JOURNAL OF CLINICAL MICROBIOLOGY, May 1992, p. 1331–1334
0095-1137/92/051331-04$02.00/0

Vol. 30, No. 5

Rapid, Simple Method for Treating Clinical Specimens Containing *Mycobacterium tuberculosis* To Remove DNA for Polymerase Chain Reaction

GEORGE E. BUCK,[1]* LILA C. O'HARA,[2] AND JAMES T. SUMMERSGILL[2]

Clinical Laboratory, Alliant Health System, Box 35070, Louisville, Kentucky 40232,[1] and Department of Medicine, University of Louisville School of Medicine, Louisville, Kentucky 40292[2]

Received 14 November 1991/Accepted 26 February 1992

Several simplified methods for treating mycobacteria to release DNA for amplification by the polymerase chain reaction (PCR) were investigated. The most effective of the methods was sonication. Samples were placed in screw-capped microcentrifuge tubes that were then placed in a plastic rack. The rack was floated in a dish of water next to the ultrasonic probe so that the ultrasonic energy was transmitted through the walls of the tubes. This allowed multiple samples to be processed safely and effectively. Forty-three clinical samples were processed by this procedure, and the crude preparations were analyzed for *Mycobacterium tuberculosis* by PCR. Twenty-six of these specimens contained *M. tuberculosis*, and 17 either had no growth or contained other species of mycobacteria. Twenty-four of the 26 (92%) positive specimens were correctly identified, and all of the negative specimens were correctly identified. This sonication procedure appears promising as a rapid, simple means of treating clinical specimens containing mycobacteria for PCR analysis.

The polymerase chain reaction (PCR) has recently been investigated for detecting *Mycobacterium tuberculosis* in clinical specimens and appears to have significant potential as a rapid, sensitive, and specific assay for diagnosing infections caused by this organism (1–6, 8–12). One of the remaining issues regarding the use of this technique is the extraction of DNA from clinical material prior to performing the PCR. Most investigators have used conventional procedures, such as phenol extraction and ethanol precipitation, to extract and purify the DNA. These procedures, however, are too laborious and would be impractical for routine use in most clinical laboratories. While more-simplified procedures have been reported (5, 9, 10), their effectiveness has not been fully evaluated. The study described here was designed to compare the effectiveness of several of these techniques in order to establish a simple procedure for disrupting mycobacteria that could be used for routine clinical specimens.

Type culture of *M. tuberculosis*. Initial experiments were performed with *M. tuberculosis* ATCC 27294. It was grown in screw-capped tubes containing 10 ml of Middlebrook 7H9 broth (BBL, Cockeysville, Md.). The tubes were incubated at 37°C in 5% CO_2 for 2 to 3 weeks. The broth above the pellet of cells at the bottom of the tube was removed very carefully in order to not disturb the pellet. This suspension was vortexed and stored at −70°C. Quantitative cultures showed that suspensions prepared in this way contained approximately 10^5 CFU/ml.

Comparison of simplified methods. A tube of the frozen suspension described above was thawed, and 10-fold dilutions were made in 7H9 broth or distilled water to produce a series of suspensions containing approximately 10^1 to 10^4 CFU/ml. One-milliliter quantities of these suspensions were placed in 1.5-ml microcentrifuge tubes. Each series of concentrations was treated by a different procedure to disrupt the bacteria, and the crude DNA preparations were amplified by PCR. The effectiveness of these methods was compared by determining the smallest number of mycobacteria detected. The procedures investigated were as follows.

(i) Treatment with proteinase K and nonionic detergents. The procedure for treatment with proteinase K and nonionic detergent was based on the method described by Higuchi (7) and explored briefly by Pierre et al. (10). The suspensions containing various concentrations of mycobacteria in 1.5-ml screw-capped microcentrifuge tubes were centrifuged at 16,000 × *g* for 5 min, washed with phosphate-buffered saline (PBS), and then centrifuged again at the same speed. After the wash solution was removed, 50 μl of lysing solution was added. This contained PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) with 0.45% Tween 20 (Sigma), 0.45% Triton X-100 (Calbiochem), and 100 μg of proteinase K (Calbiochem) per ml. The suspensions were incubated at 55°C for 1, 3, 6, or 12 h, then boiled for 10 min, and centrifuged for 20 s.

(ii) Boiling with nonionic detergents. Suspensions were centrifuged and washed once with PBS as described above and then resuspended in PCR buffer containing either 0.45% Triton X-100 and 0.45% Tween 20 or 0.45% Tween 20 alone. The suspensions were boiled for 30 min and then centrifuged for 20 s.

