohol (IAA) (25:24:1) were added and shaken vigorously for 1 min using a bead beater (Biospec Products). After centrifugation for 5 min, the aqueous phase was collected and boiled for 10 min to destroy DNase. DNA was then precipitated with one-tenth volume of 3 M sodium acetate and three volumes of cold ethanol on ice. Finally, the precipitated DNA was resuspended in 10 μl of distilled water and used for PCR.

**Amplification of *M. tuberculosis* DNA by PCR**

The primers amplifying the 123 bp (Eisenach et al. 1990) of the IS 6110 repetitive sequence were prepared using a DNA synthesizer (Applied Biosystems, Foster City, Calif.). The sequences of the primers were 5'-CCTGCGAAGCGTAGGCCCTGGT3' (P1) and 5'-CTCGTCCAGCGCCGCTTTCGG-3' (P2). The reaction mixture in 50 μl consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 200 μM of each of four dNTPs, 1.25 unit of Taq DNA polymerase (Perkin-
PCR for Diagnosis of Tuberculosis

Elmer Cetus, Norwalk, Conn.), 0.5 μM of each primer, DNA prepared from sputum samples. DNA purified from M. tuberculosis H37Rv and distilled water were included as positive and negative controls in each experiment. PCR was then carried in a thermocycler (Ericomp, Inc., San Diego, Calif.) for 32 cycles consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and primer extension at 72 °C for 3 min. The denaturation time was extended to 6 min in the first cycle and the primer extension time was 10 min in the last cycle. A 40 μl portion of the reaction mixture was the run in 8% polyacrylamide gel. After electrophoresis, the gel was stained with ethidium bromide and the 123 bp DNA band was examined under UV illumination.

RESULTS

Detection of M. tuberculosis DNA in sputum samples by PCR

A total of 220 sputum specimens from tuberculosis patients and controls were examined for the presence of M. tuberculosis by PCR, and the results were then compared with the culture findings. Fig. 1A shows a typical electrophoresis pattern of the 123 bp amplified from M. tuberculosis in sputum samples.

Examples of PCR positive results from culture negative samples and of PCR negative results from sputum specimens containing mycobacteria other than M. tuberculosis are shown in Fig. 1B. When culture-negative sputum samples from 17 healthy controls with no evidence of tuberculosis by chest X-ray and no history of family contacts were examined, none were PCR positive, thus indicating that the primers used in PCR and highly specific for detecting M. tuberculosis DNA (Table 1).

Of 109 active pulmonary tuberculosis patients, 88 (80.7%) had M. tuberculosis DNA detectable by PCR (Table 1). This PCR positive rate was significantly higher than the culture method which gave positive results in 63 (57.8%) specimens (p<0.001, McNemar test). Interestingly, the 123 bp DNA was also amplified in about 40% of sputum specimens obtained from inactive patients who visited the chest clinic with pulmonary disorders and from patients diagnosed as having pulmonary diseases other than tuberculosis. In contrast, in clinical specimens submitted for M. tuberculosis culture from 23 patients who were initially admitted with diseases involving other organs, only 2 (8.7%) showed PCR positive results. Of the two patients, one was diagnosed as tuberculous enteritis and the other patient

![Fig. 1. Amplification of an 123 bp fragment (arrows) from clinical specimens.](image)

A: a typical example of an experiment containing distilled water control in lane 1, clinical specimens in lanes 2-10, purified DNA from 100 tubercle bacilli in lane 11, and 123 bp DNA ladder size marker in lane M.

