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Detection of *Mycobacterium tuberculosis* in Clinical Specimens by Polymerase Chain Reaction and Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test

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The polymerase chain reaction (PCR) using oligonucleotides based on the repetitive sequence (IS*986*) of *Mycobacterium tuberculosis* as a primer and the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (MTD), which combines an *M. tuberculosis* rRNA amplification method with the hybridization protection assay format, were evaluated for detection of *M. tuberculosis* in clinical samples. The detection limits of these two assay systems based on nucleic acid amplification for cultured *M. tuberculosis* were less than 10 cells per reaction. A total of 135 sputum specimens were examined by the two assay systems. The PCR and the MTD systems for detection of *M. tuberculosis* gave overall positivity rates of 84.2% (32 of 38) and 91.9% (34 of 37), respectively, as compared with 71.9% (23 of 32) by smear and 96.9% (31 of 32) by culture in the liquid medium MB-Check. Procedures for sample preparation used in the two methods were different. Although the sensitivities of the PCR and MTD appeared to be similar to that of culture with the MB-Check system, the two methods based on nucleic acid amplification should be very useful for rapid detection of *M. tuberculosis* infections without the long time required for culture of *M. tuberculosis*.

Bacteriological diagnosis of mycobacterial infections usually requires a long time, and there is an urgent need for a rapid and reliable diagnostic test. Culturing of organisms has a specificity that approaches 100% and permits drug susceptibility testing of the isolates, but the slow growth of most pathogenic mycobacteria (3 to 6 weeks) results in delay of definitive diagnosis. Examination of direct smears for acid-fast bacilli is the most rapid method for the detection of mycobacteria. However, it lacks specificity and a relatively large number of bacteria ($>10^4$/ml) must be present in the sample for detection. The radiometric BACTEC system and the biphasic MB-Check system for mycobacterial cultures have shortened the time for detection and increased the rate of recovery of mycobacteria (1, 6, 12). As with conventional culture procedures, these systems are also dependent on the growth of mycobacteria.

The DNA or RNA hybridization tests with labeled specific probes which have been described so far are not sensitive enough to be used for clinical specimens without prior culturing (6, 16).

The polymerase chain reaction (PCR) as a rapid diagnostic technique has a potential to overcome the limitations of sensitivity and specificity (4). The need for rapid identification of mycobacteria has increased, especially because of their roles as opportunistic pathogens in patients with AIDS (17). In addition to therapeutic and diagnostic considerations, rapid identification of mycobacteria may facilitate follow-up of the contacts of patients in whom *Mycobacterium tuberculosis* has been identified.

In this study, PCR using oligonucleotides based on the repetitive sequence IS*986* and the newly developed Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (MTD), which is based on rRNA amplification, were compared with each other as well as with conventional smear and culture methods for specificity and sensitivity of detection of *M. tuberculosis* in clinical specimens.

MATERIALS AND METHODS

Bacterial strains and clinical specimens. Reference strains of 23 *Mycobacterium* species and 10 other bacterial species were used for nucleic acid amplification experiments (Table 1). Sputum specimens were obtained from 135 different patients admitted to Fukujuji Hospital (Japan Anti-Tuberculosis Association, Kiyose-shi, Tokyo) with symptoms of pulmonary diseases.

Smear examination of clinical specimens. Direct smears prepared from sputum specimens were stained by the Ziehl-Neelsen method. The smears were examined under the oil immersion objective lens of the microscope (×1,000). Thirty-two smear-positive and 103 smear-negative specimens were included in the study.

Processing of specimens. All specimens were decontaminated by the *N*-acetyl-L-cysteine-NaOH (NALC-NaOH) method (8). Two volumes of NALC-NaOH solution (2% NaOH, 1.45% Na-citrate, 0.5% NALC) were mixed with the specimen on a test tube mixer for digestion, and the mixtures were allowed to stand for 15 min at room temperature. Ten volumes of 10 mM phosphate buffer (pH 7.4) were added for dilution, and the mixtures were centrifuged at 3,000 × *g* for 20 min at 4°C. After the supernatant fluids were carefully decanted, the resulting sediments were suspended in 1 ml of the same buffer, 0.3 ml of the suspension was inoculated into an MB-Check bottle (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan), and 0.1 ml was inoculated on Ogawa egg medium. The remainder of each treated sample was stored at −20°C until used for nucleic acid amplification experiments.