(iii) Freezing and thawing. Suspensions were centrifuged at 16,000 × *g* for 5 min, and the fluid was poured off. Fifty microliters of either distilled water or 2% Triton X-100 in PCR buffer was added to each tube. The tubes were placed in a bath of acetone with dry ice for 1 min and then in a water bath at 85°C for 2 min. This freeze-thaw treatment was repeated for a total of eight times. The tubes were then boiled for 10 min and centrifuged for 20 s.

(iv) Sonication. The suspensions were centrifuged as described above for method iii, washed twice with distilled water, and then resuspended in the residual water (approximately 25 μl). The tubes were placed in a plastic rack that was floated in a dish of water next to the sonicator probe (Sonics & Materials, Danbury, Conn.) and sonicated for 30

* Corresponding author.

min at 45 W. After sonication the samples were boiled for 10 min and centrifuged for 20 s.

Reference method for extraction and purification of DNA. The simplified methods described above were compared with a more extensive method for extraction and purification of DNA. The procedure used was described by Eisenach et al. (4). With this technique, these investigators were able to detect between 10 and 100 cells of *M. tuberculosis*. The suspensions of mycobacteria were centrifuged at 16,000 × *g* for 5 min, and the pellets were resuspended in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) containing 20 mg of lysozyme per ml and incubated at 37°C for 2 h. NaOH and sodium dodecyl sulfate were then added to final concentrations of 0.5 N and 1%, respectively, and the tubes were boiled for 5 min. The samples were allowed to cool to room temperature and neutralized with HCl. The DNA was then removed by binding to powdered glass (GeneClean; BIO 101, Inc., La Jolla, Calif.) and eluted into 10 μl of distilled water.

PCR. The PCR procedure described by Eisenach et al. (3, 4) was used. This amplifies a 123-bp sequence found in members of the *M. tuberculosis* complex (*M. tuberculosis* and *Mycobacterium bovis*). The final reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 180 μM (each) deoxynucleoside triphosphates, and 1 U of *Taq* polymerase. In addition, the mixture also contained DNA that when amplified produces a 600-bp product with the same set of primers (4). This acts as an internal control to verify that each reaction tube has appropriate conditions for PCR. The volume of sample added to the reaction mixture varied from 5 to 25 μl. The amount of water was adjusted in the reaction mixture to make the final volume a total of 55 μl. Oligonucleotide primers used for the amplification were generously supplied by K. Eisenach, Department of Pathology, University of Arkansas for Medical Sciences. The reaction was performed in an automated thermal cycler (Perkin-Elmer Cetus model 480). The samples were first denatured by heating at 94°C for 5 min and then incubated for 25 cycles at 94°C for 2 min, 68°C for 2 min, and 72°C for 2 min. For initial experiments 25 cycles were performed. This was increased to 30 cycles for clinical specimens. The extension time was increased by 5 s with each cycle. After amplification, 10 to 20 μl of the reaction mixtures was analyzed on 12% polyacrylamide gels with Tris-borate buffer (89 mM Tris-borate, 89 mM boric acid). The gels were run at 150 V for 30 to 40 min, stained with ethidium bromide, and visualized by UV transillumination. The presence of a 123-bp band indicated successful amplification.

Patient specimens. Two groups of specimens were examined. The first consisted of smear-positive specimens from the Mycobacteriology Laboratory, Kentucky State Department of Health Services. The specimens were decontaminated by the standard sodium hydroxide-*N*-acetyl-L-cysteine procedure, and the sediments were resuspended in 0.2% bovine serum albumin, cultured, and examined by acid-fast staining. These specimens were stored at 4°C until examined by PCR. The second group consisted of specimens submitted to the Mycobacteriology Laboratory of the Alliant Health System. Past experience indicates that most of these specimens can be expected to be negative. These specimens were processed as described above and then examined by PCR, and the results were correlated after the culture results became available. To provide blinding, the person performing the PCR analysis was unaware of the results of cultures or acid-fast stains.