B: results of PCR from a pair of specimens with culture positive for M. tuberculosis (lanes 1 & 2), culture negative (lanes 3 & 4), and culture positive for mycobacteria other than tuberculosis (lanes 5 & 6).
Table 1. Comparison of culture and PCR in the detection of *M. tuberculosis* in clinical samples

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No. examined</th>
<th>Culture positive</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>(%)</td>
<td>No.</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>17</td>
<td>0 (0)</td>
<td>0</td>
</tr>
<tr>
<td>Active tuberculosis patients</td>
<td>108</td>
<td>63 (57.8)</td>
<td>88 (80.7)</td>
</tr>
<tr>
<td>Inactive tuberculosis patients</td>
<td>41</td>
<td>0 (0)</td>
<td>16 (39.0)</td>
</tr>
<tr>
<td>Patients with other pulmonary diseases</td>
<td>30</td>
<td>0 (0)</td>
<td>14 (46.7)</td>
</tr>
<tr>
<td>Patients with other diseases*</td>
<td>23</td>
<td>0 (0)</td>
<td>2* (8.7)</td>
</tr>
</tbody>
</table>

* Diseases involved in other organs.
* One from patient with tuberculous enteritis; the other from patient with history of hemoptysis.

Table 2. Comparison of culture and PCR in detecting *M. tuberculosis* in sputum samples from active tuberculosis patients

<table>
<thead>
<tr>
<th>Culture</th>
<th>PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>No.</td>
<td>(%)</td>
</tr>
<tr>
<td>Positive</td>
<td>59 (54.1)</td>
<td>4 (3.7)</td>
</tr>
<tr>
<td>Negative</td>
<td>29 (26.6)</td>
<td>17 (15.6)</td>
</tr>
<tr>
<td>Total</td>
<td>88 (80.7)</td>
<td>21 (19.3)</td>
</tr>
</tbody>
</table>

had experience of hemoptysis. All of the specimens from inactive patients and patients with other diseases were culture-negative.

**PCR results in specimens from active pulmonary tuberculosis patients**

The PCR and culture results from active pulmonary tuberculosis patients were analyzed in detail. Of 109 active patients, 59 (54.1%) had positive results as measured by both culture and PCR, while 20 (15.6%) were negative by both methods—showing the agreement rate of 69.7% (Table 2). As expectedly, of 46 specimens with culture negative, 29 (63.0%) were PCR positive, indicating that PCR is much more sensitive in detecting *M. tuberculosis* in clinical specimens from active tuberculosis patients under chemotherapy. Interestingly, four (6.3%) of 63 culture positive specimens were negative by PCR.

When the 46 culture negative specimens were reviewed, 35 were obtained from patients with culture negative conversion after chemotherapy; however, 22 (62.9%) of these patients still had *M. tuberculosis* DNA detectable by PCR in their sputum samples (Table 3). Interestingly, seven (63.6%) of 11 sputum specimens from pulmonary patients who...
PCR for Diagnosis of Tuberculosis

Table 4. PCR for detecting M. tuberculosis in specimens from inactive tuberculosis patients

<table>
<thead>
<tr>
<th>Duration after chemotherapy (yr.)</th>
<th>No examined</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1</td>
<td>11</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>2 ~ &lt;5</td>
<td>8</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td>≥ 5</td>
<td>22</td>
<td>11 (50.0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>41</strong></td>
<td><strong>16 (39.0)</strong></td>
</tr>
</tbody>
</table>

Patients visited to chest clinic because of chest problems and the specimens submitted for M. tuberculosis culture.

Table 5. Presumptive diagnosis of patients whose specimens were culture negative but PCR positive

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchitis with or without bronchiectasis</td>
<td>7</td>
</tr>
<tr>
<td>Pneumonia with unknown etiology</td>
<td>3</td>
</tr>
<tr>
<td>Diffuse interstitial lung disease</td>
<td>1</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>1</td>
</tr>
<tr>
<td>Bronchial asthma</td>
<td>1</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14</strong></td>
</tr>
</tbody>
</table>

were culture-negative at admission were also PCR positive.