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TABLE 1. Organisms tested by PCR and MTD systems

Organism	Description	PCR	MTD
M. tuberculosis	KK11-20[a] (H37Rv)	+	+
M. bovis	ATCC 19210[b]	+	+
M. africanum	ATCC 25420	+	+
M. microti	ATCC 19422	+	+
M. kansasii	ATCC 12478	−	−
M. marinum	ATCC 927	−	−
M. simiae	ATCC 25275	−	−
M. scrofulaceum	ATCC 19981	−	−
M. szulgai	KK32-01	−	−
M. gordonae	ATCC 14470	−	−
M. avium	ATCC 25291	−	−
M. intracellulare	ATCC 13950	−	−
M. xenopi	ATCC 19250	−	−
M. malmoense	ATCC 29571	−	−
M. haemophilum	ATCC 29548	−	−
M. ulcerans	KK43-01	−	−
M. gastri	ATCC 15754	−	−
M. nonchromogenicum	ATCC 19530	−	−
M. terrae	ATCC 15755	−	−
M. triviale	ATCC 23292	−	−
M. fortuitum	ATCC 6841	−	−
M. chelonae subsp. *chelonae*	ATCC 35752	−	−
M. chelonae subsp. *abscessus*	ATCC 19977	−	−
Acinetobacter anitratus	Clinical isolate	ND[c]	−
Candida albicans	Clinical isolate	ND	−
Escherichia coli	Clinical isolate	ND	−
Haemophilus influenzae	Clinical isolate	ND	−
Klebsiella pneumoniae	Clinical isolate	ND	−
Pseudomonas aeruginosa	Clinical isolate	ND	−
Serratia marcescens	Clinical isolate	ND	−
Staphylococcus aureus	Clinical isolate	ND	−
Staphylococcus haemolyticus	Clinical isolate	ND	−
Streptococcus pneumoniae	Clinical isolate	ND	−

[a] Mycobacteria Collection, Research Institute of Tuberculosis, Tokyo, Japan.
[b] American Type Culture Collection.
[c] ND, not determined.

Detection of *M. tuberculosis* DNA by PCR. For extraction and purification of *M. tuberculosis* DNA, we used a method (3) based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium thiocyanate (GuSCN) (Fluka Chemie AG, Buchs, Switzerland) and the nucleic acid-binding properties of diatoms (Sigma Chemical Co., St. Louis, Mo.) in the presence of GuSCN. Briefly, 100 μl of the pretreated sample was pipetted into a reaction vessel containing 900 μl of a GuSCN-containing lysis buffer (500 mM GuSCN, 50 mM Tris-HCl, 20 mM EDTA, 0.1 mM Triton X-100) and 20 μl of a diatom suspension (10 g of high-purity diatomaceous earth in 50 ml of H_2O and 500 μl of 32% HCl). The mixture was immediately vortexed for 5 s. After being held for 10 min at room temperature, the mixture was vortexed again and centrifuged in an Eppendorf microcentrifuge at 12,000 × *g* for 15 s, and the supernatant was removed by suction. The diatom-nucleic acid pellet was washed twice with washing buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl [pH 6.4]), twice with 70% ethanol, and once with acetone. After the acetone supernatant was removed, the vessels were dried at 56°C for 10 min with lids open. The nucleic acid binding to the diatom in the vessels was eluted by incubation in 100 μl of an aqueous low-salt buffer (1 mM EDTA in 10 mM Tris-HCl [pH 8.0]) at 56°C for 10 min.

