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Detection of *Mycobacterium tuberculosis* in Clinical Samples by Two-Step Polymerase Chain Reaction and Nonisotopic Hybridization Methods

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Detection of *Mycobacterium tuberculosis* in clinical specimens by the polymerase chain reaction (PCR) was compared with detection by culture. A 317-bp segment within the *M. tuberculosis*-specific insertion sequence IS*6110* was amplified. The detection limit of the PCR assay for cultured mycobacteria was 50 cells per reaction by ethidium bromide-stained agarose gel electrophoresis and 5 cells per reaction by hybridization with an oligonucleotide probe conjugated with either digoxigenin or alkaline phosphatase (AP). This sensitivity was reduced fivefold in sputum specimens seeded with *M. tuberculosis*. Seventy-six clinical specimens were amplified and examined by the three detection methods. Both the digoxigenin and AP procedures were found to be more sensitive than agarose gel electrophoresis, but they were occasionally associated with a high background. An additional 308 specimens were examined only by agarose gel electrophoresis and the AP procedure. Of 71 specimens found to contain *M. tuberculosis*, amplified products were detected from 56 (79%) samples by agarose gel electrophoresis and/or the AP procedure. Of the additional 313 specimens that were culture negative for *M. tuberculosis*, 19 (6%) had amplified products detectable by agarose gel electrophoresis and/or the AP procedure. Compared with culture, PCR showed sensitivities and specificities of 55 and 98%, respectively, for agarose gel electrophoresis and 74 and 95%, respectively, for the AP procedure. Despite this low sensitivity, a rapid positive PCR result was accurate and clinically useful.

Detection of *Mycobacterium tuberculosis* in culture is required for the definitive diagnosis of tuberculosis. Diagnosis of tuberculosis made on the basis of clinical, radiologic, and other laboratory findings or the presence of acid-fast bacilli in clinical samples is presumptive. In recent years, the polymerase chain reaction (PCR) was reported to be useful in the direct diagnosis of tuberculosis infections directly from a variety of clinical specimens (2, 5, 11, 13, 15, 19, 23) with a diversity of genetic elements used as a target templates. Those include rRNA (1), single-copy genes encoding for structural proteins such as those of 65 and 38 kDa (2, 11, 15, 20), and highly variable, multiple-copy sequences such as insertion element IS*6110* and P36 (5, 6, 23). Other species-specific DNA fragments have also been used (7, 8, 14). In addition to differences in the target nucleic acid sequences, studies differed in the number and type of samples used, the DNA release technique, the number of amplification cycles, and the methods used to detect amplified products. Although use of radioactively labeled probes in conjunction with agarose gel electrophoresis has been the most prevalent method of detecting amplified products, a few investigators have reported the use of nonradioactively labeled probes (10, 21). The sensitivities and specificities varied from 60 to 100% (2–5, 10, 13, 15, 21, 23). Detection limits of 1 to 1,000 cells in suspensions of known isolates (1, 8, 9, 12, 13) and clinical specimens (5, 10, 25) have been reported.

In a clinical laboratory, it is desirable to use detection methods that have low complexity and that are relatively simple and safe. In the study described here, we evaluated the direct detection of *M. tuberculosis* from clinical samples through amplification and examination by agarose gel electrophoresis or two different nonisotopic hybridization methods. The effects of variables in the routine processing of clinical specimens on PCR results are also reported.

MATERIALS AND METHODS

DNA extraction from bacterial strains and clinical specimens. Suspensions of clinical isolates of *M. tuberculosis*, *M. avium*, *M. chelonae*, and *M. kansasii* were prepared in 0.85% saline containing 0.01% Tween 80 and were adjusted to a 0.5 McFarland standard. Tenfold serial dilutions (10^{-1} to 10^{-8}) were made in the same solution. The number of CFU of the original suspension per milliliter was estimated by routine plating and colony counting by using triplicate 7H10 agar plates. Clinical specimens were processed by standard microbiological methods (16) and were decontaminated by the *N*-acetyl-L-cysteine sodium hydroxide method, as appropriate (e.g., sputum), or were used directly (e.g., cerebrospinal fluid). Two-thirds of each sample was used for routine smears and culture (BACTEC radiometric bottle, Lowenstein-Jensen and Middlebrook agar slants), 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 test.

