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Evaluation of Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test and PCR for Direct Detection of *Mycobacterium tuberculosis* in Clinical Specimens

NANCIMAE MILLER, SANDRA G. HERNANDEZ, AND TIMOTHY J. CLEARY*

Department of Pathology, University of Miami, Miami, Florida 33136

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The Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (AMTD) is a direct specimen assay for the identification of *Mycobacterium tuberculosis* from respiratory samples. rRNA is amplified, and the product is detected with a specific chemiluminescent probe. We performed a retrospective evaluation of three separate respiratory specimens from each of 250 patients by using the AMTD and compared the results with those of microscopy, culturing, and a patient chart review. From the latter results, 198 patients (594 specimens) were found negative for *M. tuberculosis* by culturing and clinical criteria. The overall specificity of the AMTD after discrepancy resolution was 98.5% (585 of 594). There were 52 patients with culture-proven and/or clinically diagnosed tuberculosis. Of these 156 specimens, the organism was cultured from 142 (91%), and acid-fast microscopy was positive for 105 (67.3%). The AMTD was positive for 142 (91%) specimens from these patients. Tuberculosis patient samples were tested by a PCR assay which uses primers for amplification of the IS*6110* insertion sequence of the *M. tuberculosis* complex. The PCR assay detected 144 of the 156 (92.3%) specimens. Overall, when three specimens per patient were examined, the AMTD found all 52 patients positive for tuberculosis, while the PCR assay found 51 patients positive by agarose gel analysis and all 52 patients positive by Southern blot hybridization.

Globally, 8 million new cases of tuberculosis are currently diagnosed annually, with a mortality rate of 3 million (15). The United States is experiencing an increase in tuberculosis cases, particularly in large metropolitan areas. This situation contrasts and reverses the downward trend that had been seen for this disease until the early 1980s. The increase in new cases of tuberculosis is also complicated by the emergence of drug-resistant strains of *Mycobacterium tuberculosis*, which can set treatment back to the preantibiotic days of tuberculosis.

The current diagnostic methods used in clinical laboratories are growth dependent and therefore can require up to 6 to 8 weeks to report a negative result. The use of BACTEC 12B bottles (Becton-Dickinson Diagnostic Instrument Systems, Sparks, Md.) in conjunction with the Accuprobe for *M. tuberculosis* (Gen-Probe Inc., San Diego, Calif.) can shorten the time for identification of this organism. These procedures still require 1 to 3 weeks to provide results from patient specimens that contain acid-fast organisms. Therefore, the ability to directly detect *M. tuberculosis* in respiratory specimens offers a rapid and specific diagnosis of tuberculosis, an obvious public health benefit. A number of investigators have reported the detection of sequences specific for *M. tuberculosis* directly in clinical specimens by PCR (1–3, 5–11, 16–22). Amplification methods provide a rapid method for detecting DNA from the slowly growing mycobacteria. In this paper, we present data from a new amplification method developed by Gen-Probe for the direct detection of *M. tuberculosis* in respiratory specimens and compare this method with PCR for patients with tuberculosis.

* Corresponding author. Mailing address: Department of Pathology (D-33), University of Miami/Jackson Memorial Medical Center, P.O. Box 016960, Miami, FL 33101. Phone: (305) 585-7851. Fax: (305) 585-0008.

MATERIALS AND METHODS

Clinical specimens. Sputum specimens were obtained from the clinical microbiology laboratory of Jackson Memorial Hospital, a large county hospital serving an urban population with a high prevalence of tuberculosis in south Florida. Three separate specimens per patient were examined for 250 patients. Specimens were digested and decontaminated by treating patient sputum with an equal volume of *N*-acetyl-L-cysteine–NaOH (5%) for 15 to 20 min and concentrated by centrifugation at 3,000 × *g* for 15 min (14). Excess fluid was poured off, and the sediment was resuspended in 2.0 ml of 0.67 M phosphate buffer (pH 5.3). All reagents used in the laboratory to process specimens were filtered through a 0.22-μm-pore-size filter prior to sterilization. Sediment from each specimen was used to prepare two smears and to inoculate a Middlebrook 7H11 plate and a BACTEC 12B bottle. Mycobacterial cultures were incubated for 6 weeks. The remaining sediments were stored at −20°C until the amplification tests were initiated and the final results were obtained.

The fixed smears were stained with auramine fluorochrome stain (14). The number of fluorescent acid-fast bacilli (AFB) was reported on the basis of the following criteria at a ×400 magnification: no AFB seen = negative, one to three per slide = rare, one to nine per 10 fields = few, one to nine per field = moderate, and greater than nine per field = many.

