JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1990, p. 2437–2441
0095-1137/90/112437-05$02.00/0

Vol. 28, No. 11

Direct Detection of *Mycobacterium tuberculosis* in Clinical Specimens by DNA Amplification

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Received 25 April 1990/Accepted 16 August 1990

A polymerase chain reaction assay was developed for the amplification of a 336-bp repetitive fragment in the chromosome of *Mycobacterium tuberculosis*. The assay is specific for *M. tuberculosis* and can be used to detect the amount of DNA present in less than 10 organisms. It was used to demonstrate *M. tuberculosis* DNA in 14 of 26 clinical specimens (from cerebrospinal fluids, pleural fluid aspirates and biopsies, pericardial fluid aspirates, and an open lung biopsy), and it was shown to be at least as sensitive as conventional culture techniques with these specimens.

The laboratory diagnosis of tuberculosis is based on the traditional method of the Ziehl-Neelsen acid-fast stain and on laboratory culture of the causative organism, *Mycobacterium tuberculosis*. The Ziehl-Neelsen stain, although rapid and inexpensive, lacks sensitivity and in a sputum specimen, for example, can only be used to detect acid-fast bacilli in concentrations of 10,000 organisms per ml or greater (14). It also lacks specificity and cannot be used to distinguish between the various members of the *Mycobacteriaceae*. In the era of immunocompromised and acquired immunodeficiency syndrome patients, this problem becomes pertinent, as infections with mycobacteria other than *M. tuberculosis* in these patients are common. The laboratory culture of *M. tuberculosis* is sensitive (10 to 100 viable organisms per sample [8]), but because of the long culture periods required, clinical and therapeutic decisions have to be made before the laboratory diagnosis becomes available. Culture techniques also require viable organisms, and this is often a problem in partially treated patients. Because of the shortcomings of these traditional methods, several rapid detection methods have been developed (2, 6, 7; C. E. Musial and G. D. Roberts, Clin. Microbiol. Newsl. **9**:89–91, 1987). However, none of these meet all the requirements of rapidity, sensitivity, and specificity, and they often require sophisticated equipment and highly trained personnel.

Several investigators have developed DNA probes for the detection of *M. tuberculosis* (4, 9, 11), and one DNA probe is commercially available (Gen-Probe, San Diego, Calif.). The sensitivity of these probes, however, is similar to that of Ziehl-Neelsen stain, and their usefulness for the detection of *M. tuberculosis* in clinical specimens is therefore questionable. This lack of sensitivity of DNA probes was the motivation, in part, for the development of the polymerase chain reaction (PCR) as a diagnostic tool (12).

PCR is now a well-developed technique and has been used extensively for the diagnosis of numerous infectious diseases, including some with a bacterial etiology (5). A few PCR assays have also been developed for *M. tuberculosis*. Brisson-Noël and colleagues (1) amplified a 383-bp DNA fragment which encodes part of the 65-kDa mycobacterial antigen. By using species-specific oligonucleotide probes, they were able to distinguish members of the *M. tuberculosis* complex from the *Mycobacterium avium-Mycobacterium intracellulare* complex and from *Mycobacterium fortuitum*. Eisenach and colleagues (3) amplified a 123-bp fragment which was repeated several times in the chromosome. This assay was also not able to distinguish members of the *M. tuberculosis* complex. Patel and colleagues (10) used low primer-annealing temperatures for their assay and demonstrated marked nonspecific amplification from nontuberculous mycobacteria. A PCR product that was unique for *M. tuberculosis* was isolated and was used as a probe in this system. In an alternative assay, Shankar and colleagues (13) amplified a 240-bp fragment which encodes part of the MPB 64 protein. They used a 17-mer oligonucleotide probe which was homologous to the central region of the amplified fragment. They were also not able to distinguish between members of the *M. tuberculosis* complex. Here we present data for an alternative PCR assay, in which the target DNA sequence is a repetitive element which is present in the *M. tuberculosis* genome. The technique is rapid, sensitive, and specific for *M. tuberculosis*.

