JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2000, p. 1559–1562 Vol. 38, No. 4
0095-1137/00/$04.00+0

Comparison of Enhanced *Mycobacterium tuberculosis* Amplified Direct Test with COBAS AMPLICOR *Mycobacterium tuberculosis* Assay for Direct Detection of *Mycobacterium tuberculosis* Complex in Respiratory and Extrapulmonary Specimens

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Received 21 October 1999/Returned for modification 1 December 1999/Accepted 20 January 2000

The new Roche COBAS AMPLICOR *Mycobacterium tuberculosis* Assay was compared to the Gen-Probe enhanced *Mycobacterium tuberculosis* Amplified Direct Test (AMTDII). A total of 486 specimens (296 respiratory and 190 extrapulmonary) collected from 323 patients were tested in parallel with both assays. Results were compared with those of acid-fast staining and culture, setting the combination of culture and clinical diagnosis as the "gold standard." After resolution of discrepant results, the sensitivity, specificity, and positive and negative predictive values for AMTDII were 85.7, 100, 100, and 90.4% for respiratory specimens and 82.9, 100, 100, and 95.5% for extrapulmonary specimens, respectively. The corresponding values for AMPLICOR were 94.2, 100, 100, and 96.6% for respiratory specimens and 85, 100, 100, and 96.1% for extrapulmonary specimens, respectively. No significant differences were observed between the results of both assays or, within each one, between respiratory and extrapulmonary specimens. The difference between AMTDII and AMPLICOR sensitivities was related to the presence of inhibitory samples, which the former assay, lacking an internal amplification control (IAC), could not detect. The overall inhibition rate for the AMPLICOR assay was 3.9% (19 specimens). It is concluded that, although both amplification assays proved to be rapid and specific for the detection of *M. tuberculosis* complex in clinical samples, AMPLICOR, by a completely automated amplification and detection procedure, was shown to be particularly feasible for a routine laboratory setting. Finally, AMTDII is potentially an excellent diagnostic technique for both respiratory and extrapulmonary specimens, provided that an IAC is included with the assay.

Since their introduction to the clinical mycobacteriology laboratory, amplification techniques have been welcomed as being able to have a strong impact on the speed and accuracy of diagnostic results. However, the promise of timely and sensitive detection of *Mycobacterium tuberculosis* complex (MTB) directly from clinical specimens is still unfulfilled because of the unsatisfactory sensitivity of current amplification assays. A number of amplification systems have been described; besides in-house assays, commercial systems have been developed with the aim of providing standardized, easy-to-use kits having the potential of "walk-away" automation. Moreover, recent evidence of inhibitory samples has brought companies to develop kits containing a second target to be used as an internal amplification control (IAC). The IAC monitors amplification and detection steps, thereby making negative test results truly reliable.

To date, a few commercial systems for the detection of MTB in clinical samples are available in Italy. Of these, the Amplified *Mycobacterium tuberculosis* Direct Test (Gen-Probe, Inc., San Diego, Calif.) has recently been upgraded, featuring a larger amount of sediment sample combined with a shorter assay time, and is marketed as the enhanced AMTD (AMTDII), whereas the COBAS AMPLICOR MTB System (Roche Diagnostic Systems, Inc., Branchburg, N.J.) exhibits an internal control for monitoring of amplification inhibitors coupled with a high degree of automation.

The purpose of this study was to carry out a comparative evaluation of these assays.

MATERIALS AND METHODS

Study design. Four hundred eighty-six clinical specimens, consecutively received for culture of acid-fast bacilli (AFB) by the Regional Mycobacteria Reference Centre in Vicenza, Italy, were used in this study. The specimens, almost entirely collected from inpatients for whom tuberculosis (TB) was strongly suspected, were submitted to the reference laboratory from different hospitals within the whole region.

Specimen collection and processing. The specimens investigated were collected from 323 patients and included 257 sputum samples, 2 bronchoalveolar lavages, 37 bronchial washings, 4 gastric aspirates, 70 urine samples, 37 normally sterile body fluid (pleural, pericardial, synovial, cerebrospinal fluid [CSF], and ascites fluid) samples, and 79 miscellaneous samples, such as pus and biopsy specimens.

