Detection of *Mycobacterium tuberculosis* in Preserved Tuberculous Lymph Nodes by Polymerase Chain Reaction

Bing Yang, Hironobu Koga, Hideaki Ohno, Kazuhiko Ogawa, Mohammad A. Hossain, Miho Fukuda, Yoichi Hirakata, Kazunori Tomono, Takayoshi Tashiro and Shigeru Kohno

*The Second Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852-8501*

Yang, B., Koga, H., Ohno, H., Ogawa, K., Hossain, M.A., Fukuda, M., Hirakata, Y., Tomono, K., Tashiro, T. and Kohno, S. *Detection of Mycobacterium tuberculosis in Preserved Tuberculous Lymph Nodes by Polymerase Chain Reaction*. Tohoku J. Exp. Med., 1998, **184** (2), 123–131 — We evaluated the usefulness of three types of polymerase chain reaction (PCR) targeting 16S rRNA, protein antigen b and IS6110 in detecting *Mycobacterium tuberculosis* in preserved tuberculous lymph nodes. The detection limit of all PCR methods was 100 colony forming unit (CFU) of *M. tuberculosis* in tissue. The test samples included eight paraffin-embedded tuberculous lymph nodes containing microscopical epithelioid cell granuloma with caseous necrosis and Langhans giant cells. Although acid-fast stained organisms in lymph node tissue were not detected in any sample, all three types of PCR tests were positive in four of eight lymph nodes. Our results suggest that PCR is not only a rapid and sensitive diagnostic method for tuberculous lymphadenitis, but also clinically significant in retrospective study for detecting *M. tuberculosis* even in some preserved lymph node tissues without evidence of acid-fast stained organisms. — Polymerase chain reaction; tuberculous lymphadenitis; paraffin-embedded tissue © 1998 Tohoku University Medical Press

Tuberculosis was until recently thought to be under control. However, it is now a worldwide disease estimated to afflict approximately 30 million people (IUAT/WHO 1982). In Japan more than 50 000 new cases per year have been reported during the last 10 years. Tuberculous lymphadenitis develops usually in otherwise healthy individuals without severe clinical symptoms or active pulmonary tuberculosis. Histologically, tuberculous lymphadenitis is characterized by the presence of epithelioid cell granuloma with caseous necrosis and

Received September 24, 1997; revision accepted for publication December 20, 1997.
Address for reprints: Shigeru Kohno, M.D., PhD, The Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
Langhans giant cells, and a majority of the cases are diagnosed by this pathological feature. However, three types of mycobacteria are responsible for mycobacterial lymphadenitis in adults. These include *M. tuberculosis* in 75%, *M. bovis* in 7.9% and by mycobacteria other than tuberculosis (MOTT) in 15.8% (Public Health Laboratory Service 1976). Since chemotherapy for MOTT is different from that for *M. tuberculosis*, identification of the exact etiological agent is important.

Detection and identification of the causative mycobacteria have been performed by conventional methods such as acid-fast staining and biochemical examination of cultured mycobacteria. However, confirmation of the presence of mycobacteria in the lymph node by these methods is often difficult. Although acid-fast staining is a simple and convenient method, the causative mycobacteria has been detected in only about 50% of the cases because this method requires a relatively large number of mycobacteria to be present in the tissue (> $10^4$/ml) (Des Prez and Heim 1990), and it does not allow identification of mycobacteria at species level. Tissue culture is also important for the identification of mycobacteria, but the incidence of positive cultures is usually less than 25% (Anno 1985), and the slow growth of mycobacteria (3 to 6 weeks) results in a delay in diagnosis.

Recent advances in molecular techniques have allowed the rapid diagnosis of mycobacteriosis by the application of polymerase chain reaction (PCR), which involves in vitro amplification of target DNA to a detectable level within a matter of hours (Saiki et al. 1988). In the present study, we evaluated the usefulness of PCR for detection and identification of *M. tuberculosis* in paraffin-embedded tuberculous lymph nodes.