MATERIALS AND METHODS

Preparation of mycobacterial DNA. A type culture strain of *M. tuberculosis* H37RV was obtained from the diagnostic Medical Microbiology laboratory at Groote Schuur Hospital, while strains of *M. avium*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. marinum*, and *M. scrofulaceum* were obtained from the Tuberculosis Research Institute (Pretoria, South Africa). *M. bovis* BCG (Tokyo strain) was obtained from the State Vaccine Institute, Pinelands, Cape Town, South Africa, and was cultured on Sautons broth, while all the other mycobacterial strains were cultured on Lowenstein-Jensen medium. The bacteria were harvested, and the DNA was extracted as follows. The cells were suspended in 2 ml of TES buffer (Tris hydrochloride, pH 8.5, 10 mM; EDTA, 1 mM; NaCl, 150 mM) and heated at 70°C for 30 min. The cells were lysed by the addition of 10% sodium dodecyl sulfate (1.5%, final concentration) and an equal volume of buffered phenol. The lysates were mixed at 37°C for 3 h on an orbital shaker to ensure complete lysis and to enhance the extraction of proteins and lipids. The lysates were centrifuged (15 min in a Beckman Microfuge), and the aqueous supernatants were extracted twice with an equal

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FIG. 1. Autoradiograph of *M. tuberculosis* chromosomal DNA digested with *Bam*HI and hybridized to the *Kpn*I-*Sma*I fragment of p36. The DNA fragments were separated by agarose gel electrophoresis and transferred onto Hybond-N membrane prior to hybridization.

volume of chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was precipitated by the addition of an equal volume of polyethylene glycol (PEG 6000; Sigma) in 2.5 M NaCl at room temperature. The DNA was harvested 15 min after the addition of the PEG by centrifugation for 15 min in a Beckman Microfuge. The DNA pellet was washed twice with 70% ethanol, dried in a Speed-Vac concentrator (Servant), and finally dissolved in sterile distilled water at a final concentration of 0.1 to 0.3 μg/μl. This procedure gave yields of 50 to 300 μg of DNA per g of wet cells, with an absorbance ratio (260 to 280 nm) of greater than 1.75.

Isolation of DNA from clinical specimens. Pleural fluid aspirates were obtained from and biopsies were performed on patients with pleural effusions in the Medical Wards of Groote Schuur Hospital, and all samples were sent for routine microscopy and culture. Samples of these were made available to us for the PCR assay. The pleural fluids (2 ml) were mixed with an equal volume of PEG as described for the extraction of mycobacterial DNA. The precipitated

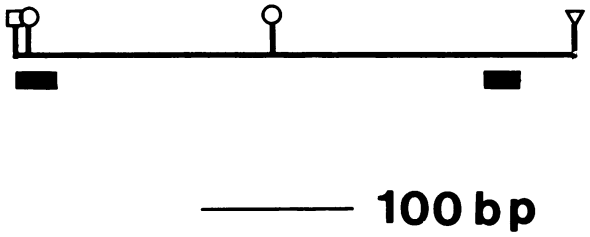

FIG. 2. Restriction enzyme map of the *Kpn*I-*Sma*I subclone of the *M. tuberculosis* recombinant, p36. The target DNA fragment for amplification by PCR is 336 bp, and it is flanked by *Kpn*I-*Sma*I restriction sites. Symbols: ○, *Ava*I; ▽, *Kpn*I; □, *Sma*I; ▬, primer binding sites.

material was dissolved in 500 μl of TES buffer. Buffered phenol (500 μl) and 10% sodium dodecyl sulfate (500 μl) were added, and the mixture was extracted by shaking at 37°C for 3 h. Following centrifugation, the aqueous supernatant fluids were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). Following a second PEG precipitation, the DNA pellets were dissolved in 30 μl of sterile distilled water.

Cerebrospinal fluids (CSF) and pericardial fluids were treated in a way similar to that for the pleural aspirates, and the lung biopsy sample was cut into small fragments prior to extraction with 10% sodium dodecyl sulfate and buffered phenol.

DNA amplification. The DNA target for amplification is a 336-bp sequence in the *M. tuberculosis* genome. We had previously cloned a 5.5-kbp *Mbo*I DNA fragment (p36) from this organism and had shown by hybridization studies that the insert hybridized to multiple bands of *M. tuberculosis* chromosomal DNA digested with a variety of restriction enzymes and separated by agarose gel electrophoresis. The element largely responsible for this phenomenon was contained on a 375-bp *Kpn*I-*Sma*I fragment of the original clone (Fig. 1). A portion of this fragment was used as the target for amplification by PCR. Two 25-mer oligonucleotide primers were synthesized by Beckman (SA) and had the following sequences: 5′-GCGGCTCGGGCGGCGTCGGTGGCTT-3′ and 5′-GCCAGAACCGACCAACCCGCCGATA-3′. The first primer hybridizes adjacent to the *Sma*I site, while the second binds 39 bp from the *Kpn*I site within the target DNA (Fig. 2).

