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Evaluation of Amplicor PCR for Direct Detection of *Mycobacterium tuberculosis* from Sputum Specimens

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We evaluated the Amplicor PCR assay (Roche Molecular Systems, Branchburg, N.J.) for direct detection of *Mycobacterium tuberculosis* in sputum. A total of 532 specimens from 270 patients were decontaminated and stored at 4 or −75°C until assayed by PCR. This assay used three-step sample preparation, biotinylated primer pairs, AmpErase, and a microtiter format for amplicon capture and detection. Amplicor PCR results were compared with clinical history, culture from a Lowenstein-Jensen slant, and results from the BACTEC TB-460 system. Eighty-seven cultures from 15 patients grew *M. tuberculosis*; of these, 83 (95%) were positive with the Amplicor PCR test. The false negatives were most likely due to sample variation and inhibitors. Of the 445 specimens from which *M. tuberculosis* was not isolated, 428 (96%) were negative with the Amplicor PCR test. Of the 17 *M. tuberculosis* culture-negative, Amplicor-positive specimens, 15 were reclassified as true positives because previous cultures grew *M. tuberculosis*. Of the 445 specimens which did not grow *M. tuberculosis*, *Mycobacterium* spp. other than *M. tuberculosis* were isolated from 150 specimens. Three of these 150 specimens were Amplicor positive; two were from a patient with a history of tuberculosis, and one specimen gave a false-positive result. We do not feel that this represents cross-reactivity, because repeated Amplicor testing of the isolate gave negative results. The microtiter plate has 96 wells. Allowing for six controls, 90 decontaminated specimens can be tested by one technologist in 7.5 h. This PCR assay took 7.5 h to complete and is a sensitive and specific, rapid method for the direct detection of *M. tuberculosis* from sputum.

Tuberculosis is again an important health problem in the United States (5). Smears lack both sensitivity and specificity for *Mycobacterium tuberculosis*, and cultures usually require weeks before the species is definitively identified (8, 13). PCR amplification can be rapidly performed by using primers for a highly conserved sequence within a gene common to all mycobacterial species to amplify minute amounts of DNA (30); specificity for *M. tuberculosis* is achieved during the detection step. PCR allows the amplification of a targeted DNA sequence over 10^6-fold through cycles of primer annealing and nucleotide extension of target sequences by DNA polymerase at specific temperatures (20, 22). Research developments with PCR for the direct detection of *M. tuberculosis* in clinical specimens have demonstrated the possible use of PCR in providing a rapid diagnosis of tuberculosis (2, 10, 11, 12, 14, 17, 18, 24–26, 29). In this study, we compared the Roche Amplicor *M. tuberculosis* test (Roche Molecular Systems [RMS], Branchburg, N.J.) with acid-fast bacillus (AFB) smear, culture, and clinical history to determine the sensitivity and specificity of direct detection of *M. tuberculosis* in concentrated sputum specimens by Amplicor PCR.

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MATERIALS AND METHODS

Specimens. A total of 532 sputum specimens from 270 patients at Thomas Jefferson University Hospital and Episcopal Hospital, Philadelphia, Pa., were submitted for AFB culture. All sputum specimens submitted for AFB culture were decontaminated and concentrated according to Centers for Disease Control and Prevention guidelines for mycobacteriology (13). The concentrated specimen pellet (approximately 0.2 ml) was resuspended in 1 ml of phosphate buffer and divided into three parts. One part (0.2 ml) was cultured onto Lowenstein-Jensen medium and used to make smears for auramine-rhodamine staining. The second part (0.5 ml) was used for routine BACTEC analysis. The third part (0.5 ml) was stored at 4°C in plastic aliquot tubes unless PCR testing was to be delayed by more than 24 h, in which case the tubes were stored at −75°C for up to 6 months. The specimens were divided into two study groups: a prospective group and a retrospective group. The prospective group consisted of 248 consecutive specimens from 129 patients; the specimens were stored at 4 or −75°C for less than 7 days. The retrospective group consisted of 284 specimens from 144 patients; these specimens were stored at −75°C for longer than 1 month. Three patients had specimens in both study groups.