The primers for PCR were based on the repetitive sequence (IS*986*) of *M. tuberculosis* (7, 11). Two primers, PT-8 and PT-9, correspond to bp 105 to 124 (5′-GTGCGGATG GTCGCAGAGAGAT-3′) and 626 to 645 (5′-CTCGATGC CCTCACGGTTCA-3′), respectively, of the IS*986* sequence. The reaction mixture for PCR (50 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM NaCl, 0.01% gelatin, 2 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dUTP), 0.01 U of uracil DNA glycosylase (UNG) (BRL, Grand Island, N.Y.), 1 U of Ampli *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 10 μl of DNA prepared from the clinical sample. For UNG activation, the reaction mixture was incubated at 22°C for 10 min and then at 50°C for 5 min, and for inactivation of the UNG, the mixture was incubated at 94°C for 10 min (9). PCR using PT-8 and PT-9 as primers was then carried out in a thermocycler (Nippon Genetics Co., Ltd., Tokyo, Japan) for 40 cycles, each consisting of denaturation at 94°C for 1.5 min, annealing at 65°C for 2 min, and primer extension at 72°C for 3 min. After amplification 10 μl of the reaction mixture was electrophoresed on a 2% agarose gel. The gels were stained with ethidium bromide and visualized by UV transillumination.

Detection of *M. tuberculosis* by amplification of rRNA and a hybridization protection assay. The MTD (Gen-Probe Incorporated, San Diego, Calif.), which combines a proprietary target (rRNA) amplification method with the hybridization protection assay format (14), was evaluated for detecting *M. tuberculosis*. For extraction of *M. tuberculosis* rRNA, 50 μl of the sample, processed by using the NALC-NaOH method, was placed into a lysing tube containing glass beads and specimen dilution buffer (200 μl). The sample was then subjected to ultrasonication at 20 kHz in a water bath sonicator for 15 min at room temperature.

The lysate (50 μl), containing free rRNA, which serves as a template for in vitro replication, was transferred to a tube which contained 25 μl of amplification reagent, including specific primers that will hybridize with *M. tuberculosis* rRNA at defined sites, and 200 μl of oil reagent overlying the contents of the tube. The reaction mixture was heated at 95°C for 15 min in a dry heat bath. After the tubes were cooled at 42°C for 5 min, 25 μl of an enzyme reagent containing reverse transcriptase, an RNA polymerase, and nucleoside triphosphates was added. The mixture was incubated at 42°C for 2 h to allow synthesis of a cDNA of the target RNA followed by synthesis of the cDNA duplex and, finally, of large numbers of RNA transcripts containing the target sequence. Twenty microliters of termination reagent was added to the tube, and incubation at 42°C was continued for 10 min.

Chemiluminescent acridinium ester-labeled DNA probes were used to specifically detect the amplicon. One hundred microliters of *M. tuberculosis* complex-specific probe reagent was added to the reaction tube, and the tube was incubated at 60°C for 15 min in a water bath to allow hybridization. After addition of selection reagent (300 μl), the tube was vortexed and incubated for an additional 10 min. After the tube was cooled at room temperature for at least 5 min, the result was read in a luminometer before 1 h had elapsed. Samples producing signals greater than or equal to the cutoff value of 30,000 relative light units were considered positive, and signals less than this cutoff value were considered negative.

Determination of the sensitivities of the PCR and MTD methods. To determine sensitivity, 10-fold serial dilutions of *M. tuberculosis* H37Rv or *M. tuberculosis* clinical isolates grown in Middlebrook 7H9 liquid medium for 10 days were made in cold water, and a portion of each dilution was used

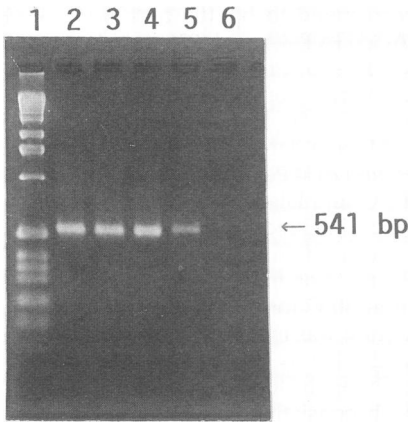

FIG. 1. Endpoint determination of the PCR amplification of purified DNA. Lane 1, *Hae*III-digested ϕX174 DNA as a molecular size marker; lanes 2 to 6, products after PCR amplification of 1 pg and 100, 10, 1, and 0.1 fg, respectively, of purified DNA from *M. tuberculosis* H37Rv.