**Material and Methods**

**Sensitivity of PCR**

The sensitivities of three types of PCR in detecting mycobacterial DNA in paraffin-embedded tissue were tested using a standard strain of *M. tuberculosis*, H37Rv. The strain was cultured in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) containing ADC supplement for two weeks at 37°C, then mycobacterial concentration was adjusted to McFarland No. 1 standard. The suspension was diluted serial 10-fold until $10^5$ times with double distilled water, and each 100 µl of $10^4$- and $10^5$-fold diluted suspension was inoculated onto Middlebrook 7H10 agar (Difco) containing OADC supplement and incubated for 3 to 4 weeks at 37°C to determine the number of viable mycobacteria. Each 1.0 ml of serial 10-fold mycobacterial suspensions was mixed with eight slices of paraffin-embedded tissue of normal BALB/c mouse lung, followed by DNA extraction and PCR in each tube using the same method applied for clinical samples (see below).
Tissue samples

Eight paraffin-embedded biopsied lymph nodes were used for clinical evaluation of PCR. Of these, four lymph nodes were obtained from the supraclavicular region, two from submaxillary, one from axillary region and one was a mesenteric lymph node. All lymph nodes were diagnosed as tuberculous lymphadenitis histologically by the presence of typical features of epithelioid cell granuloma with caseous necrosis and Langhans giant cells. In addition to the lymph nodes, lung tissue samples from three patients with small cell lung cancer and two patients with non-tuberculous lung diseases (pneumonia and pulmonary hemorrhage) were used as negative controls.

DNA extraction from paraffin-embedded tissue

Each tissue block was cut into 10 slices of 10 μm thickness. A new disposable microtome blade was used before cutting each tissue block to prevent cross-contamination of M. tuberculosis. Eight slices were placed into microtubes for DNA extraction, while the other two were used for acid-fast staining. The slices in the microtubes were extracted three times with xylene to remove paraffin, washed twice with 100% ethanol and once with each of 90%, 80% and 70% ethanol to remove the solvent, and then washed twice with TE buffer (10 mM Tris hydrochloride, 1.0 mM EDTA, pH 8.0). After centrifugation, the pellet was homogenized (Pellet Pestle Motor; Kontes Co., Vineland, NJ, USA) and resuspended again in 500 μl of TE buffer. In the next step, lysozyme (Wako Pure Chemical Co., Osaka) was added to a final concentration of 1.0 mg/ml and incubated at 37°C for 4 hours. Proteinase K and SDS (Wako) to a final concentration of 1.0 mg/ml and 1%, respectively, were added to the solution and incubated at 60°C for another 18 hours with continuous shaking. An equal volume of phenol and chloroform (1:1) was added, and the mixtures were centrifuged at 10,000 g for 3 minutes at room temperature. The supernatant was transferred to a fresh microtube, and the same procedure was performed with chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 4 μl of 2 mg/ml glycogen (Boehringer, Mannheim, Germany) and pure ethanol at −80°C over 30 minutes, and then rinsed with 1.0 ml of 70% ethanol at 4°C. After centrifugation, the DNA was dried and dissolved in 100 μl of TE buffer. Ten μl of DNA solution was used for PCR amplification. Two control samples were always used with the clinical samples, including one of distilled water (negative control) and another of H37Rv (positive control).

PCR

Four sets of PCR primers for amplification of four types of DNA fragments encoding 16S rRNA, protein antigen b (Pab), IS6110 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were synthesized by a 380B RNA/DNA
synthesizer (Applied Biosystems, Foster City, CA, USA) and used. The gene encoding 16S rRNA is a specific gene for mycobacteria species including M. tuberculosis complex, M. avium, M. intracellulare, etc. We used primers reported by Hashimoto et al. (1996), including 5'-CATGCAAGTCAACCGAAGAAG-3' and 5'-CGGTGCTTTCTCCACCATTA-3' for the first PCR, and 5'-TACTCGAGTTGCAGAAGGCT-3' and 5'-CGGACCTTCGATCGGTGA-3' for the second, nested PCR. The gene encoding 38 kDa protein, Pab, is a specific gene for M. tuberculosis complex reported by Sjöbring et al. (1990), and the nested PCR for this gene was performed as described by Miyazaki et al. (1993). IS6110 is an insertion sequence discovered in M. tuberculosis complex (Thierry et al. 1990), and the following primers reported by Noordhoek et al. (1994) were used: PGF1 (5'-TCATCAGGGCCACCGAGG-3') and PGF3 (5'-CCGTTCGACGGTGCACT-3'). The GAPDH gene, a universal gene of the human cell, was used as an internal control. The sequences of the primers were 5'-ACCATGGAAGGCTGGGG-3' and 5'-CAAAAGTTGTCATGGATGACC-3' (D'Addario et al. 1990). The four types of PCR products were 368 bp, 322 bp, 271 bp and 196 bp for 16S rRNA, Pab, IS6110 and GAPDH, respectively.

