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Detection of *Mycobacterium tuberculosis* DNA in Clinical Samples by Using a Simple Lysis Method and Polymerase Chain Reaction

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We have evaluated the polymerase chain reaction for detection of *Mycobacterium tuberculosis* in clinical samples from patients with tuberculous infection. Two simple methods for mycobacterial DNA release have been compared: sonication and lysis with nonionic detergents and proteinase K. The more effective method was the enzymatic technique. By using this protocol with 75 specimens we detected *M. tuberculosis* DNA in all of the samples, whereas only 48 and 71 samples were positive by acid-fast staining and culture, respectively.

Tuberculosis is still one of the most important infectious diseases worldwide; moreover, the incidence seems to be increasing, due largely to the AIDS epidemic. The definitive diagnosis continues to rely on microscopy and culture. The development of rapid procedures for the diagnosis of tuberculosis has been a long-standing goal; however, both the immunological detection of mycobacterial antigens (8, 16) and the use of nucleic acid probes (10, 12) lack the sensitivity to be useful for testing clinical specimens (4). The introduction of the polymerase chain reaction (PCR) has opened new diagnostic possibilities in infectious diseases, and its application to tuberculosis (1–3, 6, 13–15) is currently under evaluation. In this study, we have applied the PCR technique to detect DNA from *Mycobacterium tuberculosis* in clinical samples from patients with tuberculosis that were simultaneously studied by acid-fast bacillus stain and culture. We have also compared two simple methods for mycobacterial rupture and DNA release: sonication and lysis with both nonionic detergents and proteinase K, both of which can avoid cumbersome nucleic acid purification procedures.

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Seventy-five specimens (48 sputum samples, 20 pleural effusions, 2 urine samples, 2 abscess aspirates, 1 sample of synovial fluid, 1 lymph node biopsy, and 1 sample of ascitic fluid) were obtained from 66 patients in whom tuberculosis had been diagnosed by conventional microbiological methods using the same sample or from patients with a previous diagnosis of tuberculosis within the first 2 months of therapy. All of the specimens were processed for direct examination by auramine-rhodamine stain and cultured by inoculation on either solid media or the Bactec system (Johnston Laboratories, Inc., Towson, Md.). Aliquots of all of the samples were frozen at −20°C until processing for PCR. Sputum and urine samples, before being cultured, were decontaminated with the standard *n*-acetylcysteine–NaOH protocol (9), and aliquots of this fluidized product were subsequently used in the study.

Six specimens (pharyngeal washings) from healthy volunteers and 12 sputum samples from patients with disease other than tuberculosis were used as negative controls.

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Treatment of samples for bacterial lysis. For the release of *M. tuberculosis* DNA from clinical samples, we evaluated two simple and rapid lysis procedures: sonication and treatment with both nonionic detergents and proteinase K. Aliquots of 500 μl each were centrifuged at 9,500 × *g* for 15 min in a microcentrifuge, and the resultant pellets were processed for sonication and enzymatic lysis. For sonication (13), the sample pellets were suspended in 50 mM Tris-HCl, pH 8 (final volume, 100 μl), boiled for 5 min, mixed with 25 μl of glass beads (Sigma Chemical Co., St. Louis, Mo.), and subjected to ultrasonication at 20 kHz in a water bath for two different durations: 5 min and 15 min at 50°C. For PCR reactions, 2.5 μl of both the pure supernatant and a 1/10 dilution of supernatant in water was used. For enzymatic lysis of sample pellets, a modification of the procedure described by Higuchi (7) was used. Briefly, concentrated samples were suspended in 100 μl of lysis buffer (50 mM Tris-HCl, pH 8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P-40) containing 100 μg of proteinase K per ml and incubated at 56°C for 3 h. Proteinase K was heat inactivated at 95°C for 10 min. As in the sonication procedure, a 2.5-μl sample of the lysate and a 1/10 dilution of supernatant in water were used for PCR to overcome the action of potential PCR inhibitors.