Gen-Probe AMTD. The Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (AMTD) was done in accordance with the instructions in the package insert and as recently described by Jonas et al. (13). In brief, a 50-μl aliquot of sediment was added to a tube containing glass beads and 200 μl of sample buffer and sonicated for 15 min at room temperature in a Lab-Line sonicator (312 W). A 50-μl aliquot of lysate was added to a tube containing 25 μl of amplification reagent and 200 μl of oil. The tube was incubated at 95°C for 15 min and then cooled to 42°C for 5

TABLE 1. Summary of the AMTD results

Tuberculosis clinical criterion	No. (%) of specimens that were:				
	Total	*M. tuberculosis* culture positive	AFB smear positive	AMTD positive	
				Initial	Final
Negative	594	0[a]	15	28 (4.7)	9 (1.5)
Positive	156	142	105	131 (83.9)	142 (91.0)

[a] One hundred eighteen of these specimens grew species of mycobacteria other than *M. tuberculosis*.

min. The amplification enzyme (25 μl) was added to the tube and incubated at 42°C for 2 h. Twenty microliters of termination reagent was added, and the reaction mixture was incubated at 42°C for 10 min. A specific acridinium ester-labeled hybridization probe was added to the tube and incubated at 60°C for 15 min. Selection reagent (300 μl) was added to each sample and incubated at 60°C for 10 min. The samples were read in a Gen-Probe luminometer. The positive cutoff value of 30,000 relative light units was used for this assay. Each run included positive and negative amplification controls at the beginning and end. Positive and negative hybridization controls were added at the end of each run. Samples giving results discrepant from culture results were tested again with a new aliquot from the same processed specimen.

PCR assay. (i) Sample preparation. A 200-μl aliquot was transferred to a 1.5-ml microcentrifuge tube, heated at 80°C for 20 min to kill the organisms, and centrifuged for 5 min at 15,000 × *g*. The supernatant fluid was carefully removed, and the pellet was resuspended in 200 μl of buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.001% gelatin. The sample was transferred to a 1.8-ml round-bottom Nunc tube containing 200 mg of acid-washed glass beads (<106 μm in diameter; Sigma Chemical Co., St. Louis, Mo.). The tube was vortexed for 5 s, sonicated for 30 min at room temperature, and boiled at 100°C for 20 min. The sample was cooled rapidly to room temperature. In the case of problem samples, the lysate was transferred to a 1.5-ml microcentrifuge tube containing 75 μl of chloroform, and the tube was vortexed for 5 s and centrifuged for 30 s to separate the phases. The aqueous phase containing the DNA was transferred to a microcentrifuge tube, and 5 μl was used for amplification.

(ii) DNA amplification. The master mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 100 μmol of each nucleotide, 10 pmol of each primer, 200 fg of internal control DNA, and 1 U of *Thermus aquaticus* (*Taq*) polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The primers amplify a 123-bp segment of the IS*6110* insertion sequence of the *M. tuberculosis* complex chromosome (8, 23). Five microliters of a sample was added to 45 μl of the master mix. The amplifications were performed with a Perkin-Elmer 9600 thermal cycler. The final amplification protocol included denaturation of DNA at 94°C for 2 min and then 30 amplification cycles. Each cycle consisted of denaturation of DNA for 30 s, annealing of primers at 68°C for 30 s, and primer extension at 72°C for 30 s, with no increase in extension time with each cycle. Our initial amplification procedure used the same temperatures, but each cycle was held for a longer time period, with an increase of 5 s for each extension cycle. The slight change in the amplification protocol did not alter results but decreased the amplification time by 75 min.

(iii) Product detection. A 15-μl aliquot of the amplified DNA was analyzed by electrophoresis in 2.0% agarose gels. The 123-bp product DNA was stained with ethidium bromide and photographed. The DNA was denatured and transferred to positively charged nylon membranes (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 0.4 N NaOH for 90 min with a vacuum blotter (Bio-Rad Laboratories, Richmond, Calif.). The manufacturer's recommendations were followed for hybridization. The membranes were hybridized at 50°C with a 20-bp oligonucleotide probe which covered positions 821 through 840 of the IS*6110* sequence (22). The probe was 3'-end labeled with digoxigenin-ddUTP (Genius System; Boehringer).

(iv) Controls. *M. tuberculosis* H37RA was used as a positive control for the 123-bp product. Control template DNA that produces a 600-bp product with the same set of primers was provided by K. Eisenach and D. Cave (University of Arkansas, Little Rock). The use of this template DNA, referred to as internal control DNA, allows verification that the reaction is not inhibited by interfering substances unique to a patient sample. A control tube containing no DNA and a tube containing internal control DNA as a sole source of template were included with each set of reactions.