Mycobacterial chromosomal DNA (10^{-14} to 10^{-8} g) or 15 μl of the clinical specimen DNA preparations was added to a PCR mixture (final volume, 100 μl). The reaction mixture consisted of 16.6 mM $(NH_4)SO_4$, 67 mM Tris hydrochloride (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.01% gelatin (Difco), 10% dimethyl sulfoxide, 200 mol of each deoxynucleoside triphosphate; and 1 to 2 μmol of each of the oligonucleotide primers. The reaction mixture was preheated at 95°C for 10 min and placed on ice, and 2.5 U of *Taq* polymerase (Cetus Corporation) was added. Thermal cycling (30 to 40 cycles) at 95° for 1 min and at 70°C for 2 min was used to amplify the target sequence. These heating cycles were performed in a custom-made thermal cycling machine.

Following amplification, the DNA was concentrated by alcohol precipitation and dissolved in 20 μl of sterile distilled water.

Agarose gel electrophoresis and autoradiography. p36 DNA was digested with *Kpn*I and *Sma*I, and the 375-bp fragment was purified from a 3% agarose gel by electroelution. The purified fragment was radiolabeled by nick translation in the

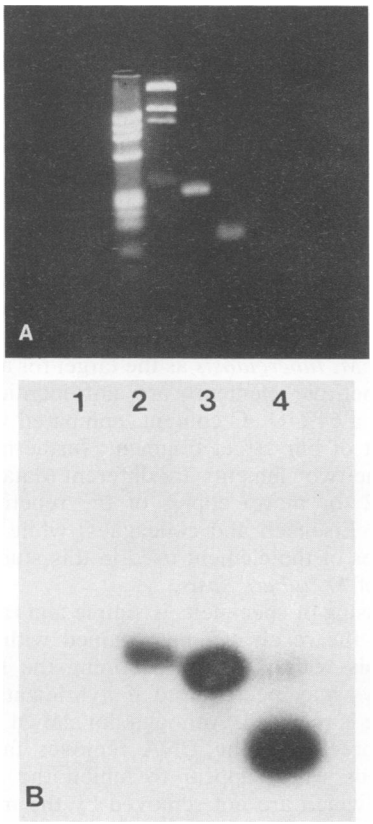

FIG. 3. Amplification of *M. tuberculosis* chromosomal target sequences. (A) Ethidium bromide-stained agarose gel. (B) Autoradiograph of the transferred DNA hybridized to the *Kpn*I-*Sma*I fragment of p36. Lanes: 1, ϕX174 DNA cut with *Hae*III; 2, recombinant plasmid p36 cut with *Kpn*I and *Sma*I; 3, target DNA fragment amplified from 10 pg of *M. tuberculosis* chromosomal DNA; 4, target DNA fragment cut with *Ava*I.

presence of [^{32}P]dCTP (Amersham) and was used as the hybridization probe.

All amplified products were analyzed by agarose gel electrophoresis (3% agarose) in TAE buffer (Tris acetate, 40 mM; EDTA, 1 mM). The DNA was transferred to a Hybond-N membrane and hybridized with the radiolabeled probe. Autoradiography of the hybridization products was for 3 to 72 h.

RESULTS

Amplification of purified mycobacterial DNA. The amplified DNA products, obtained from 1 ng of purified genomic *M. tuberculosis* DNA, were separated by agarose gel electrophoresis and transferred to Hybond-N membranes (Fig. 3). The amplified material migrated as a 336-bp band and contained an *Ava*I site, as predicted by the restriction enzyme map (Fig. 3A). It also hybridized to the DNA probe prepared from p36, confirming that the amplified product was the target DNA fragment (Fig. 3B).

The number of DNA bands amplified from *M. tuberculosis* DNA was dependent on the temperature of primer annealing. One band was amplified at an annealing temperature of 70°C, while at an annealing temperature of 55°C, ten bands were visible. More bands were present when annealing was performed at 37 or 45°C (data not shown). All subsequent amplifications were performed with a primer-annealing temperature of 70°C to ensure maximum specificity.