Prior to extraction, samples were washed in lysis buffer (1% Triton X-100, 10 mM Tris [pH 8.0], 1 mM EDTA) and were sedimented by centrifugation at 12,600 × *g* for 5 min. Each sediment was suspended in 100 μl of lysis buffer, and the mixture was incubated at 100°C for 30 min to release the DNA. Samples were centrifuged at 12,600 × *g* for 3 min, and the supernatants were stored at −70°C until use.

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Variables of processing clinical specimens. Random testing of liquefied and decontaminated clinical samples showed a pH range of 8.5 to 9.5. The effect of storage time and pH on detection of *M. tuberculosis* in sputum specimens was examined by seeding two samples of pooled sputum with 3×10^3 and 3×10^2 CFU/ml, respectively. After routine decontamination, each sample was divided into two sets of five tubes each. The pH of each set was adjusted to 8 and 10, respectively, and the tubes were stored at 4°C. After 1, 2, 4, 7, and 14 days of storage, the DNA was extracted as described above.

Amplification of mycobacterial DNA. Two oligonucleotide primers within the IS*6110* sequence (24), designated primer 41 (20 bp; 5′-CCTGCGAGCGTAGGCGTCGG-3′; located at nucleotides 935 to 954) and primer 43 (21 bp; 5′-TCAGCC GCGTCCACGCCGCCA-3′; located at nucleotides 638 to 658) were synthesized at the Centers for Disease Control (Atlanta, Ga.). The amplification procedure was performed as follows. A total of 85 μl of reaction mixture containing 1.5 mM magnesium chloride, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 50 mM potassium chloride, 200 μM (each) nucleotide (dATP, dCTP, dGTP, and dTTP), 0.5 μM (each) primer, and 2.5 U of *Taq* polymerase (Promega Corp., Madison, Wis.) was overlaid with 50 μL of mineral oil prior to the addition of 25 μl of sample DNA. Extracts from serial dilutions of *M. tuberculosis* were amplified at 30, 35, and 40 cycles to determine the optimum number of cycles. Each cycle consisted of 94°C for 2.2 min for denaturation and 68°C for 3.25 min for annealing and primer extension. On the basis of these optimization studies, extracts from clinical samples were subjected to 35 cycles of amplification. Negative and positive controls were included in each amplification experiment.

Agarose electrophoresis and hybridization analysis. The oligonucleotide probe (5′-CTCGTCCAGCGCCGCTTCGG-3′) located at nucleotides 832 to 851 of the IS*6110* sequence and within the amplified product was labeled either with digoxigenin (DIG) by using terminal transferase enzyme according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, Ind.) or directly with alkaline phosphatase (AP) following the manufacturer's procedure (Cambridge Research Biochemicals, Wilmington, Del.).

Twenty-five microliters of each amplified reaction was analyzed by ethidium bromide-stained agarose gel electrophoresis. The amplified product of the expected 317-bp fragment was considered positive for the presence of *M. tuberculosis*. The specificity and the limit of detection were determined by Southern blotting (17).

To prepare dot blots, 25 μl of amplified product was denatured with an equal volume of 0.8 N NaOH containing 20 mM EDTA at 100°C for 10 min, and the denatured product was spotted onto Zetaprobe nylon filters (Bio-Rad, Richmond, Calif.) by using a minifold system (Schleicher & Schuell, Inc., Keene, N.H.). Filters were rinsed in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.15 M sodium citrate [pH 7.0]) and were microwaved for 4 min. Hybridization by the AP procedure was performed by placing filters in prehybridization solution (5× SSC, 0.5% sodium dodecyl sulfate [SDS], 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone) for 2 h at 50°C. After hybridization for 20 min at the same temperature in a similar solution containing AP-labeled probe, filters were washed twice at 50°C (5 min each time) in 1× SSC–0.5% SDS and twice (5 min each time) at the same temperature in 0.25× SSC–0.5% SDS. To capture the AP activity, filters were sprayed with Lumi-Phos530 [4-methoxy-4-(3-phosphatephenyl)-spiro(1,2-dioxetane-3,2′-adamantane)], placed between two sheets of transparent acetate paper, and exposed to X-ray film for 1 h at 37°C. The intensity of each dot was compared with those of negative controls and with those of various dilutions of positive controls. Results were read visually and by computerized video image analysis (Jandel Scientific, Corte Madera, Calif.). Except for washing at the end of hybridization (in 6× SSC; twice at 37°C and twice at 50°C for 15 min each time), DIG hybridization and immunological detection were performed according to the manufacturer's instructions (Boehringer Mannheim). Filters were examined for the presence of AP activity as described above for the AP procedure.