RESULTS

Table 1 presents the AMTD results for the 750 specimens from the 250 patients. Of these patients, 198 were found negative for *M. tuberculosis* by culturing and clinical criteria. *Mycobacterium* species other than *M. tuberculosis* were cultured from 118 of these specimens. On the initial run, 28 of 594 (4.7%) specimens were found positive by the AMTD. These 28 positive specimens from tuberculosis-negative patients were retested with a new aliquot from the sample. Only nine samples (seven patients) were positive in the repeat run. After discrepancy analyses, the specificity of the test was found to be 98.5%. For the 52 patients with culture-proven and/or clinically diagnosed tuberculosis, 131 of 156 (83.9%) specimens were found positive on the initial run. Repeat testing of specimens with a different aliquot from the same processed sediment increased detection to 142 of 156 (91.0%) specimens.

All tuberculosis-positive patient samples were tested by PCR, as were any AMTD discrepant samples. Table 2 presents a summary of the results for the samples from the 52 patients with culture-proven and/or clinically diagnosed tuberculosis. The PCR assay detected 122 of 156 (78.2%) specimens upon initial testing. The initial testing for the PCR assay was done with the crude lysate prior to chloroform extraction of the lysate. Chloroform extraction of the lysate was necessary to eliminate inhibitors of amplification in the sample. An additional 21 specimens were amplified by PCR after extraction of the original samples with chloroform. The sensitivity of the PCR was 92.3%. For specimens that were

TABLE 2. Summary of AMTD and PCR assay results in comparison with AFB smear and culture results

Smear result[a]	No. of specimens (total no. tested) that were:				
	Culture positive	AMTD positive		PCR positive	
		Initial	Repeat[b]	Initial	Repeat[c]
0	37 (51)	32	37	21	41
1	22 (22)	20	22	19	20
2	28 (28)	26	28	28	28
3	21 (21)	20	21	20	21
4	34 (34)	33	34	34	34
Total	142 (156)	131	142	122	144

[a] 0, no AFB seen; 1, rare AFB seen; 2, a few AFB seen; 3, moderate numbers of AFB seen; 4, many AFB seen.
[b] Negative samples were retested with a different aliquot of the patient specimen.
[c] The same aliquot of the patient specimen was retested subsequent to chloroform extraction.

AFB smear positive, the initial assay was positive for 99 of 105 (94.3%) specimens in the AMTD and 101 of 105 (96.2%) specimens in the PCR. After repeat testing, the AMTD detected all AFB smear-positive specimens, whereas the PCR detected 98%.

The 51 AFB smear-negative samples were from 25 different patients. Nine patients were AFB smear-negative for all three specimens, eight patients had two AFB smear-negative specimens, and one specimen was AFB smear negative for eight patients. The AMTD detected 63% of the AFB smear-negative samples on the initial run; this value was increased to 72.5% upon repeat testing. For the PCR, initial testing of crude lysates detected 41% of the AFB smear-negative samples; this value was increased to 78% by the addition of chloroform extraction to the lysis protocol.

An analysis of the first submitted specimen showed that 32 of 52 (61.5%) were AFB smear positive. For these specimens, 45 patients were found positive by the AMTD and 47 were found positive by the PCR assay. When three specimens per patients were evaluated for laboratory diagnosis, all 52 patients were found positive by the AMTD, 51 patients were found positive by the PCR assay with ethidium bromide staining of product DNA, and all 52 patients were found positive by the PCR assay when Southern blot hybridization was also performed. Culturing, the AMTD, and the PCR assay showed approximately the same sensitivities after discrepancy resolution. Of the 156 patient specimens, 67% were smear positive, 91% were culture positive, 91% were AMTD positive, and 92% were PCR positive.

DISCUSSION

The recent increase in new cases of tuberculosis has prompted the development of rapid diagnostic assays for *M. tuberculosis*. Nucleic acid amplification assays, such as PCR, are rapid specific tests that can be done directly on patient specimens, but this methodology is currently not practical for routine use in the average clinical laboratory. The AMTD overcomes many of the challenges of the PCR procedure. The packaged kit contains all of the tubes and specific reagents needed for specimen lysis, amplification, and product detection. The AMTD specimen lysis procedure is a single-tube method in which the tube is entered only twice, once to add the patient sample prior to sonication and then again to withdraw a sample for the amplification reaction. This simple lysis method is important for controlling the potential for cross-contamination between specimens. The PCR specimen lysis procedure that we use is relatively simple; however, the chloroform extraction step adds the need to transfer the sample to another tube. A number of sample preparation methods for PCR assays for mycobacteria have been reported in the literature (2, 3, 5, 9, 10, 12, 16, 19). Many of these methods include the use of a variety of detergents or proteolytic enzymes to disrupt the organisms and then ethanol precipitation or DNA capture to purify the DNA after lysis (2, 5, 9, 12, 19).

