

Rapid Detection of *Mycobacterium tuberculosis* in Clinical Samples by Polymerase Chain Reaction

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MENEVŞE, A., ÜLKÜER, M. and SULTAN, N. *Rapid Detection of Mycobacterium tuberculosis in Clinical Samples by Polymerase Chain Reaction*. Tohoku J. Exp. Med., 1996, 179 (1), 1-9 — A polymerase chain reaction for the rapid and specific detection of *Mycobacterium tuberculosis* has been used and evaluated for clinical applicability. Two oligonucleotide primers derived from the nucleotide sequence of a immunogenic protein MPB 64 amplified DNA from *M. tuberculosis* and *M. bovis*. No amplification was observed from any of ten different mycobacterial strains. A total of 126 clinical samples were amplified and tested by both dot blot hybridization and restriction enzyme analysis. *M. tuberculosis* was detected by PCR in 38 smear and 42 culture positive cases. An additional 16 culture negative specimens were PCR positive yielding an overall *M. tuberculosis* positivity rate of 46.0% (58/126) compared to 33.3% (42/126) by culture. The superior sensitivity of PCR to culture was more evident in non-pulmonary cases where PCR picked up 10 cases in addition to 6 culture positives out of 46 specimens. On the other hand, out of 80 pulmonary specimens only 6 cases in addition to 36 culture positives were picked up by PCR. The specificity of PCR was confirmed with dot blot hybridization and restriction enzyme analysis. This study corroborates that PCR offers a more sensitive and rapid alternative for the detection of *M. tuberculosis* to culture, and that it can be used in uncultured clinical specimens. ——— *M. tuberculosis*; diagnosis; PCR; restriction enzyme

Tuberculosis is a disease of increasing importance. In developing countries it remains a major health problem, causing an estimated annual rate of greater than 2,500,000. The human immunodeficiency virus (HIV) epidemic is also having a profound impact on the tuberculosis problem in both industrialized and developing countries. Improved case detection and effective treatment are two of the key factors required to better control mycobacterial diseases (Styblo 1989).

The conventional methods of laboratory diagnosis of tuberculosis are smear examination for acid-fast bacilli and cultural identification. Though these have been very useful, they have serious limitations. Smear examination requires a minimum of about 10,000 organisms/ml for detectability and does not

differentiate the pathogenic mycobacterium from contaminant. Culture is much more sensitive than direct smear examination and provides definitive identification, but requires 6–8 weeks. Furthermore, in paucibacillary cases such as non-pulmonary tuberculosis, the efficacy of cultural detection is rather low (Bates 1979). The radiometric BACTEC system (Cormican et al. 1992) and the enzyme immunoassay for the identification of mycobacteria with monoclonal antibodies (Kadival et al. 1989) have reduced the time needed for detection, but they are still dependent on the growth of bacteria.

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 (Eisenstein 1990). Many primers which amplify specifically the DNA of *Mycobacterium tuberculosis* have been designed and successfully used for identification of this microorganism from culture and also from clinical specimens (Fiss et al. 1992; Shawar et al. 1993). The necessity of further efforts to establish the sensitivity of this technology and to develop routine and simple procedures for application in developing countries is important. In the present study, we evaluated the direct detection of *M. tuberculosis* from clinical specimens through PCR amplification followed by restriction enzyme digestion and dot blot hybridization of PCR products to detect and speciate mycobacteria in uncultured specimens.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains were provided from the strain collection of the Refik Saydam Institute, Ankara. Mycobacterial strains used in this study include *M. tuberculosis*, *M. bovis*, *M. tuberculosis H37Ra*, *M. tuberculosis H37Rv*, *M. avium*, *M. chelonae*, *M. flavescens*, *M. fortuitum*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. smegmatis*, and *M. vaccae*.