PCR. The mycobacterial DNA was amplified as previously described (5). The assay detects a 123-bp region from the *M. tuberculosis* complex-specific insertion sequence IS*6110*. The amplification reactions were performed in a final volume of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.625 U of *Taq* polymerase (Promega, Madison, Wis.), 0.2 mM each of the deoxynucleotides, primers (1 μM each) (5), and 2.5 μl of clinical sample. The PCR was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, Conn.). The samples were denatured at 94°C for 5 min, and then 30 amplification cycles were performed as follows: 2 min of denaturation at 94°C, 2 min of annealing at 68°C, and 2 min of primer extension at 72°C. The extension time was increased by 5 s with each subsequent cycle.

After amplification, 10 μl of the reaction mixtures was electrophoresed on ethidium bromide-containing 2% agarose gels (Nusieve GTC agarose; FMC BioProducts, Rockland, Maine) and visualized by UV transillumination. The DNA was transferred to nylon membranes (ZetaProbe; BioRad, Richmond, Calif.) by alkaline blotting (11). Hybridization

TABLE 1. Comparison of *M. tuberculosis* PCR results after sonication or enzymatic lysis of 25 clinical samples

Lysis procedure	PCR result (no. of positive samples)					
	Visualization on agarose gel			After hybridization		
	Undiluted lysates	Diluted[a] lysates	Total[b]	Undiluted lysates	Diluted[a] lysates	Total[b]
Sonication for 15 min	1	3	3	6	12	12
Sonication for 5 min	5	4	6	17	16	22
Enzymatic lysis	4	10	10	21	25	25

[a] 1/10 dilution of the final lysis product.
[b] Overall number of positive samples by using either diluted or undiluted lysates.

was done in 5× SSPE (0.75 M NaCl, 50 mM sodium phosphate [pH 7.7], 5 mM EDTA)–1× Denhardt's solution–1% sodium dodecyl sulfate–10% dextran sulfate at 55°C overnight with 10^6 cpm of ^{32}P-5′-labeled probe per ml. Subsequently, the membranes were washed in 2× SSPE–0.1% sodium dodecyl sulfate at 55°C and exposed to radiographic films in cassettes containing amplifying screens for 2 and 24 h at −70°C.

Initially, 25 culture-positive samples (16 sputum samples, 8 pleural effusions, and 1 sample of ascitic fluid) were treated by both sonication, for 5 and 15 min, and enzymatic lysis. Comparative results of PCR in these samples are shown in Table 1. Because the buffer lysis method was shown to be clearly superior to sonication for the detection of *M. tuberculosis* DNA, it was used in all subsequent experiments. A total of 75 samples from 66 patients with documented tuberculous infection were processed for microscopy, culture, and PCR. Results are summarized in Table 2 and Fig. 1. An acid-fast bacillus stain was positive in 48 samples (64%), and *M. tuberculosis* was grown in culture in 71 specimens (94.7%). When we amplified *M. tuberculosis* DNA in undiluted lysates, the target fragment was visible on the ethidium bromide-stained agarose gel in 21 specimens (28%) and after hybridization with the radiolabeled probe was visible in 68 specimens (90.7%). Including results with diluted samples, overall 29 (38.7%) samples had the target fragment visualized on gel. After hybridization, the PCR was positive for all 75 specimens, including all 4 samples (1 sputum sample and 3 pleural effusions) from four patients diagnosed with tuberculosis who were in their first two months of treatment, at which time cultures for mycobacteria were negative. The presence of unidentified inhibitors was suspected in the seven samples (8%) that became positive by PCR after the 1/10 dilution. Finally, none of the nontuberculous control specimens was positive by DNA amplification. Some investigators (2, 3, 6) have reported that direct visualization of electrophoresed PCR products achieves an acceptable diagnostic sensitivity in clinical samples. On the contrary, we have found that hybridization is a step needed to obtain maximal sensitivity, especially in clinical samples containing a small number of bacilli, such as pleural effusions and ascitic and synovial fluids, that are precisely the specimens in which the conventional staining and culture methods show less sensitivity.