**PCR results in specimens from inactive cases**

Sputum specimens were obtained from inactive tuberculosis patients who visited the chest clinic with pulmonary disorders. These patients completed the prescribed treatment and had culture-negative conversion at the time of completion. Of 11 patients who had completed the treatment less than one year, 2 (18.2%) were PCR positive (Table 4). Interestingly, 11 (50%) of 22 patients who had completed the treatment more than 5 years previously had M. tuberculosis DNA detectable by PCR. However, all of these sputum specimens were culture negative.

**PCR results in specimens from patients with other pulmonary diseases**

Almost 50% of the sputum specimens obtained from patients who visited the chest clinic with chest complains showed PCR positivity. When the clinical records of the patients were reviewed, the initial diagnosis of the patients included bronchitis with or without bronchiectasis, pneumonia with unknown etiology, diffused interstitial lung disease, lung cancer, etc. (Table 5).

**DISCUSSION**

Specificity of IS 6110 repetitive sequence has been discussed in detail by Eisenach et al. (1990 & 1991) and Thierry et al. (1990), who reported that the primers amplifying a portion of the IS 6110 DNA were specific to mycobacteria belonging to the M. tuberculosis complex. We also found that the 123 bp DNA was not amplified from DNA purified from three other mycobacterial species and six other bacterial species which could be found in sputum specimens (Yoon et al. 1991). This was consistent with that no 123 bp DNA was amplified from sputum samples from 17 controls in this study. Therefore, the 123 bp DNAs amplified from sputum samples in this study were from M. tuberculosis, because the other mycobacterial species of the M. tuberculosis complex were not likely present in the sputum samples from the study patients.

Sensitivity also has been an important factor in the application of PCR for detecting M. tuberculosis from clinical samples. Since the IS 6110 element is present in 10 ~ 20 sites of each chromosomal DNA (Eisenach et al. 1990; Thierry et al. 1990), the primers targeting this repetitive sequence will give greater sensitivity than those targeting the chromosomal DNA. In the reports, 1 ~ 10 fg of purified DNA, corresponding to 0.3 ~ 3 genomes, gave the PCR positive results.

We were also able to amplify the 123 bp DNA from 1 ~ 10 organisms which were diluted from a digested sputum suspension whose AFB numbers were determined under a microscope (Yoon et al. 1991). This indicated that the primers and PCR conditions employed in this study were highly sensitive in detecting M. tuberculosis from sputum samples.

However, when the results obtained from clinical samples analyzed for diagnostic purposes, the spe-
cificity and sensitivity of PCR could be markedly different depending on standard diagnostic criteria. Tuberculosis has been confirmed by culture of tubercle bacilli in clinical specimens from patients with illness compatible with the disease. However, culture of *M. tuberculosis* has failed in many occasions despite strong indication by chest X-ray and clinical findings, sometimes even from smear-positive samples (Warring and Sutramongkole 1970; Blair et al. 1976). Therefore, the PCR positive rate of about 80% among active pulmonary tuberculosis patients under chemotherapy was not surprising, but was still significantly greater than culture rate of 58%. In fact, about 42% of these active patients were culture-negative either from negative conversion by chemotherapy or from negative culture results before starting chemotherapy. Interestingly, a substantial portion (about 63%) of the culture negative samples from active tuberculosis patients had *M. tuberculosis* DNA detectable by PCR. This can be explained in two ways; firstly, the tubercle bacilli might be killed either during chemotherapy or during the decontamination process and secondly, culture conditions could not support small number of organisms (Krasnow and Wayne 1966; Warring and Sutramongkole 1970; Blair et al. 1976; Kim et al. 1984).

The interpretation of PCR result obtained from patients with a previous history of pulmonary tuberculosis seemed complicated. Considering that these inactive patients initially came to the chest with pulmonary disorders, some of them might be under a reactivation process from treatment failure. These patients were not enrolled for re-treatment because of negative culture results. However, about 40% of these patients gave PCR positive results. It is difficult to determine if *M. tuberculosis* DNA detectable by PCR were originated from live tubercle bacilli or residual dead bacilli shedding continuously from previous chemotherapy. Interestingly, the PCR positive rate was higher among the specimens from patients who had completed the prescribed treatment the longer time previous to our study. In order to support this finding, however, one has to examine the sputum specimens from inactive cases without any pulmonary problem matched with the duration since finishing chemotherapy by PCR.