Clinical specimens

Expectorated sputum and other specimens were obtained at random from 126 patients who were admitted to the Atatürk Chest Diseases and Chest Surgery Center and the Gazi Hospital with suspected of having tuberculosis. Clinical specimens included 70 sputa, 10 pleural fluid, 11 cerebrospinal fluid (CSF), 13 lymph nodules, 15 urine specimens and 6 endometrial biopsies. All the specimens were processed for direct smear examination and culture on Lowenstein-Jensen medium by standard methods (Clarridge et al. 1993), and were decontaminated by the N-acetyl-L-cysteine sodium hydroxide as appropriate (e.g., sputum), or were used directly (e.g., CSF). Two-thirds of each sample was used for routine smears and culture, and one-third of each sample (approximately 1 ml) was subjected to lysis for DNA extraction. Suspensions of known cultures were used to evaluate the efficiency of lysis and the sensitivity and specificity of the PCR test. None

of the patients had a positive result in the serological test for HIV or had anti-tuberculous drugs. Twenty-nine sputum samples from patients with diseases other tuberculosis were used as controls.

DNA extraction from bacterial strains and clinical specimens

DNA was extracted from bacterial cultures as described previously (Pao et al. 1988). Briefly, the bacteria were suspended in a solution containing 2 mg/ml lysozyme, 0.15 M NaCl, 25% sucrose, 0.1 M EDTA and 50 mM Tris HCl, pH 8.0. After incubation at 37°C for 30 min, 8 volumes of 0.1 M Tris HCl buffer, pH 9.0 containing 0.1% sodium lauryl sulfate (SDS) and 0.1 M NaCl, were added. After repeated phenol-chloroform extractions, the DNA was ethanol-precipitated. The DNA concentration was determined by spectrophotometry and confirmed by agarose gel electrophoresis. Ten ng of each DNA was used for PCR.

For sputum specimens, a portion of the concentrated material was taken for DNA extraction. For all body fluids, 1 ml of the fluid was spun and the sediment was used. These were suspended in a solution containing 0.1 M NaOH, 1.0 M NaCl, 0.5% SDS. The tubes were incubated in a 95°C water bath for 15 min following which phenol-chloroform extraction and ethanol-precipitation were done.

The tissue samples were minced and digested with proteinase K (100 µg/ml) at 37°C overnight in a solution containing 100 mM NaCl, 10 mM Tris HCl, pH 8.0, 25 mM EDTA, and 0.5% SDS. This was followed by phenol-chloroform extraction and ethanol precipitation. The DNA pellets were dissolved in 30 µl of distilled water and 10 µl of this was used for the PCR reaction.

Amplification of DNA

A 240 base pair region (nts 460-700) from MPB 64 protein coding gene (Yamaguchi et al. 1989) was selected for amplification. Two oligonucleotide primers within the MPB64 sequence, designated primer MT1 (5'-TCCGCTGC-CAGTCGTCTTCC-3') and MT2 (5'-GTCCTCGCGAGTCTAGGCCA-3') were synthesized by using a DNA synthesizer (Promega Corp., Madison, WI, USA). Amplification was performed by using a DNA Thermal Cycler and a Gene Amp kit with Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA).

Aliquots (5 µl) of DNA samples were amplified, as previously described (Forbes and Hicks 1993), in a 100 µl reaction mixture contained 50 mmol KCl, 10 mmol Tris HCl, pH 8.3, 1.5 mmol MgCl₂, 0.01% gelatin (w/v), 0.1% Triton X-100, 200 µmol of each of the four dNTPs, 100 ng of each of the two primers, and 2.5 U of Taq polymerase. Amplification was carried out for 30 cycles (2 min 94°C, 2 min at 60°C and 2 min at 72°C). Positive and negative controls were included in each set of specimens tested. With each series of tests a negative control containing all the ingredients except DNA template was included. *M. bovis* DNA was used as a positive control.

Analysis of amplified products

After amplification, 10 μ l of PCR reaction mixture was run in a 2% agarose gel. After being stained with ethidium bromide, PCR products were visualized and photographed under uv transillumination. Hae III fragments of ϕ X 174 were used as molecular weight standards.