TABLE 1. Comparison of culture and PCR results for clinical specimens

Specimen no.	Culture result[a]	PCR result
1	4+ *M. tuberculosis*	+
2	2+ *M. tuberculosis*	+
3	1+ *M. tuberculosis*	+
4	4+ *M. tuberculosis*	+
5	NG	−
6	1+ *M. tuberculosis*	+
7	4+ *M. tuberculosis*	−
8	NG	−
9	NG	−
10	3 colonies of *M. tuberculosis*	−
11	4+ *M. tuberculosis*	+
12	2+ *M. tuberculosis*	+
13	1+ *M. tuberculosis*	+
14	3 colonies of *M. tuberculosis*	+
15	4+ *M. tuberculosis*	+
16	2+ *M. tuberculosis*	+
17	2+ *M. tuberculosis*	+
18	2 colonies of *M. tuberculosis*	+
19	*Mycobacterium gordonae*	−
20	NG	−
21	1+ *M. tuberculosis*	+
22	4+ *M. tuberculosis*	+
23	4+ *M. tuberculosis*	+
24	4+ *Mycobacterium avium-M. intracellulare*	−
25	2 colonies of *M. tuberculosis*	+
26	NG	−
27	NG	−
28	NG	−
29	4+ *Mycobacterium fortuitum*	−
30	4+ *M. avium-M. intracellulare*	−
31	3+ *Mycobacterium kansasii*	−
32	4+ *M. tuberculosis*	+
33	NG	−
34	1+ *M. tuberculosis*	+
35	NG	−
36	*M. avium-M. intracellulare*	−
37	*Mycobacterium kansasii*	−
38	4+ *M. tuberculosis*	+
39	1+ *M. tuberculosis*	+
40	1+ *M. tuberculosis*	+
41	Culture negative; previous culture was positive	+
42	2+ *M. tuberculosis*	+
43	NG	−

[a] NG, no growth; 1+, 50 to 100 colonies; 2+, 100 to 200 colonies; 3+, moderate growth; 4+, heavy growth.

In the preliminary comparison of methods, the sonication procedure produced the most promising results. Between 10 and 100 organisms could be detected by this procedure, and this compared favorably with the reference method. The other methods investigated were much less efficient. Treatment with proteinase K and nonionic detergents produced DNA that could be amplified only if the suspensions contained 10^3 or more organisms. Freezing and thawing in 2% Triton X-100 produced similar results. No amplification occurred when suspensions were treated by freezing and thawing in distilled water or boiling with nonionic detergents.

The sonication procedure was therefore investigated further with actual clinical specimens. A total of 43 specimens were examined. Twenty-six contained *M. tuberculosis* in various quantities. An additional 17 specimens had no mycobacteria or had species of mycobacteria other than *M.*

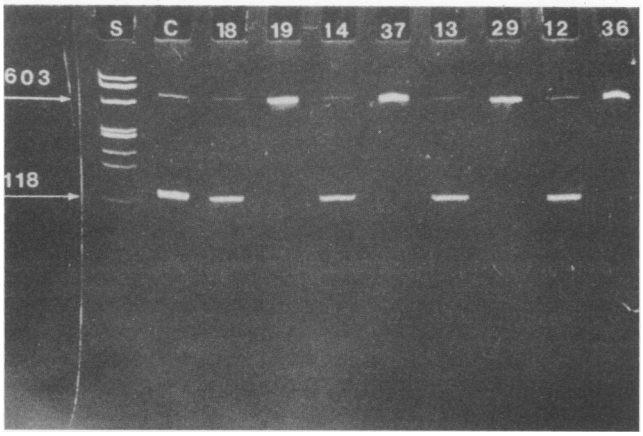

FIG. 1. Ethidium-bromide stained polyacrylamide gel with amplification products from clinical specimens. After amplification a portion of each reaction mixture was mixed with sample buffer, and 10 μl was added to the wells of a 12% polyacrylamide gel. The gel was run at 150 V for 40 min, stained with ethidium bromide, and examined on a UV transilluminator. Lane S contains a molecular size standard with polynucleotides with 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp. Lane C is a positive control, containing the amplified product from a pure culture of *M. tuberculosis*. The other lanes contain amplified products from the clinical specimens; the numbers correspond to the specimen numbers shown in Table 1. Specimens containing *M. tuberculosis* show the 123-bp band. Specimens containing other species of mycobacteria do not show this band. All of the specimens show the 600-bp internal control band. This is a DNA fragment added to the reaction mixture that amplifies with the same primers as the 123-bp fragment, as described by Eisenach et al. (4), thus ensuring that the conditions are adequate for amplification in each reaction mixture.

tuberculosis (Table 1). Twenty-four of the 26 positive specimens (92%) could be detected by PCR using the rapid technique to process the specimens. None of the specimens that had other species of mycobacteria or no growth were positive by PCR. An example of the results from various types of specimens is shown in Fig. 1.

These results confirm, to some extent, results published by previous investigators. Pierre et al. (10) used Triton X-100, Tween 20, and proteinase K to treat clinical specimens. Hermans et al. (5) used repeated freezing and thawing to treat specimens for PCR analysis. Patel et al. (9) investigated PCR with pure cultures of mycobacteria and reported amplification when suspensions were either added directly to the reaction mixture or sonicated prior being added to the reaction mixture. None of these investigators, however, quantitatively assessed the efficiency of these methods. Our results confirm that these simplified methods are capable of releasing DNA for amplification but suggest that these methods are relatively ineffective, since the sensitivity of detection was only down to about 10^3 organisms.