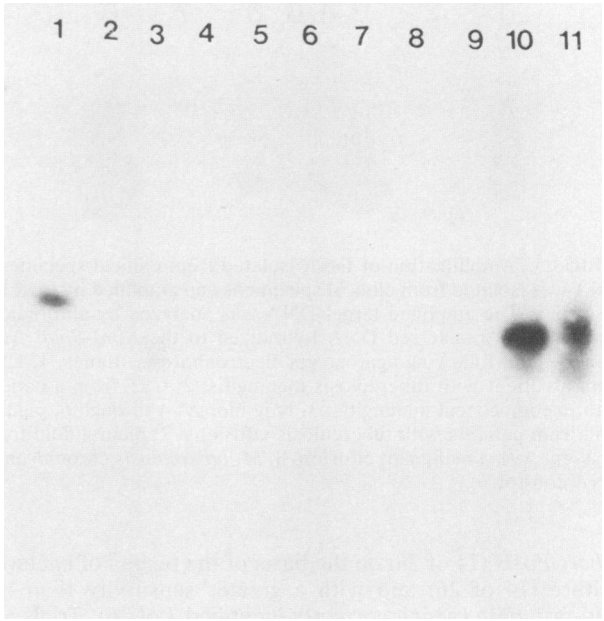

FIG. 4. Amplification of DNA isolated from other members of the *Mycobacterium* genus. Chromosomal DNA (10 ng) was amplified for 30 cycles at 95°C for 1 min and 70°C for 2 min. The amplified target DNA was analyzed by autoradiography of the transferred DNA hybridized to the *Kpn*I-*Sma*I fragment of p36, following agarose gel electrophoresis. Lanes: 1, recombinant plasmid p36 cut with *Kpn*I and *Sma*I; 2, *M. avium*; 3, BCG; 4, *M. fortuitum*; 5, *M. gordonae*; 6, *M. intracellulare*; 7, *M. kansasii*; 8, *M. marinum*; 9, *M. scrofulaceum*; 10, *M. tuberculosis* (10 pg of chromosomal DNA); 11, *M. tuberculosis* (1 pg of chromosomal DNA).

To determine the specificity of the primers for *M. tuberculosis*, 10 ng of chromosomal DNA, prepared from eight other mycobacterial species, was also used as the template for the PCR. With the exception of strain BCG, no amplification products were detected with the non-*M. tuberculosis* DNA by either agarose gel electrophoresis or hybridization (Fig. 4). A band with a mobility corresponding to a size of 260 bp was present in the BCG assay. It is therefore possible to distinguish the amplification products obtained from *M. tuberculosis* and BCG in this case. The lower limit of detection of *M. tuberculosis* DNA in the assay was determined by diluting the DNA to 10^{-14} g per assay. An amplification product was obtained at this dilution, which corresponds to the amount of DNA present in less than 10 microorganisms (9). With 1 pg of chromosomal DNA, the amplified product could be detected by agarose gel electrophoresis alone, while when 10 fg of chromosomal DNA was used, DNA hybridization and autoradiography were necessary for the detection of the amplified target DNA (data not shown).

Detection of *M. tuberculosis* DNA in clinical specimens. An example of the amplification of *M. tuberculosis* DNA in three types of clinical specimens (CSF, pleural effusion, and lung biopsy) is shown in Fig. 5. These and additional results are summarized in Table 1. The amplified target fragment is not always visible in the ethidium bromide-stained agarose gel and in some cases is masked by nonspecific amplified DNA. The target is clearly demonstrated in the autoradiographs of the transferred DNA hybridized with the radiolabeled probe (Fig. 5). In the examples given in Table 1, the PCR technique correctly identified samples containing *M.*

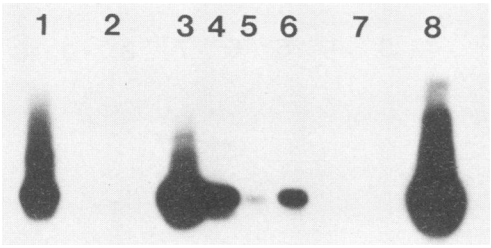

FIG. 5. Amplification of DNA isolated from clinical specimens. DNA was isolated from clinical specimens and amplified by PCR for 30 cycles. The amplified target DNA was analyzed by autoradiography of the transferred DNA hybridized to the *Kpn*I-*Sma*I fragment of p36, following agarose gel electrophoresis. Lanes, 1, CSF from a patient with tuberculosis meningitis; 2, CSF from a patient with pneumococcal meningitis; 3, lung biopsy; 4 through 6, pleural fluid from patients with tuberculosis effusions; 7, pleural fluid from a patient with a malignant effusion; 8, *M. tuberculosis* chromosomal DNA control.

tuberculosis (14 of 26) on the basis of the results of bacterial culture (14 of 26) and with a greater sensitivity than the acid-fast stain (which correctly identified 3 of 26). Trials are being instituted to determine the sensitivity and specificity of the technique and to determine its usefulness in clinical situations.