Respiratory specimens were liquefied and decontaminated by the standard *N*-acetyl-L-cysteine–sodium hydroxide (NALC-NaOH) method (6). Extrapulmonary specimens, such as urine, gastric aspirates (which were neutralized upon receipt with 0.067 M phosphate-buffered saline [PBS; pH 6.8]), and pleural and other similar body fluids (pericardial, synovial, and ascites fluid), were centrifuged at 3,300 × *g* for 15 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 ml of sterile water and decontaminated with NALC-NaOH. Part of the sediment from each specimen was inoculated onto the culture media and used for acid-fast staining, while the remainder was aliquoted and stored at −80°C until the amplification techniques were performed. CSF specimens were cultured without prior decontamination.

Pretreatment of selected clinical specimens for amplification. (i) Pretreatment of CSF. CSF was treated with NALC-NaOH and centrifuged at 12,000 × *g* for 10 min. The pellet was resuspended in PBS and frozen in aliquots until the amplification techniques were performed.

(ii) Pretreatment of pleural and other sterile fluids. After decontamination with NALC-NaOH, the sediment was washed twice with sterile distilled water before being stocked for amplification assays.

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TABLE 1. Comparison of AMTDII and AMPLICOR amplification assays with smear and culture results and clinical data for respiratory specimens[a]

Patient status (no. of specimens)	No. of specimens positive by[b]:	
	AMTDII	AMPLICOR
Smear and culture positive (97)	89 [7][a]	95 (2)
Smear negative, culture positive (17)	13 [2]	13 (4)
Smear and culture negative with final diagnosis of TB (12)	6 [1]	5 (2)
All TB positive (126)	108 [10]	113 (8)
Smear and culture negative, non-TB pulmonary disease (151)	0	0
Isolation of NTM (19)	0	0

[a] *P* values were not significant.
[b] Values in brackets represent the number of specimens which turned out to be positive when retrospectively tested after dilution. Values in parentheses represent the number of specimens showing a negative amplification control which turned out to be positive when retested after dilution.

Culture. A 0.5-ml portion of the processed sediment was cultivated by the radiometric BACTEC technique (Becton-Dickinson Diagnostic Instrument Systems) and with Löwenstein-Jensen (LJ) solid medium. All media were incubated for 6 weeks at 36 ± 1°C. BACTEC 12B culture vials were tested for growth twice a week for the first 3 weeks and weekly thereafter. The radiometric growth index (GI) was recorded by the BACTEC instrument; a GI of >50 was considered suspect, and smears were made daily to confirm the presence of AFB.

LJ slants were inspected weekly for growth, and acid fastness from suspect colonies was confirmed by Ziehl-Neelsen staining.

Microscopy. To detect AFB, smears were stained with auramine-rhodamine fluorescent stain.

Identification of mycobacteria. Isolates were identified by specific DNA probes (Accuprobe; Gen-Probe, Inc., San Diego, Calif.), by standard biochemical tests, and by the high-performance liquid chromatography method (6).

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

Gen-Probe AMTDII. The Gen-Probe AMTD assay (Gen-Probe, Inc., San Diego, Calif.) was performed according to the instructions supplied by the manufacturer. Each run included positive and negative amplification controls: the former was prepared from a 10^{-4} to 10^{-5} dilution of a 1 McFarland nephelometric standard suspension of *M. tuberculosis* ATCC 27294, while the latter was made from a similarly prepared suspension of *Mycobacterium gordonae* ATCC 14470. A cutoff value of 30,000 relative light units was used for positive specimens. To tentatively detect inhibitory substances, frozen aliquots of discrepant samples were tested retrospectively after 1:5 and 1:10 dilution with PBS.

COBAS AMPLICOR. The COBAS AMPLICOR test was done by following the instructions supplied by the manufacturer. The procedure, starting from a 100-μl sediment sample portion, consisted of two steps: specimen preparation and combined, fully automated amplification and detection. The IAC DNA sequence contained primer-binding regions identical to those of the MTB target sequence. A unique probe-binding region differentiated the IAC from the target amplicon. The IAC was introduced into each amplification reaction and was coamplified with the possible target DNA from the clinical specimen. In addition, each run included positive and negative amplification controls. A colorimetric reading exhibiting absorbance values greater than 0.350 optical density units was considered as positive. Specimens showing IAC inhibition were repeated after 1:5 and 1:10 dilutions of the sample with a mixture of 50% Respiratory Lysys Reagent and 50% Neutralizing Reagent.