Preparation of samples for PCR analysis. All reagents and equipment for performance of the Amplicor PCR assay were supplied by RMS. This test was performed according to draft package insert instructions for the Amplicor *M. tuberculosis* kit (23). The technologist was blinded to all smear and culture results for the prospective study group. Sputum preparation, for both study groups, consisted of the addition of 500 μl of a sputum wash solution containing Tris-HCl, 0.05% sodium azide, and 1% solubilizer to 100 μl of fresh or thawed decontaminated sputum in a 1.5-ml screw-cap tube. The tubes were vortexed and centrifuged at 12,500 × *g* for 10 min. The supernatant was aspirated, and 100 μl of *M. tuberculosis* specimen lysis reagent containing 0.4% sodium hydroxide and 0.05% sodium azide was added to the pellet. The pellet was vortexed to resuspend it. Positive and negative controls were supplied with the kit; control stocks were prepared by pipetting 100 μl of positive or negative controls into a tube with 400 μl of *M. tuberculosis* specimen lysis reagent and vortexing. A 100-μl aliquot was pipetted from each control stock and placed in a 1.5-ml screw-cap tube to be processed. The specimens and controls were incubated in a 60°C dry heat block (containing 0.5 cm of sand) for 45 min. The tubes were removed from the heat block and pulse centrifuged for 5 s to obtain a pellet. A 100-μl aliquot of *M. tuberculosis* specimen neutralization reagent containing Tris-HCl with 0.05% sodium azide was added to the pellet and vortexed. Master mix reagent was prepared by the addition of 100 μl of uracil *N*-glycosylase (UNG) enzyme (AmpErase) to the master mix reagent containing the deoxynucleotide triphosphates dATP, dCTP, dGTP, and dUTP, biotinylated primers, and thermostable *Taq* polymerase just prior to the amplification process. The genus-specific primers used amplify a 584-bp sequence located in a highly conserved region of the 16S rRNA gene of *Mycobacterium* spp. (3, 28). Fifty microliters of the master mix reagent was pipetted into each reaction tube of a tray (MicroAmp; Perkin-Elmer, Norwalk, Conn.). This was followed by the addition of 50 μl of prepared specimen or controls. Single-well amplification was performed with each sputum specimen. The reaction tubes were sealed with plastic cap strips and placed in a

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Perkin-Elmer 9600 thermocycler programmed for 2 cycles of 20 s of denaturation at 98°C, 20 s of annealing at 62°C, and 45 s of extension at 72°C followed by 35 cycles of 20 s at 94°C, 20 s at 62°C, and 45 s at 72°C. After amplification, 100 μl of denaturation solution containing 1.6% sodium hydroxide and EDTA was added to each PCR tube and incubated at room temperature for 10 min. A 100-μl aliquot of hybridization solution was added to each well of a 96-well microtiter plate coated with specific oligonucleotide probes for *M. tuberculosis*. This was followed by the addition of 25 μl of denatured amplification sample. The plate was incubated for 1.5 h at 37°C and then washed five times with a buffered wash solution by using a microwell plate washer. A 100-μl aliquot of avidin-horseradish peroxidase conjugate was added to each well and incubated for 15 min at 37°C. The microtiter plate was again washed five times as described above. A 100-μl aliquot of prepared substrate containing 12.0 ml of substrate A (hydrogen peroxide) and 3.0 ml of substrate B (tetramethylbenzidine) was added to each microtiter well and incubated at room temperature for 10 min. Following incubation, each well received 100 μl of stop reagent (4.9% sulfuric acid). The optical density at 450 nm (OD_{450}) was measured for each well, and samples were initially classified as negative (OD_{450}, <0.300), equivocal (OD_{450}, 0.300 to 0.600, inclusive), or positive (OD_{450}, >0.600). Amplicor PCR was repeated once for specimens in the equivocal range; if the repeat value was ≥0.350, the specimen was positive, and if the repeat value was <0.350, the specimen was negative. (According to the draft of the package insert, specimens with OD_{450} readings of less than 0.35 are negative and specimens with readings of 0.35 or greater are positive.)