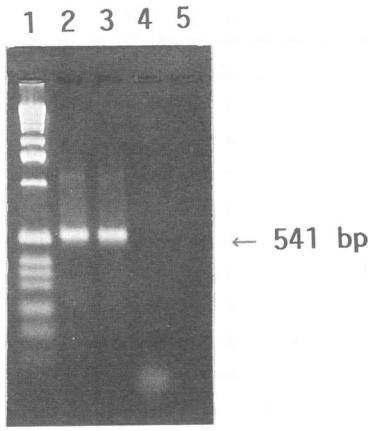

FIG. 2. Agarose gel analysis of PCR-amplified DNA from two subsequent runs, using incorporation of dUTP and incubation with UNG. Ten microliters of amplified *M. tuberculosis* and *M. bovis* DNAs (lanes 2 and 3, respectively) from the first PCR runs were treated with dUTP and UNG and used for the second PCR runs for reamplification (lanes 4 and 5, respectively). Lane 1, *Hae*III-digested ϕX174 DNA as a molecular size marker.

for extraction of DNA or rRNA to be used in PCR or MTD. Another portion of the same dilution used for nucleic acid extraction was cultured on Ogawa egg and Middlebrook 7H11 media for 4 to 6 weeks to determine the number of bacteria.

RESULTS

Specificity and sensitivity of PCR for detection of *M. tuberculosis* DNA. The specificity of PCR for detection of *M. tuberculosis* was tested by using the reference strains of 23 *Mycobacterium* species. The 541-bp amplification product was seen only when DNAs from the bacteria belonging to the *M. tuberculosis* complex (*M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. microti*) were used as templates (Table 1).

M. tuberculosis H37Rv grown in Middlebrook 7H9 liquid medium was used to determine the sensitivity of PCR. The limit of detection of *M. tuberculosis* by PCR was less than 10 CFU (data not shown). In another experiment, 10-fold serial dilutions containing between 10^{-13} and 10^{-16} g of purified *M. tuberculosis* H37Rv DNA were made and used in a PCR amplification with 40 cycles. As shown in Fig. 1, the lower limit for the visualization of the amplified product on ethidium bromide-stained gels was 10^{-15} g (1 fg), which corresponds to the amount of DNA present in about one microorganism (15).

Of the sources commonly considered to contaminate the DNA preparations, carryover contamination is the major source. We determined the effectiveness of the use of dUTP and UNG in preventing carryover contamination during PCR. The 541-bp DNA was amplified by PCR using DNAs from *M. tuberculosis* H37Rv and *M. bovis* as templates. Ten microliters of a solution of the amplicon was added to a new PCR mixture not containing a mycobacterial DNA template, and after the mixture was incubated at 22°C for 10 min and then at 50°C for 5 min, PCR was performed. As shown in Fig. 2, no amplification was observed in the second amplification (lanes 4 and 5), indicating that PCR based on incorporation of dUTP yields amplicons which are completely degraded after incubation with UNG.

Specificity and sensitivity of the MTD system. A total of 33 strains were tested by the Gen-Probe MTD system. These were the reference strains of 23 *Mycobacterium* species and 10 strains of other bacterial species which are frequently associated with pulmonary diseases. Only the organisms belonging to the *M. tuberculosis* complex showed a positive result in the assay system (Table 1).