The PCR reaction solution was prepared by mixing 40 μl of a previously mixed reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl₂, 100 μg/ml of gelatin, 0.25 mM of each four types of deoxynucleotide, 50 pmol of each oligonucleotide primer, 1.25 U of Taq polymerase (Takara Shuzo, Co., Ltd., Shiga) and 10 μl of the template DNA to create a total of 50 μl in a tube. For the second PCR, 10 μl of the reaction solution containing the amplified first PCR product was sampled and mixed with 40 μl of a freshly prepared reaction mixture. As for the PCR conditions, the temperature and time of denaturation, annealing and extension, and number of cycle were as follows: 94°C/60 sec, 63°C/90 sec, 72°C/60 sec, 35 cycles for both the first and second PCR for 16S rRNA and Pab, 94°C/60 sec, 63°C/60 sec, 72°C/60 sec, 40 cycles for IS6110, and 94°C/60 sec, 60°C/60 sec, 72°C/90 sec, 40 cycles for GAPDH. The PCR products were electrophoresed in 2% agarose gel (Seakem ME agarose, FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide and visualized by UV transillumination.

Results

The PCR results and clinical records regarding to bacteriological examination, period between the onset of lymphadenitis and biopsy, presence of antituberculous therapy before biopsy, and efficacy of therapy in all eight cases were summarised in Table 1. Although mycobacterial culture was not performed in all eight lymph nodes when those were biopsied, two strains of M. tuberculosis were cultured from sputum and ascites in each one patient, respectively. Consequently, tuberculous lymphadenitis in all patients was cured by antituberculous therapy.

The sensitivity of each type of PCR for 16S rRNA, Pab and IS6110 was
<table>
<thead>
<tr>
<th>Sample</th>
<th>Region of lymph nodes</th>
<th>Bacteriological examination(^a)</th>
<th>Period between onset and biopsy(^b) (month)</th>
<th>Antituberculous therapy before biopsy</th>
<th>PCR results</th>
<th>Clinical efficacy of chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Right supraclavicular</td>
<td>Smear (+) in sputum</td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>Cured</td>
</tr>
<tr>
<td>2</td>
<td>Right supraclavicular</td>
<td>Smear (−) and culture (−) in sputum</td>
<td>1</td>
<td>−</td>
<td>+</td>
<td>Cured</td>
</tr>
<tr>
<td>3</td>
<td>Mesentery</td>
<td>Smear (+) and culture (+) in ascites</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>Cured</td>
</tr>
<tr>
<td>4</td>
<td>Right and left supraclavicular</td>
<td>Smear (−) and culture (−) in sputum, bone marrow, stool and urine</td>
<td>1</td>
<td>−</td>
<td>+</td>
<td>Cured</td>
</tr>
<tr>
<td>5</td>
<td>Right submaxilla</td>
<td>Smear (−) and culture (−) in sputum, TBLB(^c) in stool and urine</td>
<td>3</td>
<td>−</td>
<td>−</td>
<td>Cured</td>
</tr>
<tr>
<td>6</td>
<td>Left axilla</td>
<td>n.d.(^d)</td>
<td>1</td>
<td>−</td>
<td>−</td>
<td>Cured</td>
</tr>
<tr>
<td>7</td>
<td>Left supraclavicular</td>
<td>n.d.</td>
<td>12</td>
<td>−</td>
<td>−</td>
<td>Cured</td>
</tr>
<tr>
<td>8</td>
<td>Left submaxilla</td>
<td>n.d.</td>
<td>4</td>
<td>−</td>
<td>−</td>
<td>Cured</td>
</tr>
</tbody>
</table>

\(^a\)Smear and culture of *M. tuberculosis* in clinical specimens other than lymph node.

\(^b\)Period between clinical onset of lymphadenitis and lymph node biopsy.

\(^c\)Transbronchial lung biopsy

\(^d\)n.d., not done.
evaluated using serial 10-fold dilutions of mycobacterial suspension mixed with paraffin-embedded tissue. The detection limit of all tested PCR was 100 CFU of H37Rv strain (data not shown).