Resolution of discrepant samples. Amplicor PCR results were classified as true positive or true negative on the basis of patient clinical history and culture correlation. Discrepancy analysis testing, performed by RMS, was used to gather additional information about the specimens' characteristics but not to determine the classification of specimens.

Amplicor PCR on *M. tuberculosis* culture-positive, PCR-negative samples was repeated in duplicate. If either of the repeat PCRs was positive, the initial Amplicor PCR result was confirmed as false negative. If the repeat Amplicor PCRs were negative, further discrepancy analysis was performed by RMS by using spike back analysis, DNA phenol extraction, and a PCR assay utilizing an alternate primer pair targeting the superoxide dismutase (SOD) gene. Spike back analysis was used to detect the presence of possible inhibitors to PCR in the specimen. In spike back analysis a known quantity of *M. tuberculosis* is added to the specimen; a negative PCR result following addition of *M. tuberculosis* to the specimen indicates the possibility of inhibitors interfering with amplification of *M. tuberculosis.*

Amplicor PCR on *M. tuberculosis* culture-negative, PCR-positive samples was repeated in duplicate. If either of the repeat Amplicor results was also positive, the sample was classified as a true positive, provided that the patient had prior cultures positive for *M. tuberculosis*. When the repeat Amplicor PCRs were negative, spike back analysis, DNA phenol extraction, and PCR with primers to SOD were performed as required to better characterize the sample. Patient follow-up and chart review were used to classify these discrepant results. SOD PCR was performed by RMS for all *M. tuberculosis* culture-negative, Amplicor PCR-positive specimens by using a modification of a previously described assay (33). The SOD alternate primer targeted DNA sequences specific to only a few of the *Mycobacterium* species, including *M. tuberculosis*; the detection step was specific for *M. tuberculosis.*

RESULTS

A total of 532 decontaminated sputum specimens were stained for AFB with auramine-rhodamine and tested for the presence of *M. tuberculosis* by Amplicor PCR and culture. Because the data for the prospective and retrospective groups were essentially the same, the data were combined. Eighty-seven specimens from 15 patients were culture positive for *M. tuberculosis*; 83 of these 87 specimens were Amplicor PCR positive (Table 1). Of the 445 specimens that were *M. tuberculosis* culture negative, 428 were PCR negative. When Amplicor PCR is compared only with culture for the detection of *M. tuberculosis*, the sensitivity and specificity for the combined study groups were 95 and 96%, respectively. In our study, we found that prolonged freezing of specimens, as in the retrospective group, did not seem to affect the sensitivity and specificity of the Amplicor PCR assay.

Of the 17 specimens that were *M. tuberculosis* culture negative and Amplicor PCR positive, 15 specimens (Table 2, specimens 5 to 11 and 13 to 20) were from four patients with a history of tuberculosis. All of these patients had had cultures positive for *M. tuberculosis* within the past 2 months, and all had been started on antituberculosis medications. Because of the prior positive cultures, 15 of the 17 *M. tuberculosis* culture-negative, PCR-positive specimens were reclassified as true positive. Additional testing of these specimens by RMS included repeating the 16S rRNA PCR twice as well as amplification of the SOD primer. Spike back analysis was performed for two of the specimens (specimens 5 and 16) because the additional 16S rRNA amplification was negative in duplicate. When *M. tuberculosis* was added to the specimens, the amplification gave a positive result, indicating that an inhibitor was probably not present. The most likely explanation for the duplicate negative results from a specimen which was initially positive is clumping of organisms and sampling artifact.

