JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 193–196 Vol. 35, No. 1
0095-1137/97/$04.00+0

Comparative Evaluation of Two Commercial Amplification Assays for Direct Detection of *Mycobacterium tuberculosis* Complex in Respiratory Specimens

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Received 1 May 1996/Returned for modification 20 August 1996/Accepted 16 October 1996

Two commercial assays detecting the presence of *Mycobacterium tuberculosis* complex in clinical specimens by rRNA target amplification (Gen-Probe Amplified *M. tuberculosis* Direct Test [AMTD]) and PCR (Amplicor) were evaluated. The tests were applied to 327 digested, decontaminated respiratory specimens collected from 236 patients. Results were compared with those of acid-fast staining and culture. The combination of culture and clinical diagnosis was considered the "gold standard." A total of 60 specimens were collected from 27 patients with a diagnosis of pulmonary tuberculosis. Thirteen of these specimens were from patients receiving standard antituberculosis therapy and therefore were not included in the comparison. Of the remaining 47 specimens, 33 were smear positive, 40 were culture positive, 45 were AMTD positive, and 39 were Amplicor positive. After resolution of discrepant results, the overall sensitivities, specificities, and positive and negative predictive values were 77, 100, 100, and 95 for staining; 87, 100, 100, and 97.4 for culture; 95.9, 98.9, 94, and 99.2 for AMTD; and 85.4, 99.6, 97.9, and 97.1 for Amplicor, respectively. Agreement between AMTD and Amplicor assay results was 96.8%. It is concluded that although both nucleic acid amplification methods are rapid and specific for the detection of *M. tuberculosis* complex in respiratory specimens, AMTD appeared to be more sensitive than Amplicor.

At the end of the 20th century, an increase in the prevalence of tuberculosis has been noted, and its incidence seems likely to increase over the next decade, mainly due to the human immunodeficiency virus epidemic. Definitive diagnosis is still based on microscopy and culture.

Unfortunately, both techniques have limitations. Microscopy, while quick and easy, has poor sensitivity. Culture on solid media is more specific and sensitive, but results require several weeks of incubation. The use of radiometric liquid medium and biphasic culture (MB-Check acid-fast bacillus [AFB]; Becton-Dickinson Microbiology Systems) in conjunction with nucleic acid probes (Accuprobe; Gen-Probe Inc., San Diego, Calif.) has considerably shortened the detection time, but even these procedures require a minimum of 2 weeks before a definitive laboratory diagnosis of tuberculosis can be made. Moreover, as with conventional culture media, these systems are entirely growth dependent.

The use of PCR to detect the presence of *Mycobacterium tuberculosis* in clinical specimens has been widely reported (3, 6, 7, 9, 15). Although PCR can be specific and sensitive, there are difficulties associated with the technique. Problems of contamination (10), the presence of amplification inhibitors (4), and, more recently, an unexpected high variation in sensitivity (14) have called the utility of "homemade" PCR in the clinical laboratory into question.

Two newly developed, ready-to-use, direct detection kits for *M. tuberculosis* complex are commercially available. The Gen-Probe Amplified *M. tuberculosis* Direct Test (AMTD) is based on the isothermal amplification of rRNA by DNA intermediates, whereas the Roche Amplicor *M. tuberculosis* test (Amplicor) (F. Hoffmann-La Roche Ltd., Basel, Switzerland) employs PCR to amplify a specific DNA sequence of the 16S rRNA gene. The aim of the present study was to evaluate these new commercial amplification methods and to compare them with conventional microscopy and culture.

MATERIALS AND METHODS

Study design. Clinical samples were collected from the microbiology laboratories of two Italian hospitals. The Clinical Microbiology Laboratory of Pordenone General Hospital serves a 950-bed regional hospital in northern Italy, whereas the Department of Clinical Microbiology of the Umberto I°-Torrette Hospital serves a 1,100-bed regional hospital in central Italy. Both laboratories receive an average of 250 specimens per month for mycobacterial culture which are obtained almost entirely from patients admitted to the Pulmonary Medicine and Infectious Diseases wards.