Tissue slices from eight paraffin-embedded lymph nodes and five lung tissue samples were stained with acid-fast (Ziehl-Neelsen) staining for microscopical examination of mycobacteria, but no acid-fast organism was detected. However, all eight lymph nodes contained microscopical epithelioid cell granuloma with caseous necrosis and Langhans giant cells as described above. On the other hand, the results of PCR of eight paraffin-embedded lymph node tissues are shown in Fig. 1. Three PCR products of the same size, as expected, were obtained from four of eight lymph node tissues as well as positive control samples, while the other four lymph nodes did not show any PCR product. However, all eight samples were positive for PCR of GAPDH, indicating that the DNA extraction was correctly performed in each sample. Furthermore, the results of PCR using DNA from cancerous and non-tuberculous lung tissues were all negative except the PCR for GAPDH (data not shown).

**Discussion**

Many investigators have recently described the rapid detection of *M. tuberculosis* in clinical samples using molecular techniques such as PCR with high specificity and sensitivity (Miyazaki et al. 1993; Noordhoek et al. 1994; Hashimoto et al. 1996; Portillo et al. 1996). In addition, detection of
mycobacteria in biopsy material such as lymph node, by similar techniques has also been reported (Narita et al. 1992; Sugita et al. 1994; Hardman et al. 1996). However, few papers have reported the usefulness of PCR for retrospective confirmation of tuberculous lymphadenitis diagnosed by pathological feature without bacteriological examination (Diaz et al. 1996; Ding et al. 1995). The present study extended these early findings by demonstrating the usefulness of PCR in the detection of *M. tuberculosis* in paraffin-preserved tuberculous lymph nodes.

The sensitivity of each type of PCR was the same, with a detection limit of 100 CFU of *M. tuberculosis*. However, our previous results showed that the detection limit in vitro was 10 fg of template DNA corresponding to 2 CFU of *M. tuberculosis* (Miyazaki et al. 1993; Hashimoto et al. 1996). The discrepancy in the sensitivity of PCR in the two studies may be due to the additional DNA extraction steps performed in paraffin-embedded tissue. These may result in a loss of DNA compared with the standard DNA extraction method using clinical samples such as the sputum. Therefore, a more efficient DNA extraction method from paraffin-embedded tissue should be developed to avoid false-negative PCR results. As for comparison of the sensitivities of the three kinds of PCR, the sensitivity was similar in all types, indicating that even a single-step PCR of *IS6110* has a good sensitivity compared with other nested PCR methods.

The DNA fragment of *M. tuberculosis* was amplified by all PCR primer sets in four of eight tuberculous lymph nodes, indicating that these samples contained *M. tuberculosis* rather than MOTT. Although the cross-contamination of the PCR products is a serious problem in laboratory, the positive results of multiple PCR sets targeting each different gene such as our study may not only distinguish the causative organisms, but support to deny the DNA contamination. The 50% (4/8) positive rate of PCR in the present study was evaluated to be considerable, because mycobacteria were not detected in any lymph node by acid-fast staining. However, since four PCR negative samples were present, we carefully analyzed the clinical records of all eight patients in order to investigate the reason of negative PCR result (Table 1). Neither the region of lymph nodes nor clinical efficacy of chemotherapy influenced on the results of PCR. The lymph node biopsy had been performed within one month from the clinical onset of lymphadenitis in three of four PCR positive cases, and within 9 months in another case of recurrent tuberculosis. Thus, the average period between the onset of lymphadenitis and biopsy was 3 months in PCR positive cases. In contrast, the average period was 5 months in PCR negative cases. Although the sample number was too small to allow for any meaningful statistical analysis, our results suggest that lymph node biopsy followed by PCR should be performed during the early stage of the disease.

We also investigated the influence of antituberculous therapy on the results of PCR. Two of the four PCR positive cases had received antituberculous
treatment before biopsy (Table 1). In contrast, the other six patients, including four PCR negative cases, were not treated before biopsy. While one cannot make a firm conclusion regarding the effect of treatment on the results of PCR, our results indicate that the PCR method is sensitive enough for the detection of \textit{M. tuberculosis} even in patients who had received antituberculous agents. Similar observations were also reported by Yuen et al. (1993) in patients with pulmonary tuberculosis.

In conclusion, our results showed that PCR is not only a rapid and sensitive diagnostic method for tuberculous lymphadenitis, but also clinically significant in retrospective study for detecting \textit{M. tuberculosis} even in some preserved lymph node tissues without evidence of acid-fast stained organisms.

References