Patients' clinical evaluation. Clinical assessment included the patients' medical history, signs, symptoms, chest X-ray, pathology, and microbiology results, as well as follow-up observations. All of the records were carefully reviewed, with the aim of setting up the combination of culture and clinical diagnosis 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; $P < 0.05$ was considered significant.

AMTDII-negative specimens, which turned out positive when frozen aliquots were tested retrospectively, were considered as false negatives according to their first assay, while AMPLICOR specimens showing IAC inhibition and a subsequent positive result on repeat testing were considered as true positives.

Moreover, samples which remained inhibited by the AMPLICOR assay despite dilution were considered uninterpretable and therefore were excluded from calculations. Sensitivity, specificity, and predictive values were determined accordingly.

RESULTS

Respiratory specimens. A total of 296 respiratory specimens collected from 194 patients were tested. Altogether, 133 specimens yielded a culture positive for AFB; 114 isolates were found to belong to the MTB, while the remaining 19 strains were identified as nontuberculous species. Amplification results with smears, cultures, and clinical data are summarized in Table 1. A total of 126 specimens were from patients with a diagnosis of tuberculosis, and 170 were from patients with nontuberculous pulmonary disease, based on clinical and microbiological findings.

Of the 97 samples which were smear and culture positive, 89 were AMTDII positive and 95 were AMPLICOR positive (the difference was statistically not significant; $P = 0.051$). Seventeen samples were smear negative for AFB, but culture positive; 13 were positive by both assays. There were 12 samples, smear and culture negative, collected from patients in whom TB was strongly suspected. Of these, six were AMTDII positive, and five were AMPLICOR positive. The cumulative difference for all TB-positive specimens (108 positive by AMTDII and 113 positive by AMPLICOR) was statistically not significant.

A total of 18 discrepant samples (AMTDII negative but collected from TB patients) were tested retrospectively after dilution. Ten samples turned out positive, while the others remained negative. On the other hand, eight specimens tested by the AMPLICOR assay showed IAC inhibition and turned out positive on repeat testing. Moreover, six samples (one smear and culture positive, three smear negative but culture positive, and two smear and culture negative, but from patients with strong TB suspicion) showing inhibition by the AMPLICOR assay remained inhibited despite dilution.

Of the 151 samples from patients with nontuberculous pulmonary disease that were smear and culture negative for AFB, none was positive by both assays. Nineteen specimens (11 smear positive) from 15 patients grew nontuberculous mycobacteria (NTM). The following species were identified: *Mycobacterium avium* complex ($n = 10$), *M. gordonae* ($n = 4$), *Mycobacterium malmoense* ($n = 3$), *Mycobacterium kansasii* ($n = 1$), and *Mycobacterium chelonae* ($n = 1$). All of these specimens were negative by both amplification assays. Table 2 shows the sensitivity, specificity, and predictive values of both amplification methods for smear-positive and smear-negative specimens compared with those of AFB smear and culture,

TABLE 2. Sensitivity, specificity, and predictive values of AMTDII and AMPLICOR assays for respiratory specimens[a]

Method	% Sensitivity	% Specificity	Predictive value (%)	
			Positive	Negative
Smear	77.0	93.5	92.0	85.4
Culture	90.5	100	100	93.4
AMTDII				
All specimens	85.7	100	100	90.4
Smear positive	91.7	100	100	95.5
Smear negative	65.5	100	100	94.4
AMPLICOR				
All specimens	94.2	100	100	96.0
Smear positive	98.9	100	100	99.4
Smear negative	75.0	100	100	96.6

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

TABLE 3. Comparison of AMTDII and AMPLICOR amplification assays with smear and culture results and clinical data for extrapulmonary specimens[a]

Patient status (no. of specimens)	No. of specimens positive by[b]:	
	AMTDII	AMPLICOR
Smear and culture positive (25)	22 [3]	23 (1)
Smear negative, culture positive (8)	7 [1]	7 (1)
Smear and culture negative with final diagnosis of TB (8)	5 [0]	4 (2)
All TB positive (41)	34 [4]	34 (4)
Smear and culture negative, non-TB disease (138)	0	0
Isolation of NTM (11)	0	0

[a] *P* values were not significant.
[b] Values in brackets represent the number of specimens which turned out to be positive when retrospectively tested after dilution. Values in parentheses represent the number of specimens showing a negative amplification control which turned out to be positive when retested after dilution.

assuming the combination of culture and clinical diagnosis as the gold standard.