DISCUSSION

The need for a sensitive, specific, and rapid test for the laboratory diagnosis of tuberculosis has long been acknowledged. The data presented here indicate that the PCR, in which a repetitive 336-bp fragment is amplified from *M. tuberculosis* chromosomal DNA, meets the requirements for such a test. The specific DNA fragment can be detected in 10 to 100 fg of purified chromosomal DNA, which represents the amount of DNA in less than 10 organisms. This sensitivity is due to both the PCR technique itself and to the repetitive nature of the DNA target fragment.

The assay is specific for *M. tuberculosis*. When chromosomal DNAs from other members of *Mycobacteriaceae* family were used, the 336-bp band was not synthesized. This is despite the presence of 10^3 to 10^4 times greater amounts of non-*M. tuberculosis* chromosomal DNA (10 ng versus 1 to 10 pg) in the assays. In the case of strain BCG, a 260-bp fragment was synthesized which could be used to distinguish between *M. tuberculosis* and BCG; both species are members of the *M. tuberculosis* complex. This distinction is not possible with DNA probes directed against the 16S ribosomal sequences of these organisms, as these target sequences are identical in both. The ribosomal sequence is the target for the commercially available *M. tuberculosis* probe (Gen Probe, San Diego, Calif.). Similarly, recently described PCR assays for *M. tuberculosis* (1, 3, 13) cannot be used to distinguish members of the *M. tuberculosis* complex. In this regard, the assay described by Patel and colleagues (10) can be used to distinguish members of the complex.

TABLE 1. Comparison of different detection methods for *M. tuberculosis* in clinical specimens

Specimen type (*n*)	No. of cultures				
	Positive				Negative (acid-fast stain and culture)
	Acid-fast stain	Bacterial culture of fluid	Bacterial culture of biopsy	PCR	
CSF (4)	1	1	NA[a]	1	3
Pleural aspirates and biopsies (16)	0	8	11	11[b]	5
Open lung biopsy (1)	1	1	1	1	0
Pericardial aspirates (5)	1	1	NA	1	4

[a] NA, Not applicable.
[b] PCR performed on aspirates alone.

As in this study, Eisenach and colleagues used a repetitive element from *M. tuberculosis* as the target for amplification. The two repetitive elements are not identical, as their fragment has a 64% G+C content, compared with the 80% G+C content of our target fragment; furthermore, the sequences of the two elements are different (data not shown). There are 12 (or more) copies of the repetitive element described by Eisenach and colleagues, while there are at least 10 copies of the element used in this study present in the genome of *M. tuberculosis*.

The processing of specimens is simple and rapid. In most cases (60%), the result can be obtained with agarose gel electrophoresis within 8 h of obtaining the specimen. A further 2 days may be required if hybridization with the specific probe is required. Although not shown here, the use of PEG to precipitate the DNA removes factors in the aspirated fluids which appear to inhibit the amplification reaction and which are not removed by the more conventional precipitation with ethanol.

The *M. tuberculosis*-specific target was detected in all of the positive culture samples (14 of 26) that were tested in this study. The type of specimen chosen for this study is representative of those obtained from patients in whom there is a diagnostic problem. Suspected tuberculous meningitis is particularly important in this regard. This assay may provide a rapid diagnosis that could prevent a delay in the initiation of antituberculous therapy in these patients and could also be used to avoid unnecessary treatment of patients who do not have tuberculosis infections. With regard to tuberculous pleural effusions, it would appear from this preliminary study that PCR of the pleural fluid has a detection limit for *M. tuberculosis* that is greater than bacterial culture of the fluid alone and is similar to that of culture of the fluid and pleural biopsy together (Table 1).

The true value of a diagnostic PCR assay for *M. tuberculosis* in a high prevalence area for tuberculosis, such as the Western Cape Province of South Africa (400 to 500 cases per 100,000 population), has to be evaluated in the appropriate, controlled trials. One trial involving the comparison of different diagnostic methods for tuberculosis in pleural effusions, including PCR, is nearing completion.

ACKNOWLEDGMENTS

We thank E. Nel of the Tuberculosis Research Institute for mycobacterial cultures, members of the Mycobacteriology section of the Department of Medical Microbiology for clinical specimens, A.-L. Williamson for constructive discussion, and B. Dalgliesh for typing the manuscript.

This work was supported by grants from the University of Cape Town and the Medical Research Council to L. M. Steyn and by Public Health Service award GM32964 from the National Institutes of Health to M. L. Sogin.

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