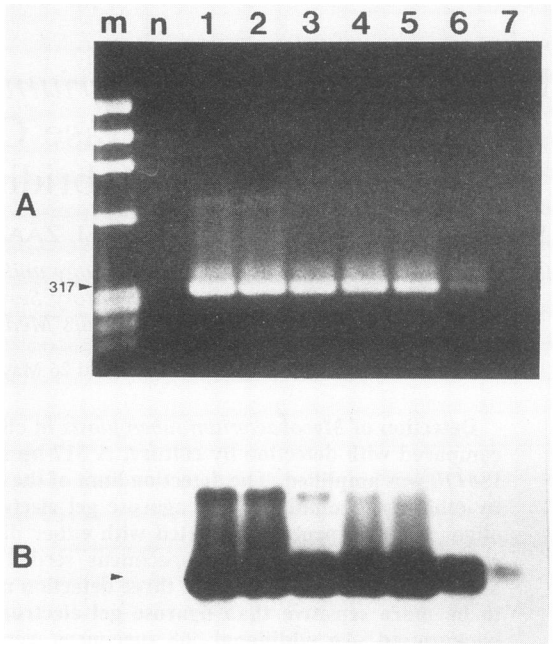

FIG. 1. Sensitivity of PCR detection by agarose gel electrophoresis (A) and Southern hybridization with AP-labeled probe (B) by using amplified products from mycobacterial cultures. Lane m, *Hae*III-digested ϕX174 used as a size marker; lane n, template-negative control; lanes 1 to 7, 10-fold serial dilutions of *M. tuberculosis* starting at 5×10^6 cells per reaction.

RESULTS

PCR and hybridization analysis. The sensitivity and specificity were determined by performing PCR on serial dilutions with known concentrations of *M. tuberculosis* and other mycobacteria. When the number of cycles was increased from 30 to 35, a 100-fold increase in the amount of product was observed. An increase in the number of cycles to 40, however, did not result in a similar increase in the amount of product. At 35 cycles of amplification, 50 organisms per reaction were detectable by agarose gel electrophoresis and 5 cells were detectable by either Southern hybridization (Fig. 1) or dot blot hybridization (Fig. 2). No amplification product was observed from pure cultures of other species of mycobacteria analyzed (*M. avium*, *M. intracellulare*, *M. chelonae*, and *M. kansasii*). A faster-migrating band observed from rare *M. avium* complex strains at 10^8 cells per reaction was shown to be nonhybridizing by Southern blotting (data not shown). Figure 2 provides the dot blot hybridization results for the four species

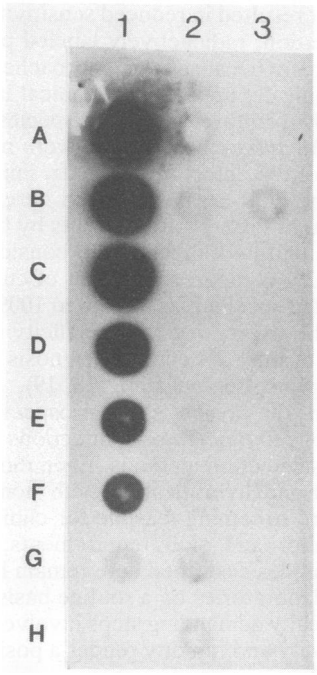

FIG. 2. Specificity and detection limit of *M. tuberculosis* DNA after 35 amplification cycles. Dot blot hybridization with AP-labeled probe was performd on amplified products from 10-fold serial dilutions starting at 5×10^5 cells of *M. tuberculosis* per reaction (1A to 1H) and at 1×10^8 cells of *M. avium* (2A to 2D), *M. intracellulare* (2E to 2H), *M. chelonae* (3A to 3D), and *M. kansasii* (3E to 3H) per reaction.

listed above. When mycobacterial suspensions were mixed with sputum, the detection limits were fivefold higher by both agarose gel electrophoresis and hybridization analysis.