Extrapulmonary specimens. A total of 190 extrapulmonary specimens collected from 129 patients were tested. Altogether, 44 specimens yielded a culture positive for AFB; 33 isolates were found to belong to the MTB, while the remaining 11 strains were identified as nontuberculous species. The amplification results with smears, cultures, and clinical data are summarized in Table 3. A total of 41 specimens were from patients with a diagnosis of extrapulmonary tuberculosis, and 149 were from nontuberculous patients, based on clinical and microbiological findings.

Of the 25 samples which were smear and culture positive, 22 were AMTDII positive and 23 were AMPLICOR positive (the difference was statistically not significant). Eight samples were smear negative for AFB, but culture positive; seven were positive by both assays. There were eight samples, smear and culture negative, collected from patients in whom TB was strongly suspected clinically. Of these, five were AMTDII positive and four were AMPLICOR positive. The cumulative difference for all TB-positive specimens was statistically not significant.

As previously reported, a total of seven discrepant samples (AMTDII negative but collected from TB patients) were tested retrospectively after dilution. Four samples turned out to be positive, while the others remained negative. On the other hand, four specimens tested by the AMPLICOR assay showed IAC inhibition and turned out to be positive on repeat testing. Moreover, one sample (smear and culture positive) showing inhibition by the AMPLICOR assay remained inhibited despite dilution.

Of the 138 samples from patients with nontuberculous disease that were smear and culture negative for AFB, none was positive by both assays. Eleven specimens (7 smear positive) from seven patients grew NTM. The following species were identified: *M. avium* complex ($n = 7$), *M. gordonae* ($n = 3$), and *M. kansasii* ($n = 1$). All of these specimens were negative by both amplification assays. Table 4 shows the sensitivity, specificity, and predictive values of both amplification methods for smear-positive and smear-negative specimens compared with those of AFB smear and culture, assuming the combination of culture and clinical diagnosis as the gold standard.

When the results of both assays were evaluated for individual patients, the specimen-associated differences disappeared. AMTDII allowed a correct diagnosis in all of the suspected TB patients ($n = 92$); the same, except for one patient, held true for AMPLICOR.

Specimen inhibition. On the basis of our retrospective study, the overall inhibition rate for AMTDII was 2.9%, ranging from 2.1 to 3.4% for extrapulmonary and respiratory specimens, respectively. The inhibition rate for AMPLICOR was slightly higher (3.9%), ranging from 2.6 to 4.7% for extrapulmonary and respiratory specimens, respectively. Proper dilution according to the manufacturer's protocol was able to overcome inhibition in 12 of 19 (63.1%) samples. AMTDII-inhibiting samples (except one) were different from those showing inhibition by the AMPLICOR assay. Almost entirely, they included sputa, biopsy specimens, and sterile fluids. Moreover, inhibitory samples were not found in all sputum specimens collected from the same patient.

DISCUSSION

The laboratory diagnosis of MTB infection by culture techniques usually requires 1 to 8 weeks. The present study demonstrates that amplification tests can detect MTB in clinical samples within a few hours. The AMTDII turnaround time is shorter than that of AMPLICOR (2.5 versus 7.5 h). However, the latter assay offers a considerable advantage in reducing hands-on time and gives the opportunity to run the system overnight. The kits contain all of the reagents needed for sample amplification and detection and appeared to fit well in the workflow of a reference laboratory performing amplification twice a week. From the analytical point of view, differences among cutoff values, positive and negative controls, and samples were broad enough to allow easy discrimination by both assays.

In comparison with the gold standard, the sensitivity and specificity of AMTDII were 85.7 and 100% for respiratory specimens and 82.9 and 100% for extrapulmonary specimens, respectively. No significant differences in sensitivity between respiratory and extrapulmonary specimens were observed. Negative results obtained from smear-positive or smear-negative MTB-yielding samples were shown to depend almost entirely on the presence of inhibitors of enzymatic amplification. In fact, most of these samples (13 of 16), which in a routine setting were likely to be misdiagnosed to contain NTM or considered as negative, turned out positive by simple dilution.