The AMTD was done on 750 specimens from 250 patients; 198 of these patients were found negative for *M. tuberculosis* by culturing and clinical criteria. The specificity of the test initially was 95.3%; after repeat testing of discrepant samples, the specificity was 98.5%. A small fraction of the patients who were included in the tuberculosis-negative group had discrepant results. Seven patients in this category had one or more specimens positive in the AMTD. The PCR assay was positive for one or more samples from all of these patients. None of these patients was admitted to the hospital with a diagnosis of tuberculosis in the 12-month period following the original sample collection. Six of the seven individuals are human immunodeficiency virus antibody positive. The specimens may have been contaminated during the digestion and decontamination procedure, but considering that many of the patients had more than one specimen positive, it is possible that these patients will return to our hospital with tuberculosis. An example of this situation occurred in our tuberculosis-positive group. One patient had a history of tuberculosis, and three specimens submitted during the study were AFB smear and culture negative; one was positive in the AMTD, and all three were positive in the PCR. Subsequent specimens submitted 6 months later were culture positive for *M. tuberculosis*.

Fifty-two patients were considered positive for *M. tuberculosis* by cultural and clinical criteria. These patient samples were also evaluated by the PCR. The initial comparative sensitivities of the AMTD and the PCR for these patients were 83.9 and 78.2%, respectively. When specimens that gave discrepant results were retested and the final PCR protocol was adapted, the sensitivities were 91 and 92.3%. Several studies have evaluated the PCR for the detection of *M. tuberculosis* in clinical specimens (1–3, 5, 6, 9, 10, 16, 18, 20–22) and reported good success with AFB smear-positive samples; however, the sensitivity for AFB smear-negative samples has been unacceptable for the PCR assay to be used routinely in a clinical setting. AFB smear-positive specimens were detected without difficulty; however, the PCR failed to amplify DNA from two specimens that were found to contain rare organisms by microscopy. For the 51 AFB

smear-negative specimens, the sensitivities of the AMTD and the PCR were 69 and 78% after our analyses of discrepant results and the use of the final PCR method. Specimens that are AFB smear negative or contain rare organisms detected by the smear technique may not contain the target organism in a particular aliquot because of natural clumping and cording of the organism. Both assays found more than 85% of patients positive with the first submitted specimen. Overall, when three separate specimens were tested, the AMTD found all 52 patients positive, and the PCR found 51 patients positive by agarose gel analysis of the ethidium bromide-stained amplified product and all 52 patients positive by Southern blot hybridization. For specimens that were AFB smear negative or contained rare AFB, testing of three specimens was necessary to obtain a reliable result. On occasion, we obtained a negative result for a previously positive specimen when we retested AFB smear-negative samples. This result indicates that there may be few targets present in AFB smear-negative samples, thus supporting the need for the testing of more than one specimen or the testing of multiple samples from the same specimen before reporting a negative result. Our recommendation for the analysis of AFB smear-negative patients would be to test three specimens from the patient before reporting a negative amplification result. This recommendation is important from an infection control standpoint, as it is better to have a patient in isolation and receiving medication while multiple specimens are tested than to rely on a single amplification result.

For the 156 specimens from the 52 tuberculosis patient, *M. fortuitum* was isolated from 24 (15.4%) of the specimens. The presence of *M. fortuitum* in the samples did not interfere with either assay, but this type of contamination does make it more difficult to isolate *M. tuberculosis* by culturing because of overgrowth by the rapidly growing *M. fortuitum* (4).

The AMTD was well suited to our busy clinical laboratory because up to 50 specimens could be amplified in a single run. Following concentration of the samples in the mycobacterium area, the total time for completion of the assay was 5 h and 15 min. This total time consisted of 3 h and 15 min of incubation time and 2 h of technical time. To process 25 specimens with the PCR assay, the total time needed for detection of the amplified product by ethidium bromide staining was 6 h. This total time consisted of 4 h of incubation time and 2 h of technical time. With the PCR assay, it was technically difficult to process more than 25 specimens in a single run. All specimens had to be extracted with chloroform prior to testing, and this step increased the initial preparation time and the potential for contamination of the assay. Additional samples could have been processed during some of the longer incubation steps, but this activity would have extended the total time for completion of the assay for 50 specimens beyond 8 h. Southern blot hybridization added an additional full day to the assay. This latter step would not be practical for a normal clinical laboratory because it would be too time-consuming for the additional benefits. Overall, sample preparation, product detection, total turnaround time, and technical hands-on time demonstrate the usefulness of the AMTD in the clinical microbiology laboratory. The cost of this procedure has not been set at this time; therefore, a cost comparison with the PCR assay cannot be made. The total expenditure for the PCR assay would include the purchase of the instrument, reagents (including primers and probes), and possible royalty fees.

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