To prepare dot blots, 10 μ l of amplified product was denatured with 0.5 M EDTA and 10 M NaOH at 37°C for 30 min. Then the denatured product was spotted on to probind 45 nylon filters by using a minifold system (Pharmacia LKB Biotechnology, Bromma, Sweden). The PCR product of *M. bovis* DNA was used as a probe after being labeled using non-isotopic methods. Briefly, non-isotopic probe was prepared by reamplification of the 240 base pair product which had been gel purified using agarose. PCR was conducted as described, except dTTP was replaced by mixture of digoxigen-labeled dUTP and dTTP (molar ratio : 0.35 dUTP : 0.65 dTTP). Hybridization and detection was performed by using methods of Boehringer-Mannheim (Genius™ kit; Boehringer-Mannheim, Indianapolis, IN, USA).

To restriction enzyme digestion, 15 μ l of the amplified reaction mixture was transferred to a fresh tube and digested with 20 units of Sma I at 30°C for overnight. The samples were fractionated by electrophoresis in a 2% agarose gel. The DNA bands were visualized by ethidium bromide fluorescence. After PCR analysis, results of conventional culture were obtained and compared.

RESULTS

Specificity and sensitivity of the PCR

The primers MT1 and MT2 were tested in the PCR with isolated or purified chromosomal DNAs from 12 different mycobacterial species, three unrelated bacterial species and one eucaryotic DNA in addition to the *M. tuberculosis* H37Ra and H37Rv genomes (Table 1). A 240 base pair amplification product was seen when *M. tuberculosis* (including H37Ra and H37Rv) and *M. bovis* DNAs were used as template: none of the other templates tested revealed any amplified band. Further, none of the non-tuberculosis control specimens was positive by PCR. An example of the results is shown in Fig. 1. These results demonstrate that the 240 base pair amplification product was found only with *M. tuberculosis* and *M. bovis*.

The sensitivity of the PCR with the primers used in this study was determined by preparing a purified *M. tuberculosis* DNA stock solution. Ten fold serial dilutions (10^1 – 10^7) were prepared in 10 mM Tris pH 8.0, 1 mM EDTA and were adjusted to a 0.5 Mc Farland standard. DNAs of dilutions could be amplified sufficiently and reproducibly to give a clearly detectable band in an agarose gel (Fig. 1). When mycobacterial suspensions were prepared on the basis of absorbance values and tested in the PCR, 10 *M. tuberculosis* bacteria gave a

TABLE 1. *Specificity of the PCR based on the DNA sequence of MPB64 gene of M. tuberculosis*

Species	Presence of the 240 base pair PCR product
<i>M. tuberculosis</i>	+
<i>M. bovis</i>	+
<i>M. tuberculosis H37Ra</i>	+
<i>M. tuberculosis H37Rv</i>	+
<i>M. avium</i>	—
<i>M. chelonae</i>	—
<i>M. flavescens</i>	—
<i>M. fortuitum</i>	—
<i>M. intracellulare</i>	—
<i>M. kansasii</i>	—
<i>M. marinum</i>	—
<i>M. scrofulaceum</i>	—
<i>M. smegmatis</i>	—
<i>M. vaccae</i>	—
<i>Staphylococcus aureus</i>	—
<i>Streptococcus pyogenes</i>	—
<i>Haemophilus influenza</i>	—
Human leucocytes	—