TABLE 1. Culture, Amplicor PCR, and smear results

AFB culture result (no. of specimens)	No. Amplicor PCR positive	No. smear positive
M. tuberculosis (84)	81	79
M. tuberculosis and other AFB (3)	2	2
M. avium-M. intracellulare complex (77)	0	7
M. gordonae with prior *M. tuberculosis*-positive culture (1)	1	1
M. gordonae (42)	1	0
Other AFB and mixed nontuberculosis AFB (30)	1	2
No growth (295)	14	8
Total (532)	100	102

With another specimen, the Amplicor PCR result was positive, but *Mycobacterium gordonae*, not *M. tuberculosis*, was isolated (Table 2, sample 21); this specimen was from a patient without clinical evidence of tuberculosis and is a false positive. No remaining specimen was available for testing, but when Amplicor PCR on the *M. gordonae* isolate was performed, it was repeatedly negative. The other PCR-positive, culture-negative specimen (Table 2, sample 12) was also from a patient lacking clinical evidence of tuberculosis. Repeat Amplicor PCR in duplicate was negative; this specimen is also a false positive.

Four specimens were *M. tuberculosis* culture positive and PCR negative; Amplicor PCR was repeated in duplicate (Table 3). One specimen (Table 3, sample 1) was positive in duplicate with repeat Amplicor PCR testing, and the patient had a past history of tuberculosis; this specimen is a false negative, which is probably due to sampling variation. This specimen was also one of the two specimens which on initial testing gave an equivocal OD_{450} reading of 0.545. Although this would have been considered a positive result according to the package insert, we repeated the PCR according to our study protocol. Our repeat OD_{450} was 0.272, and the specimen was classified as negative by PCR because the repeat test value was less than 0.350.

Duplicate repeat Amplicor PCRs on sample 2 gave one positive and one negative result; spike back analysis showed a lack of inhibitors. This false-negative result was also due to sampling variation.

One specimen (Table 3, sample 3) was still negative with duplicate repeat Amplicor PCR testing. Spike back analyses in duplicate were negative, indicating the possible presence of a PCR inhibitor. Phenol extraction to remove inhibitors was also performed on the specimen; the results with the Amplicor test and SOD alternate primer assay following phenol extraction were all positive in duplicate, providing additional evidence that the false-negative result was due to an inhibitor. This patient also had a past history of tuberculosis.

TABLE 2. Analysis of culture-negative, Amplicor-positive specimens

Sample no.	OD_{450} (interpretation[a]) with the following analysis:						Prior culture with *M. tuberculosis*
	Additional 16S rRNA PCR testing		Spike back		SOD alternate primer		
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	
5	0.089 (−)	0.111 (−)	3.951 (+)	3.036 (+)	0.092 (−)	0.76 (+)	Yes
6	0.084 (−)	1.577 (+)	ND[b]	ND	0.103 (−)	0.080 (−)	Yes
7	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
8	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
9	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
10	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
11	3.923 (+)	2.725 (+)	ND	ND	1.583 (+)	4 (+)	Yes
12	0.078 (−)	0.113 (−)	4	4	0.113 (−)	4 (+)	No
13	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
14	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
15	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
16	0.079 (−)	0.074 (−)	3.042 (+)	3.316 (+)	0.086 (−)	0.055 (−)	Yes
17	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
18	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
19	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
20	4 (+)	4 (+)	ND	ND	4 (+)	4 (+)	Yes
21[c]	ND	ND	ND	ND	ND	ND	No

[a] −, negative; +, positive.
[b] ND, not done.
[c] No specimen was available for additional studies; repeat Amplicor PCR on the isolate was negative.

Duplicate repeat Amplicor PCR testing on specimen 4 (Table 3) gave one positive and one negative result. Spike back analysis was negative in duplicate, indicating the possible presence of a PCR inhibitor. Phenol extraction followed by Amplicor and SOD PCR testing gave mixed results. The false-negative result for this specimen was probably due to both sampling variation and an inhibitor. This specimen is the second of the two specimens which initially gave an equivocal reading. The initial reading was 0.339; the repeat reading was 0.295, and the specimen was classified as negative by initial PCR testing.