The sonication procedure, on the other hand, was capable of detecting as few as 10 to 100 organisms. It appears that enough ultrasonic energy is transmitted through the walls of the microcentrifuge tubes to effectively disrupt the mycobacteria. This is a procedure that could be used in almost any laboratory setting, and a number of specimens can be treated simultaneously. Since the tubes remain closed during the process, dissemination of infectious aerosols is prevented. It may be possible to increase the sensitivity of this method even further by using the Southern blot technique for detecting amplified DNA, rather than visualization on a gel.

There were only two clinical specimens in which PCR failed to detect the mycobacteria present. In one of these there were only a few colonies of *M. tuberculosis* isolated in culture (Table 1, specimen 10), and apparently this specimen was false negative because of the small number of mycobacteria present. The other false-negative specimen had 4+ growth on culture (Table 1, specimen 7). Additional specimens from the same patient were negative when treated by sonication but PCR positive when purified DNA was extracted. This suggests that the viscous nature of the specimen caused inhibition of the PCR when crude lysates were used. If found consistently, this could be a limiting feature of the sonication procedure. One means of circumventing this problem, however, might be to process most specimens by the sonication procedure and use more-cumbersome conventional DNA extraction procedures for extremely viscous specimens. It may also be possible to modify the sonication procedure in some way to prevent inhibition of the PCR.

In summary, the sonication procedure described here appears to be a simple, rapid means for disrupting clinical specimens containing *M. tuberculosis* to remove DNA for amplification. Further studies are necessary to evaluate this procedure more fully. Such studies should include a larger number of specimens and specimens that are smear negative. On the basis of results presented here, however, additional studies seem warranted.

This work was supported by an Alliant Community Trust Fund grant.

We are indebted to Norma Carlin of the Kentucky State Department of Health Services for making sputum specimens available to us for study. We also thank Frederick Luzzio of the Department of Chemistry, University of Louisville, for allowing the use of the sonication equipment and for helpful advice in the sonication experiments.

REFERENCES

1. **Brisson-Noel, A., B. Gicquel, D. Lecossier, V. Levy-Frebault, S. Nassif, and A. J. Hance.** 1989. Rapid diagnosis of tuberculosis by amplification of mycobacterial DNA in clinical samples. Lancet **ii**:1069–1071.
2. **DeWit, D., L. Steyn, S. Shoemaker, and M. Sogin.** 1990. Direct detection of *Mycobacterium tuberculosis* in clinical specimens by DNA amplification. J. Clin. Microbiol. **28**:2437–2441.
3. **Eisenach, K. D., M. D. Cave, J. H. Bates, and J. T. Crawford.** 1990. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J. Infect. Dis. **161**:977–981.
4. **Eisenach, K. D., M. D. Sifford, M. D. Cave, J. H. Bates, and J. T. Crawford.** 1991. Detection of *Mycobacterium tuberculosis* in sputum samples using a polymerase chain reaction. Am. Rev. Respir. Dis. **144**:1160–1163.
5. **Hermans, P. W., R. A. Hartskeerl, J. E. Thole, and P. R. Klatser.** 1990. Development of diagnostic tests for leprosy and tuberculosis. Trop. Med. Parasitol. **41**:301–303.
6. **Hermans, P. W. M., A. R. J. Schuitema, D. Van Soolingen, C. P. H. J. Verstynen, E. M. Bik, J. E. R. Thole, A. H. J. Kolk, and J. D. A. Van Embden.** 1990. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. J. Clin. Microbiol. **28**:1204–1213.
7. **Higuchi, R.** 1989. Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications **2**:1–3.
8. **Pao, C. C., T. S. B. Yen, J.-B. You, J.-S. Maa, E. H. Fiss, and C.-H. Chang.** 1990. Detection and identification of *Mycobacterium tuberculosis* by DNA amplification. J. Clin. Microbiol. **28**:1877–1880.
9. **Patel, R. J., J. W. U. Fries, W. F. Piessens, and D. F. Wirth.** 1990. Sequence analysis and amplification by polymerase chain reaction of a cloned DNA fragment for identification of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **28**:513–518.

10. **Pierre, C., D. Lecossier, Y. Boussougant, D. Bocart, V. Joly, P. Yeni, and A. J. Hance.** 1991. Use of a reamplification protocol improves sensitivity of detection of *Mycobacterium tuberculosis* in clinical samples by amplification of DNA. J. Clin. Microbiol. **29:**712–717.
11. **Shankar, P., N. Manjunath, K. K. Mohan, K. Prasad, M. Behari, Shriniwas, and G. K. Ahuja.** 1991. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. Lancet **337:**5–7.
12. **Sjobring, U., M. Mecklenburg, A. B. Andersen, and H. Miorner.** 1990. Polymerase chain reaction for detection of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **28:**2200–2204.