TABLE 4. Sensitivity, specificity, and predictive values of AMTDII and AMPLICOR assays for extrapulmonary specimens[a]

Method	% Sensitivity	% Specificity	Predictive value (%)	
			Positive	Negative
Smear	61.0	95.3	85.4	90.3
Culture	80.5	100	100	94.9
AMTDII				
All specimens	82.9	100	100	95.5
Smear positive	88.0	100	100	98.0
Smear negative	75.0	100	100	96.1
AMPLICOR				
All specimens	85.0	100	100	96.1
Smear positive	95.8	100	100	99.3
Smear negative	68.7	100	100	96.7

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

A considerable increase in the specimen volume (from 50 to 450 μl) in comparison with the former version has been claimed as the most likely explanation of this previously undescribed inhibition (5).

In our view, an overall inhibition rate for MTB-yielding samples of 8.8% stresses the importance of monitoring inhibitory substances in clinical specimens. Systematic inclusion of the IAC would greatly contribute to the accuracy of the assay, also providing important information when testing nonapproved types of samples.

In this context, removal of inhibitory substances may be an alternative approach to improve sensitivity (P. Singer and F. Vlaspolder, Abstr. 20th Annu. Conf. Eur. Soc. Mycobacteriol., p. 76, 1999). However, because the nature of the inhibition is still unclear and probably affects amplification techniques unevenly, it seems unlikely we will be able to find a routine-fitting procedure able to remove all inhibitory substances (3, 4).

The sensitivity and specificity of AMPLICOR were 94.2 and 100% for respiratory specimens and 85.0 and 100% for extrapulmonary specimens, respectively. No significant differences in sensitivity between respiratory and extrapulmonary specimens were observed. Data from the literature about AMPLICOR sensitivity and specificity are in agreement with our findings (Bodmer et al. [1], 92.6 and 99.6%; Rajalahti et al. [8], 83 and 99%; Wang and Tay [10], 96.1 and 100%; and Reischl et al. [9], 83.5 and 98.8%, respectively) and document that the automated AMPLICOR assay exhibits higher sensitivity and specificity than those obtained by the manual version (7).

Using different kinds of clinical samples for amplification, we observed an overall inhibition rate of 3.9%. No significant difference between respiratory (4.7%) and extrapulmonary specimens (2.6%) was found. False-negative inhibitory samples were easily detected and soon reclassified as true positive or, when repeatedly inhibitory, as uninterpretable. Moreover, a positive IAC strengthens the predictive value of negative tests.

Negative results obtained by the AMPLICOR assay for two smear-positive, MTB-yielding noninhibitory samples remain unexplained. The same results were obtained upon a repeat assay, when a frozen aliquot of both samples was tested retrospectively. For smear-negative, culture-positive noninhibitory samples, a low number of mycobacteria, unequally distributed in the test suspension, is perhaps the most likely explanation.

Of the 20 samples (12 respiratory and 8 extrapulmonary) that were both smear and culture negative, obtained from patients strongly suspected of having TB, 11 were AMTDII positive and nine were AMPLICOR positive.

None of the specimens from patients found negative for TB by culture and clinical criteria or yielding NTM was positive by any assay.

We can conclude that, although at present, amplification assays cannot replace culture techniques, AMTDII and AMPLICOR were shown to be rapid and specific for the detection of MTB in clinical samples. Their protocols were easy to perform and suitable for a routine microbiology laboratory's workflow. On the basis of our data, the difference between AMPLICOR and AMTDII sensitivities was found to depend on the use of the IAC, which was lacking in the latter assay. Evidence supporting the IAC as an essential feature of commercial amplification assays is thriving, and it is likely to represent the landmark of the "second generation" kits. Moreover, the testing of three consecutive specimens per patient has to be considered as the minimum requirement, especially if IAC is not available.

In our opinion, transcription-mediated amplification (TMA) is potentially an excellent diagnostic technique for both respiratory and extrapulmonary specimens, provided that an IAC is included with the assay. In this context, the recent development of the VIDAS Probe MTB System (bioMerieux, Inc., Rockland, Mass.) featuring TMA automation coupled with IAC highlights a new technological challenge to current assays (2).

Finally, companies should make every effort to improve the sensitivity of amplification assays for smear-negative, culture-positive noninhibitory samples.

ACKNOWLEDGMENTS

This study is part of the scientific activity undertaken by the AMCLI (Italian Association of Clinical Microbiology) Committee of Mycobacteriology.

We thank bioMerieux Diagnostici (Rome, Italy) and Roche Diagnostici (Milan, Italy) for providing reagents and instrumentation.

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