Specimen collection and processing. A total of 327 sequential specimens from 236 patients were investigated. Specimens were limited to sputa, bronchoalveolar lavages, bronchial washings, and gastric aspirates. All were liquefied with dithiothreitol (Sputasol; Unipath, Basingstoke, United Kingdom) and decontaminated for 15 min with NaOH (final concentration, 1.5%). After decontamination, an equal volume of phosphate-buffered saline (PBS) (pH 6.8) was added, and the specimens were centrifuged at 3,000 × *g* for 20 min at 4°C. Then the sediment was resuspended in 2 ml of PBS and neutralized with 1 N HCl. The culture medium was inoculated with part of the sediment from each specimen and used for acid-fast staining, while the remaining sediment was aliquoted and stored at −80°C until the amplification techniques were performed.

Culture. The processed sediment (0.5 ml) was cultivated by using a radiometric BACTEC technique (Becton-Dickinson Diagnostic Instrument Systems) and MB-Check AFB culture bottles (Becton-Dickinson Microbiology Systems). In addition, Löwenstein-Jensen medium was inoculated with 0.2 ml of the sediment. The Löwenstein-Jensen tubes and the MB-Check AFB bottles were incubated at 35 to 37°C for 8 weeks and inspected for growth twice a week for the first 4 weeks and weekly thereafter. The radiometric growth index of the 7H12 vials was recorded with a BACTEC instrument twice a week for 6 weeks. A growth index of >10 was considered positive, and smears were made to confirm the presence of AFB.

Microscopy. Smears were stained by the Ziehl-Neelsen method and examined under the oil immersion objective lens of a microscope (magnification, ×1,000).

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TABLE 1. Distribution of specimens that were tested for *M. tuberculosis* by AMTD and Amplicor

Culture result	No. of samples from the indicated source				
	Sputum	Bronchial washing	BAL[a]	Gastric aspirate	Total
Positive	30	10	0	0	40
Negative	182	90	3	12	287

[a] BAL, bronchoalveolar lavage.

Identification of mycobacteria. Isolates were identified by use of specific DNA probes (Accuprobe; Gen-Probe) and standard procedures (13).

Amplification procedures. Amplification assays were run in three separate areas which had been set up in two rooms.

Gen-Probe AMTD. The Gen-Probe AMTD was performed according to the instructions supplied by the manufacturer. Briefly, a 50-μl aliquot of sediment was added to a tube containing glass beads and sample buffer and sonicated for 15 min in a water bath sonicator at room temperature. 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 min. An enzyme reagent mix was added, and the mixture was incubated at 42°C for 2 h. Termination reagent was added, and the reaction mixture was further incubated at 42°C for 10 min. For detection, a specific labelled hybridization probe was added to the tube and the mixture was incubated at 60°C for 15 min; then the selection reagent was added to each sample, and the mixtures were incubated at 60°C for 10 min. The sample results were read in a Leader 50 luminometer (Gen-Probe); a cutoff value of 30,000 relative light units (RLUs) was used for positive specimens. Each run included positive and negative amplification controls as well as positive and negative hybridization controls. Moreover, two smear-positive sediment samples of clinical origin, previously collected and stored at −80°C, were also included as controls in each run.

Roche Amplicor PCR. The Roche Amplicor PCR was done according to the manufacturer's instructions. It consists of three steps: specimen preparation, amplification, and detection. In brief, a 100-μl aliquot of the sediment sample was mixed with wash solution and centrifuged (13,000 × *g*) for 10 min. After centrifugation, the supernatant was removed and lysis reagent was added to the pellet. After being vortexed, the suspension was incubated at 60°C for 45 min to complete lysis of the mycobacteria. The lysed material was then neutralized by the addition of neutralization reagent. For amplification, 50 μl of the neutralized specimen was added to 50 μl of the master mix reagent. The latter was prepared by the addition of 100 μl of uracil *N*-glycosylase enzyme (Amperase; Roche Molecular Systems, Inc.) to an amplification mixture containing nucleotides, biotinylated primers, and thermostable *Taq* polymerase just prior to the amplification process. The primers used amplify a 584-bp sequence located in a highly conserved region of the 16S rRNA gene of *Mycobacterium* spp. The PCR procedure was carried out by using a 37-cycle program in a Thermocycler TC 9600 (Perkin-Elmer Cetus, Norwalk, Conn.). After amplification, the amplified nucleotide sequences were detected by hybridization to a DNA probe specific for *M. tuberculosis* complex. Hybrid detection was then accomplished with an avidin-horseradish peroxidase conjugate-tetramethylbenzidine substrate system. The reaction was stopped by the addition of hydrosulfuric acid, and the absorbances were read at a wavelength of 450 nm. Specimens giving an absorbance value (AV) of >0.350 were considered positive. Positive and negative amplification controls, as well as two smear-positive sediment samples of clinical origin, were included in each run.