Table 1 presents the smear results, AFB species identification, and Amplicor results. Mycobacteria were isolated from 237 of the 532 specimens; 102 of the 237 specimens (43%) were smear positive. Of the 87 specimens culture positive for *M. tuberculosis*, 81 (93%) were smear positive; three of the specimens which were smear negative and from which *M. tuberculosis* was isolated were Amplicor PCR positive. PCR was negative for all 77 specimens containing *Mycobacterium avium-M. intracellulare* complex, which is frequently isolated from AIDS patients. Two of the 43 specimens culture positive for *M. gordonae* were Amplicor PCR positive. One of these specimens came from a patient with a clinical history of and cultures positive for tuberculosis; this amplification result was classified as a true positive. Additional discrepancy testing could not be done for the other specimen with *M. gordonae* and a positive Amplicor result because additional specimen was not available; however, as mentioned above, Amplicor PCR testing of the *M. gordonae* isolate from this specimen was repeatedly negative, and so the initial Amplicor PCR result for the specimen was a false positive.

DISCUSSION

The BACTEC TB system (Becton Dickinson Instrument Systems) and Septi-Chek AFB (Becton Dickinson Microbiology Systems) are AFB culture systems that can detect positive specimens more rapidly than does conventional culture (31). However, the time needed to detect growth can be up to 15 to 22 days with BACTEC or Septi-Chek and 22 to 26 days with conventional culture (31). The Centers for Disease Control and Prevention is recommending that growth of mycobacteria be detected within 14 days of specimen receipt in the clinical laboratory (4), emphasizing the increased demand for rapid diagnostic tests for *M. tuberculosis*. The College of American Pathologists recommends that acid-fast stain results be re-

TABLE 3. Analysis of culture-positive, Amplicor-negative specimens

Sample no.	OD_{450} (interpretation[a]) with the following analysis:								Past *M. tuberculosis*-positive culture
	Additional 16S rRNA PCR testing		Spike back		Phenol extraction and 16S PCR		Phenol extraction and SOD PCR		
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	
1	2.406 (+)	>3.00 (+)	ND[b]	ND	ND	ND	ND	ND	Yes
2	0.07 (−)	0.739 (+)	4	4	ND	ND	ND	ND	Yes
3	0.085 (−)	0.108 (−)	0.09	0.14	4 (+)	4 (+)	0.76 (+)	1.157 (+)	Yes
4	0.065 (−)	0.527 (+)	0.069	0.083	1.021 (+)	0.065 (−)	1.822 (+)	1.637 (+)	Yes

[a] +, positive; −, negative.
[b] ND, not done.

ported within 24 h of specimen receipt by the laboratory (7, 32).

The Amplicor *M. tuberculosis* test is a rapid, specific test that can be done directly on patient specimens, with the potential for same-day laboratory results. The Amplicor PCR test uses prepared, specific reagents needed for specimen preparation, amplification, and amplicon detection. The specimen lysis method uses a single-tube system, which is important for minimizing cross-contamination among specimens (16).

The addition of AmpErase to specimens prior to amplification also minimizes false-positive results due to amplicon contamination. Amplicons contain uracil instead of the native thymine. AmpErase prevents any residual uracil-containing amplicons, created during previous amplification cycles, from being amplified again; the AmpErase cuts the DNA strand where uracil is located. Contamination caused by amplicons, resulting in false positives, may occur after some time in laboratories that routinely do PCR (15). The significance of false positivity in the detection of *M. tuberculosis* by PCR has been addressed by Noordhoek et al. (19) in a study comparing several laboratories; each laboratory used its own protocol for sample treatment, DNA extraction, and detection. False-positivity rates were reported to be as high as 77%, indicating the need for an established PCR protocol that would use the same preparation treatment for samples and controls (19). dUTP and UNG are important features of the Amplicor PCR test system. The ability of dUTP and UNG to detect and prevent carryover contamination in PCR amplification was demonstrated by Abe et al. (1); amplicons and UNG were mixed and PCR was performed, but no amplification occurred.