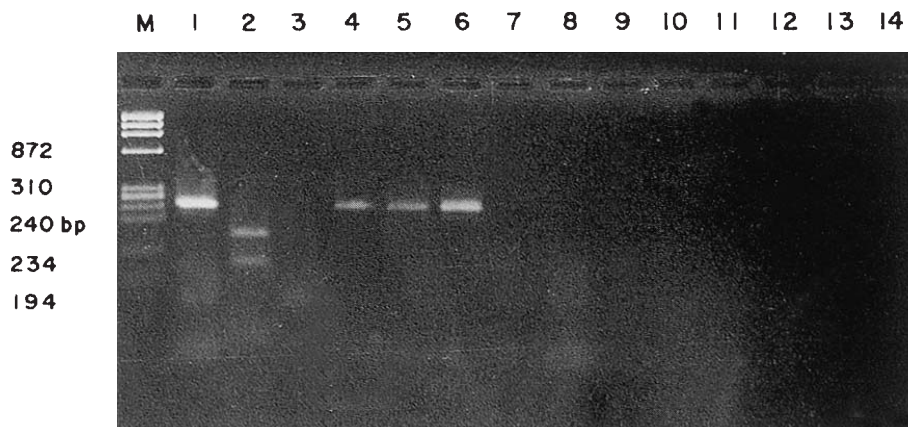


Fig. 1. Analysis of PCR products by agarose gel electrophoresis. Numbers at the left indicate the sizes of DNA fragments (in base pairs). Lane M, Hae III-digested ϕ X174 used as a size marker; Lane 1, *M. bovis* DNA (uncut PCR product); Lane 2, Sma I digests of PCR products from *M. bovis*; Lane 3, template-negative control; Lane 4 to 6, 10, 100 and 1,000 *M. tuberculosis* bacilli per PCR vial, respectively (made from a series of 10 fold dilution steps of an *M. tuberculosis* suspension); Lane 7, *M. avium*; Lane 8, *M. flavescens*; Lane 9, *M. intracellulare*; Lane 10, *M. kansasii*; Lane 11, *M. marinum*; Lane 12, *M. scrofulaceum*; Lane 13, *M. smegmatis*; Lane 14, *M. vaccae*.

TABLE 2. *Comparison of PCR with conventional methods for detection of M. tuberculosis in clinical specimens*

Source of specimen	Number tested	Smear (+)	Culture (+)		Culture (–)	
			PCR (+)	PCR (–)	PCR (+)	PCR (–)
Pulmonary	80	35	36	0	6	38
Non-pulmonary	46	3	6	0	10	30
Total	126	38	42	0	16	68

clearly detectable band on agarose gels. Concentration of bacilli in these solutions was confirmed by culturing corresponding samples and counting colonies (data not shown).

Clinical specimens

Sputum samples from 29 patients with bronchiectasia and chronic bronchitis other than tuberculosis were all negative in the PCR, indicating the absence of false-positive results. Table 2 provides the results of the PCR, culture, and smear of various pulmonary and non-pulmonary specimens from 126 different patients with suspected tuberculosis. The PCR was done without knowledge of the clinical data or the results of smear and or culture, since those data were obtained elsewhere. A total of 38 specimens were positive on smear examination and 42 were positive by culture. All the 42 specimens which yielded *M. tuberculosis* by culture were also positive by PCR.

PCR was positive in an additional 16 specimens which were negative by smear and culture, giving an overall positivity rate of 46.0% (58/126) as compared to 33.3% (42/126) by culture. When the clinical specimens were divided into those from pulmonary and from non-pulmonary cases, the high sensitivity of PCR over culture was more evident in non-pulmonary specimens. While PCR detected 6 additional cases over 36 culture positives out of 80 pulmonary specimens. Ten additional cases over 6 *M. tuberculosis* cases were picked up by PCR out of 46 non-pulmonary specimens. In other words, PCR increased detectability of pulmonary cases by 7.5%, and of non-pulmonary cases by 21.7%. Using MT1 and MT2 as primers for PCR, we obtained specific amplification of 240 base pair DNA fragment, coding for a portion of MPB64 protein (Fig. 1). The amplified fragment contains an internal Sma I restriction site which permits the confirmation of the amplified fragment by simple digestion. In this way, to confirm that the amplified 240 base pair region corresponds to original nucleotide sequence, we digested the PCR product of *M. bovis* with Sma I, and obtained fragments of the expected sizes as 90 base pair and 150 base pair bands (Fig. 1). The specificity of the 240 base pair product was also confirmed by dot blot hybridization using the digoxigenin-labeled amplified product (data not shown).

No additional positives were detected by hybridization.