Two problems have been recognized as obstacles to successful PCR amplification: difficulties with mycobacterial cell rupture and DNA extraction and the presence of PCR inhibitors. Our work addressed these difficulties. In regard to cell rupture and DNA extraction, most protocols published have used conventional procedures, such as phenol extraction and precipitation with ethanol, to purify mycobacterial DNA for PCR. These procedures are laborious for routine

TABLE 2. Comparison of different diagnostic methods used for detection of *M. tuberculosis* in 75 clinical specimens

Specimen type (*n*)	No. of samples positive for mycobacteria by:		
	Microscopy[a]	Culture[b]	PCR[c]
Sputum (48)	46	47	48
Pleural effusion (20)	0	17	20
Urine (2)	1	2	2
Abscess aspirate (2)	1	2	2
Synovial fluid (1)	0	1	1
Lymph node biopsy (1)	0	1	1
Ascitic fluid (1)	0	1	1
Negative control samples (18)	0	0	0
Total positive samples	48	71	75

[a] Auramine-rhodamine stain.
[b] Culture on Lowenstein-Jensen medium or Bactec system.
[c] Both diluted and undiluted enzymatic lysates were used for PCR.

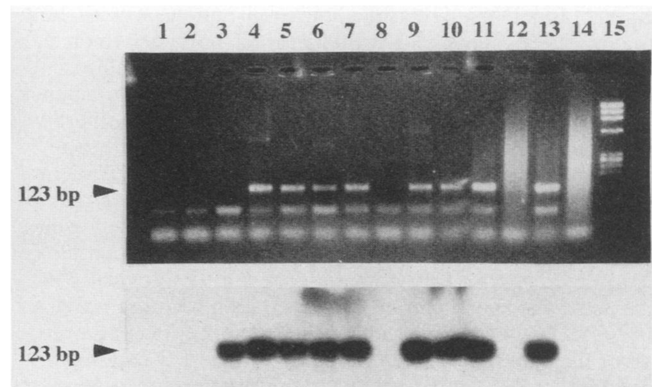

FIG. 1. Representative results of PCR amplification of clinical specimens. The amplification products were analyzed on 2% agarose gels stained with ethidium bromide (top) and by hybridization (bottom). Lanes 1, negative control without mycobacterial DNA; lanes 3, pleural effusion smear and culture negative; lanes 5, pleural effusion smear negative and culture positive; lanes 6, *M. tuberculosis* DNA-positive control; lanes 8, abscess aspirate smear and culture positive; lanes 10, sputum sample smear negative and culture positive; lanes 12 and 14; sputum samples positive by both direct examination and culture; lanes 15, ϕX174 hydrolyzed with *Hae*III. Lanes 2, 4, 7, 9, 11, and 13, diluted samples (dilution ratio, 1/10) from lanes 3, 5, 8, 10, 12, and 14, respectively. Lanes 8, 12, and 14 illustrate the removal of PCR inhibitors when samples were diluted 10-fold in distilled water. The 123-bp amplified fragment is indicated by an arrow.

use in the clinical diagnostic laboratory, and furthermore, the manipulations required are prone to cross-contamination of samples. Some investigators (2, 13) have reported that sonication was the most effective method for lysing clinical samples. However, we have found a poor sensitivity for sonication with and without glass beads (data not shown). An excessive mechanical rupture of DNA strands by sonication could account for this lack of sensitivity since we have obtained better results in our study by using shorter sonication times.

In order to minimize the presence of PCR inhibitors that seem to be abundant (1, 15) in clinical specimens, all the samples were diluted 10-fold in distilled water and tested simultaneously with the undiluted samples. Although an overall higher sensitivity was obtained with diluted samples, indicating dilution of inhibitors, a few, probably those with a very small number of mycobacteria, were negative by PCR when diluted 1/10. Our results suggest that amplification of diluted and undiluted lysates is convenient to ensure the highest sensitivity. Interestingly, *M. tuberculosis* DNA was detected by PCR in four samples that were acid-fast bacillus and culture negative that were obtained from patients receiving treatment for tuberculosis for as long as 2 months. The value of this PCR assay to monitor antituberculous treatment deserves to be investigated further. In summary, this PCR assay, which combines a simple specimen treatment, along with the use of hybridization with a radiolabeled probe, appears to be a sensitive approach to the diagnosis of tuberculous infection, especially in specimens with a small number of mycobacteria.

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