Patient clinical evaluation. Clinical assessment included each patient's medical history, signs, symptoms, chest X ray, microbiological results, and follow-up observations, as well as the results obtained from additional specimens collected during the follow-up. All of the considered records were reviewed by a tuberculosis expert, enabling us to set the combination of culture and clinical diagnosis with high suspicion as the "gold standard." After this analysis, amplification results were reclassified as appropriate.

Statistical analysis. Statistical comparisons were calculated by using the chi-square test; a P of <0.05 was considered significant.

RESULTS

Analytical performance of Gen-Probe AMTD and Roche Amplicor PCR. Positive and negative results could be clearly distinguished by the magnitude of both RLUs and AVs. The majority of samples with positive results had >1,500,000 RLUs for AMTD and >1.500 AVs for Amplicor. Samples with negative results had values far below the cutoffs of 30,000 RLUs for AMTD and 0.350 AV for Amplicor.

Clinical results. A total of 327 respiratory specimens collected from 236 patients were included in this study. Altogether, 40 specimens yielded cultures positive for *M. tuberculosis* (Table 1). A comparison of amplification results with smears, cultures, and clinical data is summarized in Table 2. A total of 60 specimens were obtained from 27 patients with diagnosis of tuberculosis, and 267 specimens were from 209 patients with nontuberculous pulmonary disease, based on clinical and radiological findings. Of the 33 samples which were smear and culture positive, all were AMTD positive and 32 were Amplicor positive. Seven samples were smear negative for AFB but culture positive; five were AMTD positive, and six were Amplicor positive. There were seven samples, smear and culture negative, collected from patients for whom tuberculosis was strongly suspected clinically. All of these were AMTD positive, but only one was Amplicor positive. Thirteen specimens were collected from patients with pulmonary tuberculosis who were receiving drug therapy. All of these were smear and/or culture positive before therapy but were both smear and culture negative at the time the samples were taken for amplification. Five of these were AMTD positive, and four were Amplicor positive. Of the 267 samples from patients with nontuberculous pulmonary disease that were smear and culture negative for AFB, three were AMTD positive and one was Amplicor positive. These samples, after resolution of discrepant results based on a negative patient culture history, were considered to be false positive. Moreover, when calculating sensitivity, specificity, and predictive values, we decided to omit samples from patients with pulmonary tuberculosis who were receiving drug therapy. In our opinion, chemotherapy may represent a potential pitfall as it modifies, in an unpredictable way, the performance of all diagnostic methods. Table 3 shows the sensitivity, specificity, and predictive values of both amplification methods compared with AFB smear and culture, assuming the combination of culture and clinical diagnosis as the gold standard.

DISCUSSION

The increased incidence of tuberculosis has stimulated the development of rapid and direct detection methods for the laboratory diagnosis of *M. tuberculosis*. Newly developed, standardized, commercial detection assays include Gen-

TABLE 2. Comparison of AMTD and Amplicor with smear and culture results and clinical data

Patient status	No. of specimens		
	Total	Positive by AMTD	Positive by Amplicor
Acid-fast smear and *M. tuberculosis* culture positive	33	33	32
Acid-fast smear negative, *M. tuberculosis* culture positive	7	5	6
Acid-fast smear and *M. tuberculosis* culture negative with high clinical suspicion of tuberculosis	7	7	1
Acid-fast smear and/or *M. tuberculosis* culture positive previously, negative during therapy	13	5	4
Acid-fast smear and *M. tuberculosis* culture negative, nontuberculosis pulmonary disease	267	3	1
Total	327	53	44

TABLE 3. Sensitivities, specificities, and predictive values of various methods[a]

Method	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
Smear	77	100	100	95
Culture	87	100	100	97.4
AMTD	95.9	98.9	94	99.2
Amplicor	85.4	99.6	97.9	97.1

[a] The combination of culture results yielding *M. tuberculosis* and clinical diagnosis of tuberculosis was considered the gold standard.