Clinical specimens. Initially, 76 specimens were amplified and examined by the three detection methods. Of the specimens that were found to contain *M. tuberculosis* on culture, 24 of 36 (66%) were positive by agarose gel electrophoresis, 28 of 36 (77%) were positive by the DIG procedure, and 26 of 34 (75%) were positive by the AP procedure. Four (11%) specimens were negative by all three detection methods, while eight (22%) were negative by agarose gel electrophoresis but gave a signal by either the AP or the DIG procedure. All 40 samples that either were culture negative or grew other mycobacteria were negative by agarose gel electrophoresis and the AP procedure, while 2 (5%) were positive by the DIG procedure. An additional 308 specimens were examined by both agarose gel electrophoresis and the AP procedure but not the DIG method because of the greater simplicity, rapidity, and lower background of the AP procedure compared with those of the DIG method. Of the specimens that were culture positive for *M. tuberculosis*, 39 of 71 (55%) were positive by agarose gel electrophoresis and 51 of 69 (74%) were positive by the AP procedure; 34 were positive by both methods (Table 1). All 80 specimens that grew mycobacteria other than *M. tuberculosis* were negative by both methods. Of the additional 233 specimens that were culture negative for acid-fast bacilli, 4 (1.7%) were positive by agarose gel electrophoresis alone, 12 (5.1%) were positive by the AP procedure alone, and 3 (1.2%) were positive by both procedures (Table 1). The sensitivity, specificity, and predictive values of PCR by agarose gel electrophoresis and AP hybridization are summarized in Table 2.

TABLE 1. Comparison of detection of *M. tuberculosis* in 384 specimens by PCR and routine culture

PCR product detection by:		No. of specimens with the following culture result[a]:		
Agarose gel electrophoresis	AP procedure	Positive for *M. tuberculosis* (71)	Positive for MOTT (80)	Negative for AFB (233)
+	+	34	0	3
−	+	17	0	12
+	NT[b]	2	0	0
+	−	3[c]	0	4
−	−	15	80	214

[a] MOTT, mycobacteria other than *M. tuberculosis*; AFB, acid-fast bacilli. Numbers in parentheses are total number of specimens.
[b] NT, not tested because of insufficient material.
[c] Samples were not available for confirmation.

Effect of pH and time of storage. Lysed and amplified sputum samples containing 2,500 cells per reaction showed a strong 317-bp band in agarose gels, regardless of the pH (8 versus 10) or time of storage prior to lysis (1, 2, 4, 7, and 14 days). Samples containing 10-fold lower inocula (250 cells per reaction) showed a faint ethidium bromide-stained band for samples that were stored for up to 7 days at pH 8 and a weaker band for samples that were stored at pH 10 (data not shown). However, at 250 cells per reaction, we detected no bands from samples stored for 14 days at either pH.

DISCUSSION

As expected, the hybridization methods examined in the present study were more sensitive than agarose gel electrophoresis. Under the conditions used in the present study, each PCR was capable of detecting as few as 50 and 5 *M. tuberculosis* cells from culture by agarose gel electrophoresis and hybridization, respectively. These detection limits seem to be an improvement over those reported by Hance et al. (9); their PCR detection limits were 1,000 cells by agarose gel electrophoresis and 100 cells by hybridization. Our results are in close parallel with reported detection limits of

TABLE 2. Detection of *M. tuberculosis* in clinical specimens by PCR and culture[a]

PCR positive by:	Culture for *M. tuberculosis*				Predictive value (%)	
	Positive		Negative			
	No. of specimens[b]	Sensitivity (%)	No. of specimens[c]	Specificity (%)	Positive test	Negative test
Agarose gel electrophoresis	39	55	7	98	85	91
AP procedure	51	74	15	95	77	94

[a] Upon correcting for three culture-positive, PCR-negative specimens that were inhibited and five culture-negative, PCR-positive specimens from former tuberculosis patients, the corrected sensitivity, specificity, and positive predictive values were as follows: 62, 99, and 96%, respectively, for agarose gel electrophoresis and 80, 97, and 86%, respectively for the AP procedure. The negative predictive values were increased only slightly.
[b] Seventy-one *M. tuberculosis* culture-positive specimens were examined by agarose gel electrophoresis and 69 specimens were examined by the AP procedure.
[c] A total of 313 culture-negative specimens were examined by agarose gel electrophoresis and the AP procedure.