To investigate the sensitivity of the assay with whole organisms, 10-fold dilutions of four *M. tuberculosis* clinical isolates grown in Middlebrook 7H9 liquid medium were made in cold distilled water after sonication for 10 s. The number of organisms was determined in a manner similar to that used for PCR. Each dilution containing *M. tuberculosis* organisms was sonicated and then assayed by the MTD system. The detection limits in the MTD system were estimated as less than 10 CFU. When the organisms from the dilution containing 10^{-1} CFU/50 μl was used for the assay, the result was positive for two samples and negative for two samples.

Detection of *M. tuberculosis* in clinical samples. A total of 135 sputum specimens were tested for the presence of *M. tuberculosis* by the nucleic acid amplification methods (PCR and MTD systems) as well as by direct smear examination and culture. Thirty-two specimens were positive for mycobacteria by smear, and 50 were positive by culture. Of the strains isolated from the latter 50 specimens, 32 were identified as belonging to the *M. tuberculosis* complex, 16 were identified as members of the *M. avium* complex (Accuprobe; Gen-Probe), and the remaining 2 isolates were identified as *M. kansasii* and *M. chelonae* subsp. *abscessus*. The MB-Check system detected 31 *M. tuberculosis* isolates among the 135 specimens, while culture on egg medium was positive in only 24 cases, confirming our previous work (1) in which two systems based on liquid media, MB-Check and BACTEC, proved to be significantly better than the egg-based solid media for the isolation of mycobacteria from clinical specimens.

As shown in Table 2, 26 of the 32 specimens which yielded *M. tuberculosis* by culture were positive for *M. tuberculosis* DNA by PCR. However, the 541-bp DNA was not detected in the remaining six specimens, which included one specimen which was smear positive and culture positive (for *M.*

TABLE 2. Comparison of two systems based on nucleic acid amplification with conventional smear and culture examinations in sensitivity of detection of *M. tuberculosis* in clinical specimens

Specimens	PCR		MTD	
	Positive	Negative	Positive	Negative
Smear positive-culture positive for *M. tuberculosis* complex	21[a]	1	22	0
Smear negative-culture positive for *M. tuberculosis* complex	5	5	7	3
MOTT[b] positive in culture	0	18	0	18
Smear positive-culture negative	1	2	1	2
Smear negative-culture negative	5	77	4	78
Total	32	103	34	101

[a] Number of specimens.
[b] MOTT, mycobacteria other than *M. tuberculosis* complex.

tuberculosis) and five specimens which were smear negative and culture positive (for *M. tuberculosis*). PCR was positive for an additional six specimens, five of which were negative by both smear and culture and one of which was smear positive and culture negative. The sensitivity, specificity, and negative and positive predictive values of the PCR test were 81.3, 94.2, 94.2, and 81.3%, respectively.

Twenty-nine of the 32 culture-positive (for *M. tuberculosis*) specimens were positive for *M. tuberculosis* by the MTD system. The assay system gave a positive result for all of the 22 specimens which were both smear and culture positive (for *M. tuberculosis*) but failed to detect *M. tuberculosis* in three specimens which were smear negative and culture positive (for *M. tuberculosis*). The MTD system detected *M. tuberculosis* complex organisms in 4 of 82 specimens which were both smear and culture negative and in one of three smear-positive and culture-negative specimens. The sensitivity, specificity, and negative and positive predictive values of the MTD test were 90.6, 95.1, 97.0, and 85.3%, respectively.

No positive results for the *M. tuberculosis* complex were obtained by either the PCR or MTD systems for the specimens which were positive for mycobacteria other than *M. tuberculosis* in culture.

All the patients whose sputum specimens were culture negative but *M. tuberculosis* positive in the PCR or MTD system had clinical signs indicative of tuberculosis, including characteristic radiographs, typical clinical manifestations of the disease, history of exposure to tuberculosis or past history of tuberculosis, and/or clinical response to antituberculosis chemotherapy.