Probe AMTD and Roche Amplicor. The kits contain all the reagents needed for sample amplification and detection, as well as controls. In our experience, the analytical performance of both amplification assays showed good reproducibility. Generally, differences between cutoff values, positive and negative controls, and samples were broad enough to permit easy discrimination. The kits were well suited for use in a routine microbiology laboratory; however, the AMTD time is shorter than Amplicor's (5 versus 6.5 h), and the procedure is easier to perform.

Thirty-eight of 40 culture-positive samples, including 7 that were smear negative and culture positive, were positive by both systems. Negative results obtained by amplification assays for culture-positive samples may be explained by the presence of inhibitors of enzymatic amplification and/or by a low number of mycobacteria, unequally distributed in the test suspension. Moreover, the observation that one smear-positive sample, which was likely to contain many AFB, was Amplicor negative emphasizes the importance of endogenous sputum inhibition as one major problem of DNA amplification by Amplicor PCR (17). Thus, it would be useful if inhibition controls were included in the amplification assays. Of the seven samples that were both smear and culture negative, obtained from patients strongly suspected of having tuberculosis, all were AMTD positive and only one was Amplicor positive, suggesting that AMTD appears to be more sensitive than Amplicor in detecting few mycobacteria or, more likely, that AMTD is less susceptible to inhibitors. During the follow-up, clinical suspicion could be confirmed for five of seven patients for which *M. tuberculosis* was grown from additional sputum specimens. A small number of samples collected from patients found negative for *M. tuberculosis* by culturing and clinical criteria showed discrepant results (Table 2). Three samples in this category were positive by AMTD and one was positive by Amplicor; however, they gave positive results for one amplification assay only, and additional specimens from the patients were also negative by any test. We conclude that these specimens may have been contaminated during the aliquoting procedures just before being frozen. Table 4 shows the sensitivity, specificity, and predictive values of both amplification methods for smear-positive and smear-negative specimens.

TABLE 4. Sensitivities, specificities, and predictive values of AMTD and Amplicor for acid-fast smear-positive and smear-negative specimens

Specimen type (*n*) and test	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
Smear positive (33)				
AMTD	100	100	100	100
Amplicor	97	100	100	99.6
Smear negative (281)				
AMTD	87.5	98.8	82.3	99.2
Amplicor	66.7	99.6	93.3	97.4

Of the 13 samples obtained from patients receiving standard antituberculosis therapy, 5 were AMTD positive and 4 were Amplicor positive. These results may be considered paradoxical, as AMTD detects rRNA, which rapidly disappears after microbial death. Probably, the high number of rRNA copies per cell (about 2,000 versus one DNA copy) and the short duration of therapy explain our data.

In summary, the sensitivity and specificity of AMTD were 95.9 and 98.9%, respectively, while Amplicor PCR reached a sensitivity of 85.4% and a specificity of 99.6%.

Data from the literature are in agreement with our findings: AMTD sensitivities and specificities ranged from 91 to 98.4% and from 96.9 to 100%, respectively (1, 11, 16, 18), while Amplicor showed sensitivities and specificities ranging from 66.7 to 86% and from 97 to 99.6% (2, 5, 12, 17). Recently, two groups performed comparative evaluations of AMTD and Amplicor; both reported AMTD as being more sensitive than Amplicor (86.2 versus 82.8% [19] and 100 versus 97.8% [8]). They also demonstrated very high specificities for both assays (100% each [19] and 99.3 and 98.9% [8]). In our study, the difference between AMTD and Amplicor sensitivities was found to be statistically significant ($P = 0.045$). However, since we could not document the detection limits of both assays in terms of the minimum number of *M. tuberculosis* CFU in clinical specimens, we suspect the significance of such a difference to be only marginal.

In conclusion, although the exact role of amplification assays has yet to be determined, AMTD and Amplicor were shown to be rapid and specific for the detection of *M. tuberculosis* in respiratory specimens and suitable for a clinical microbiology laboratory's work flow. The good performance of AMTD makes it a useful tool for tuberculosis diagnosis.

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