Among patients with other pulmonary diseases, the PCR positive rate was also quite high, reaching about 47%. This was in contrast to the PCR positive rate of 8.7% among sputum samples submitted from culture from patients with illness in other organs to rule out tuberculosis. This finding suggested that tubercle bacilli were present in culture negative but PCR positive specimens from patients with other pulmonary diseases, but these patients were not diagnosed as having tuberculosis because of culture negative results. The presumptive diagnosis of the patients with PCR positive results included bronchitis, pneumonia, bronchial asthma, etc. whose clinical symptoms could be also found among tuberculosis patients (Des Perz and Craig 1990).

However, it may be too early to tell that the patients with culture negative but PCR positive results really have tuberculosis, because *M. tuberculosis* might be simply present for a short period of time without causing any tissue damage. Considering the tuberculin positive rate of about 80% among the populations at age over 20 years old regardless of BCG vaccination history and the national prevalence rate of 1.8% in the study area, the subclinical carrier of *M. tuberculosis* would be also high. Pao et al. (1990) also reported that *M. tuberculosis* was detectable by PCR in about 29% of culture negative specimens from patients with pulmonary diseases suspected having tuberculosis radiographs, clinical findings, history of exposure to tuberculosis patients, or past history of tuberculosis, etc. These patients were also from a country where tuberculosis is prevalent. The culture negative but PCR positive findings had been reported in many occasions in tuberculosis (Brisson-Noel et al. 1991; Thierry et al. 1990; Eisenach et al. 1991) and in other infectious diseases. For examples, about 14% of culture negative specimens gave PCR positive results in detecting *Bordetella pertussis* (Glare et al. 1990). *Chlamydia trachomatis* was also detectable by PCR in 17% of culture negative specimens (Ostergaard et al. 1990). Therefore, the PCR positive results from *M. tuberculosis* culture negative specimens were fully expected considering confounding factors in culture such as killing during decontamination process (Krasnow and Wayne 1966) and frequency of culture attempts (Blair et al. 1976).

Surprisingly, *M. tuberculosis* DNA was not amplified by PCR in 4 (6.3%) of 63 culture positive samples. Since any internal control was not inculded from each sample in this study, it was not known whether there was any PCR inhibitor or not (Eisenach et al. 1991). The presence of PCR inhibitors had been noted in about 5% clinical specimens (Brisson-Noel et al. 1991). Another explanation would be that relatively smaller portion (one-fifth) of original suspension was used in PCR compared to the volume (four-fifths) for culture in this study. If
there were only a few bacilli, most of them might be used for culture. In fact, we noted only colony growing in the media inoculated with specimens in many occasions. This was apparent from our experience that about 30% of aliquots from culture positive specimens were PCR negative when each suspension was divided into 3 tubes for repeated tests (Yoon et al. 1991). Therefore, it may be important to process as large a volume of specimen as possible in clinical applications.

From this study, we noted that PCR using the primers targeting the IS 6110 repetitive sequence is very sensitive and specific in detecting M. tuberculosis from clinical specimens. However, the interpretation of the presence of M. tuberculosis DNA detectable by PCR in clinical samples should be very carefully made for each individual, particularly in the areas with a high prevalence of tuberculosis. From any patient with PCR positive but culture negative results, repeated culture efforts should be made for a certain period of time before any conclusive decision on the diagnosis of tuberculosis. Therefore, further follow-up studies particularly on patients with culture negative but PCR positive results are desirable to understand the clinical course of the patients.

REFERENCES


Patel RJ, Fries JWW, Piessens WF, Wirth DF: Sequence analysis and amplification by polymerase chain reaction of cloned DNA fragment for identification of