In our study, the discrepant results of Amplicor PCR and culture were attributed to many factors. These include the presence of inhibitors, low organism numbers resulting in sampling variation, and the detection of nonviable organisms by PCR, as well as combinations of these. The importance of proper sample preparation for PCR to eliminate inhibitors has been demonstrated in several studies; suitable lysis of tubercle bacilli is necessary (6), and the wash step is critical to rid the sample of proteins and salts produced by decontamination with NaOH (14). It has been suggested that heparin, hemoglobin, phenol, and sodium dodecyl sulfate may also be potent inhibitors (6). In addition, Soini et al. (27) suggest that infection due to *M. tuberculosis* may lead to the introduction of inhibitory substances into sputum as well as to decreased sputum production. A study by Clarridge et al. (6), with a large-volume work load, determined that specimen inhibition was not necessarily patient related, because other specimens from the same patients could be amplified.

Clarridge et al. (6) also examined whether a lack of amplification in a culture-positive specimen was a property of the organism itself and found that all strains could be amplified from isolates of the organism. Likewise, in our study, RMS performed Amplicor PCR on the *M. tuberculosis* isolates from samples 1 to 3 (Table 3); three of the four isolates from the false-negative specimens could be amplified. The isolate from the fourth false-negative specimen was not amplified because a repeatable positive PCR result was obtained from the specimen.

There have been a number of sample preparation methods described for PCR assays that involve extensive DNA extraction steps (9, 21, 24, 26, 29), such as phenol extraction and use of proteolytic enzymes; these preparations may eliminate interfering substances in specimens.

In our study, some of our results clearly showed that the organisms were not evenly distributed. The natural clumping of the organism makes it difficult to get uniform sampling even with vortexing and specimen-lysing procedures. In addition, the small sample volume (0.1 μl) used contributed to sampling variations with the Amplicor PCR assay.

Fifteen of the 17 PCR-positive, *M. tuberculosis* culture-negative specimens were from four patients with a history of antituberculous therapy. All four of these patients had had cultures positive for *M. tuberculosis* within 2 months of producing specimens which were culture negative and PCR positive. Kolk et al. (15) also found that most of the PCR-positive, culture-negative samples were from patients who responded to antituberculosis therapy. Successful therapy will kill the organisms and cause subsequent cultures to be negative; the remnants of these killed organisms can still be amplified and detected by PCR. Cultures are designed to detect viable organisms by providing necessary nutrients for metabolism, survival, and replication. PCR, however, is capable of amplifying DNA from viable as well as nonviable organisms; *M. tuberculosis* can still be detected by PCR after therapy has been initiated. It will be important to decide when to perform *M. tuberculosis* PCR testing in addition to routine cultures; both systems have different objectives with their own limitations. The lack of a "gold standard" for diagnosing *M. tuberculosis* infection makes the evaluation of a new diagnostic technique very complex (17). Patients with a history of tuberculosis will not always give uniformly positive PCR results; one patient in our study had a history of *M. tuberculosis* but had mixed Amplicor PCR results. The usefulness of this assay will be in the initial positive diagnosis of tuberculosis; once the assay is qualitative, rather than quantitative, one can explore the hypothesis that the assay would be useful for tracking the efficacy of antituberculous therapy.

AFB smears can detect the organism, but for patients who have a small number of organisms, the smear loses sensitivity. In our study, 87 cultures were positive for *M. tuberculosis*; 81 (93%) were smear positive, and 83 (95%) were PCR positive. Six samples were smear and culture negative but PCR positive; all of these patients had prior cultures with *M. tuberculosis.*

In summary, the Amplicor PCR assay should be very useful for the rapid detection of *M. tuberculosis* respiratory infections. The microtiter plate has 96 wells. Allowing for six controls, 90 decontaminated specimens can be tested by one technologist in 7.5 h; the technique will be particularly useful in laboratories serving populations with a high incidence of tuberculosis. It will also be very useful for populations with a high incidence of other mycobacterial respiratory infections; a reliably negative Amplicor PCR result for a specimen with a positive smear could save needless therapy and patient isolation. The assay provides a very sensitive and specific, rapid method of detecting *M. tuberculosis.*

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