The results of the PCR test after resolution of discrepant results (by patient history) were a sensitivity of 84.2% and a specificity of 100%. The MTD test exhibited a sensitivity and specificity of 91.9 and 100%, respectively.

DISCUSSION

Two oligonucleotides based on the sequence of IS*986*, whose presence is restricted to organisms belonging to the *M. tuberculosis* complex, were used as primers for PCR. The number of copies of the IS*986* element varies from 1 to 19, although most strains have between 8 and 15 copies (18). The use of these oligonucleotides may be useful for increasing PCR sensitivity.

To increase the sensitivity of the PCR or MTD system, we considered it crucial to use the best method for extraction and purification of nucleic acids from clinical samples. The method used for the PCR testing in this study was different from that used for the MTD system. The sensitivities of these two tests, however, were similar; the detection limit of these tests for cultured *M. tuberculosis* was less than 10 cells per reaction. These results were similar to those reported by others (2, 7, 10, 19) for PCR. However, since clumping is a well-known problem with mycobacteria, this estimate may not be entirely accurate. The high specificity of the PCR and MTD systems was revealed by testing cultures of 19 *Mycobacterium* species other than *M. tuberculosis*. The specificity of the PCR and MTD systems was also confirmed by the results obtained with the clinical specimens.

The MTD system detected *M. tuberculosis* in all smear-positive and culture-positive and in 7 of 10 smear-negative and culture-positive specimens (Table 2). In repeated experiments, one of the three negative samples gave a positive result in the MTD system (data not shown). Furthermore, four samples which were both smear and culture negative and one sample which was smear positive and culture negative were positive by the MTD system, indicating the high sensitivity of the Gen-Probe system. The PCR test was only a little less sensitive than the MTD system, although the sample preparation procedure used in the two tests was different. The PCR and MTD systems failed to detect *M. tuberculosis* from six and three culture-positive clinical samples, respectively. It is likely that the larger inoculum (more than 10 times) for culture may account for some of these differences in sensitivity. These results also indicate the limitations of the nucleic acid amplification methods and suggest that further improvement in both assay systems, perhaps including methods of processing of clinical specimens, is necessary.

There was a 97.0% correlation between the PCR and MTD systems in the detection of *M. tuberculosis* from clinical samples, and only 4 (2.9%) of the 135 samples tested gave different results in the two systems. Of these, three were specimens that were smear negative and yielded *M. tuberculosis* only by culture.

The Gen-Probe MTD system provides several distinct technological and clinical advantages compared with traditional probe assays and other amplification methods. The most notable points are as follows. (i) Since rRNA is present in thousands of copies per cell (13), the likelihood of initiating amplification is greater than when a single copy of DNA per cell is used as the target. This advantage can be very important when the organisms to be detected are present in very low numbers; that is when target amplification methods are most useful. (ii) The procedure of the MTD system is simple to perform and gives rapid generation of the target sequence present in the sample. (iii) The RNA product of the amplification system is much more labile outside the reaction tube than is the DNA product made by some other amplification systems. The risk of laboratory contamination and false-positive results should therefore be reduced. (iv) Reagents are only added to the amplification reaction tube, never removed or transferred. This again minimizes the chance of cross contamination and false-positive results.

The PCR and MTD systems for the detection of *M. tuberculosis* in clinical specimens gave overall sensitivities (after resolution of discrepant results) of 84.2% (32 of 38) and 91.9% (32 of 37), respectively, as compared with 71.9% (23 of 32) by smear examination. The MB-Check detected *M. tuberculosis* complex organisms in 31 of 32 specimens.

These results appear to indicate that the sensitivities of the PCR and MTD systems, based on nucleic acid amplification for detection of *M. tuberculosis*, were similar to the sensitivity of the culture method using the MB-Check system. Both the DNA and rRNA amplification methods for the *M. tuberculosis* complex should be very useful for rapid detection of mycobacterial infections, especially for the culture-positive but smear-negative specimens which usually require the long time necessary for culture of *M. tuberculosis*.

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