10 to 200 cells determined on the basis of hybridization with radioactively labeled probes (10, 12, 13). The sensitivity of detection in sputum specimens seeded with *M. tuberculosis* was fivefold lower than that in suspensions of cultured organisms. This reduction is in concordance with another study (10) reporting 5- to 20-fold lower sensitivities of detection in clinical specimens compared with those obtained by using cultured organisms. Nonetheless, the sensitivity of detection from clinical specimens was higher with hybridization than with agarose gel electrophoresis (Table 1). PCR achieved a sensitivity of 74% and a specificity of 95% by hybridization (Table 2). Our results show that direct detection of *M. tuberculosis* in clinical specimens by PCR is not as sensitive as that by culture methods used in our laboratory. This is in contrast to some reports (2, 5, 12) in which the sensitivity of PCR was found to exceed that of culture. Nevertheless, our results compare favorably with other reports demonstrating PCR sensitivities of 50% (20), 63% (15), and 75% (20).

In our experience, the low sensitivity of detection by PCR is correlated to the low number of colonies recovered by culture. Cultures of smear-negative, agarose gel electrophoresis-negative samples that were positive by the AP procedure exhibited growth of only 100 to 200 colonies. This was calculated to be 200 to 400 CFU/ml or 50 to 100 cells per PCR. These findings support our experimental results with seeded sputum specimens in which the detection limits were 250 and 25 cells per PCR by agarose gel electrophoresis and the AP procedure, respectively. The possible influence of low numbers is also reflected by the observation that the sensitivity of PCR by the AP procedure was 53% for smear-negative specimens and 90% for smear-positive specimens (data not shown). These results are in accordance with the findings by Sun et al. (22), in that PCR was 50% sensitive for smear-negative culture-positive samples.

Some of the discrepant results can be explained as follows. Of the 15 specimens that were PCR negative but culture positive, 9 exhibited sparse growth. However, six PCR-negative specimens were smear positive and showed numerous colonies on culture. Of these, three specimens contained inhibitors (negative results even after a spiking reaction with a known quantity of *M. tuberculosis* DNA; data not shown), while the results for three specimens remain unexplained. The three agarose gel electrophoresis-positive samples that failed to hybridize were not available for further testing. Of 19 culture-negative, PCR-positive samples, 5 had other positive *M. tuberculosis* cultures. It could be argued that the remaining 14 may represent a "true" PCR result because of occult infection. Future follow-up of these patients may help to resolve this discrepancy. If these factors are taken into consideration, the corrected sensitivities for agarose gel electrophoresis and the AP procedure become 62 and 80%, respectively, with positive predictive values of 96 and 86%, respectively (Table 2).

Research attempts to increase the sensitivity of detection by PCR have focused on the use of methods that enhance the sensitivity of detection, such as extraction and purification of DNA (1, 5, 9, 10), the use of isotopically labeled probes (2, 5, 15, 18), and reamplification of negative samples by using nested primers (15). Pierre et al. (15) have shown that the use of reamplification along with radioactive hybridization results in increased sensitivity (from 63 to 100%) without affecting the specificity. The recent report by Victor et al. (25) describing purification of organisms prior to lysis shows that the method has promise in removing the effects of inhibitors, but it resulted in reduced sensitivity. We have not tried reamplification, radioactively labeled probes, or DNA purification, mostly because such approaches are expensive and/or not technically appealing to clinical laboratories.

By using known cultures from four species of mycobacteria other than *M. tuberculosis*, there were no false-positive results by any of the detection methods, indicating the high specificity of the PCR assay described here. In the clinical comparison trial, if false-positive results by both agarose gel electrophoresis and hybridization are considered, the specificity falls to 92% compared with that of culture. This is in line with reported specificities of 62.6 to 100% (3, 13, 15, 18, 19, 20). Note, however, that the specificity in some studies was calculated against the clinical diagnosis and not against culture, which was often negative (18, 19).

In conclusion, the present study prospectively compared the diagnosis of *M. tuberculosis* infections by culture and PCR by various detection methods. Even though agarose gel electrophoresis and hybridization with nonisotopically labeled probes are becoming feasible for clinical laboratories in terms of labor and skill requirements, the PCR and detection techniques described here remain hard to incorporate in clinical laboratories on a routine basis because of the multiple technically demanding steps involved. Nonetheless, the high specificity and rapidity render a positive PCR result clinically useful.

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