DISCUSSION

The capacity of PCR specifically to amplify fragments of DNA has been extensively investigated for the diagnosis of the infectious diseases. It seems to be particularly suitable for the detection of pathogens present in small numbers, or which are difficult or impossible to culture. Tuberculosis represents such a challenge and has stimulated several research groups to investigate the application of PCR in this area (Kolk et al. 1992). In PCR, the specificity is ensured by the choice of the amplified sequence (Lassence et al. 1992). The data presented here indicate that the PCR, in which a 240 base pair fragment is amplified from *M. tuberculosis* chromosomal DNA, meets a sensitive, specific, and rapid test requirements. The specific DNA fragment can be detected in small amount of purified DNA, which represents the amount of DNA in only 10 organisms. The PCR test is specific for *M. tuberculosis*. In this respect, the amplified 240 base pair fragment seems to be species specific for *M. tuberculosis* and *M. bovis*, for it was not present in any of the different mycobacterial species that we assayed in the present study (Fig. 1). Amplification of this fragment has also been attempted with other organismic DNAs, and the results appear to confirm the presence of this DNA fragment exclusively within the *M. tuberculosis* genome (Table 1).

The sensitivity of the PCR is also dependent on the efficiency of the DNA extraction procedures. Although not shown here, preparing samples (especially sputum) by boiling simplifies the test so that it can be used routine clinical practice.

The high degree of sensitivity was also evident in comparative studies since PCR detected 58/126 cases while culture was positive in 42 cases (Table 2). PCR for rapid diagnosis of tuberculosis has also been used by Manjunath et al. (1991) and Noel et al. (1991). These authors also detected all the 17/117 and 15/34 culture-positive cases, and additional 14 and 2 culture-negative cases by PCR, respectively. All culture-positive specimens, including four that were smear negative, were positive with PCR. Of the 84 smear and culture-negative, obtained from patients clinically suspected of tuberculosis, 16 were positive with PCR, suggesting that the PCR is more sensitive than the culture by detecting fewer viable mycobacteria. However, in cases where the sensitivity is increased beyond the gold standard of culture, specificity must be carefully ascertained to rule out false positivity. The specificity of PCR is controlled by the DNA segment chosen for amplification. We used primers for MPB64 protein coding gene because this gene has been sequenced by the elegant method of Yamaguchi et al. (1989) and shown to be highly specific to the *M. tuberculosis* complex (Shankar et al. 1990). A comparison of protocols involving three mycobacterial DNA sequences, IS6110, 65kDa antigen and MPB64 protein have recently indicated that although the protocols involving the three DNA sequences were able to

detect tuberculosis in the specimens, the repeat copy sequence IS6110, and the 65kDa gave unacceptably large numbers of false positive results, 62% and 33%, respectively (Lee et al. 1994). In the present study, the use of a species-specific sequence of MPB64 protein coding gene prevents the need for further confirmation. In fact, restriction enzyme digestion of the PCR product by Sma I (Fig. 1) and dot blot hybridization (not shown) by using the digoxigenin-labeled amplified product neither increased the sensitivity nor specificity over agarose gel electrophoresis. The fact that a specific diagnosis could be made by simple agarose gel electrophoresis would make the PCR test especially suitable for the use in developing countries. The mean time required to achieve a positive culture result is 2–3 weeks on Lowenstein-Jensen medium in contrast to the PCR test requiring maximum only two to three days.

In non-pulmonary tuberculosis cases tuberculosis culturing often produces negative results and causes an unacceptable delay in diagnosis. The high sensitivity of PCR raises hope for more rapid diagnosis of these infections. In our study, PCR increased the detectability rate by 7.5% in pulmonary cases and 21.6% in non-pulmonary cases suggesting the practical use of PCR for diagnosis of non-pulmonary tuberculosis in a case difficult to diagnose conventionally (Table 2).

In conclusion, PCR is superior to the currently available methods for diagnosis of tuberculosis in terms of specificity, sensitivity and rapidity. It is feasible for use in developing countries since detection can be achieved by simple agarose gel electrophoresis.

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