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Detection and Identification of *Mycobacterium tuberculosis* Directly from Sputum Sediments by Ligase Chain Reaction

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Sputum specimens received for the diagnosis of tuberculosis or other mycobacterial infections were tested by a ligase chain reaction (LCR)-based assay and acid-fast stain and culture techniques. Results from the LCR assay (Abbott LCx *Mycobacterium tuberculosis* [MTB] Assay) were compared to results from standard culture techniques held for 6 weeks. Four hundred ninety-three specimens from 205 patients suspected of pulmonary tuberculosis were included in the prospective study. Thirty-four (6.9%) of the specimens were culture positive for *M. tuberculosis*, and 13 (38%) of these were also fluorochrome stain positive. LCR sensitivities and specificities compared to culture were 74 and 98%, respectively. LCR sensitivity was 100% for fluorochrome stain-positive specimens and 57% for fluorochrome stain-negative specimens. Nine LCR-negative, culture-positive specimens were the result of low concentrations of *M. tuberculosis*. No inhibitors were detected in any of these specimens. Of the eight LCR-positive, culture-negative specimens, five were from patients with active tuberculosis. With these considered culture misses, final LCR sensitivity, specificity, positive predictive value, and negative predictive value were 77, 99, 91, and 98%, respectively. The same performance values for the fluorochrome acid-fast bacillus smear were 33, 98, 62, and 94%, respectively. After normal laboratory sputum processing, the Abbott LCx MTB Assay can be completed in 6 h. Thus, it is possible to have results available within 8 h of specimen submission.

Laboratory diagnosis of *Mycobacterium tuberculosis* currently depends on acid-fast staining and culture of processed sputum specimens, methods that have been utilized for decades in the United States (20). While these techniques have been continuously refined and improved, they still have severe limitations. Microscopic examination of acid-fast smears has sensitivity and positive predictive values low enough to be useful only as a presumptive screening test. However, because acid-fast smear results are available rapidly and correlate with patient infectivity, they are utilized for patient management and public health decisions (5, 14, 17, 22, 31). Culture, considered the most accurate test due to high sensitivity and specificity, is labor-intensive and slow. Clinical laboratories hold cultures 6 to 8 weeks to achieve maximum sensitivity (21). Radiometric liquid culture (Bactec), the most rapid culture technique widely utilized, requires an average of 13 days to become positive (2). The most sensitive and rapid culture and staining techniques available are not utilized by all laboratories due to funding, staffing, and training difficulties (18). The recent increase in tuberculosis cases in the United States and the emergence of multidrug-resistant strains have demonstrated the weaknesses in the currently utilized techniques and underscored the need for more rapid and accurate methods of laboratory diagnosis (9).

Many investigators have demonstrated the utility of nucleic acid amplification tests (NAAT) to supplant acid-fast bacillus (AFB) smear and culture for laboratory diagnosis of *M. tuberculosis*. Studies have focused on the rapid detection of *M. tuberculosis* for the initial diagnosis of pulmonary tuberculosis. Assays which amplify either DNA or RNA have been utilized for this purpose. The performance characteristics of transcription-mediated amplification (amplification of rRNA) (1, 7, 19, 23, 25, 26, 29, 30) as well as laboratory-based PCR (11, 15, 16, 32) and commercially based PCR (4, 6, 10, 12, 13, 24) have been demonstrated. Sensitivity ranging from 82 to 100% and specificity ranging from 98 to 100% have been documented (1, 4, 6, 7, 10–13, 15, 16, 19, 23–26, 29, 30, 32). A new commercial assay based on ligase chain reaction (LCR) for diagnosis of pulmonary tuberculosis is presently under study; two reports have indicated sensitivity of >90% for this test (3, 28). However, several factors could have affected the results and may have led to this high reported sensitivity. The patient population included in these studies had high prevalence rates, and the sensitivities of AFB smear in these two studies was 78.7 and 82.6%, indicating a preponderance of specimens with high concentrations of *M. tuberculosis*, a situation not seen in all laboratories. This study describes a prospective clinical trial of the Abbott LCx *Mycobacterium tuberculosis* (MTB) Assay (Abbott Laboratories, Abbott Park, Ill.), a commercially developed LCR assay for *M. tuberculosis*, for detection of new tuberculosis cases in a public health clinic population.

MATERIALS AND METHODS

Clinical specimens and culture techniques. Sequential specimens were collected from patients being screened for tuberculosis or other pulmonary mycobacterial disease at the Orange County Health Care Agency Pulmonary Disease Clinic. All specimens were identified as being collected for the initial diagnosis of tuberculosis by the clinic staff. Specimens submitted for the follow-up of patients on antimicrobial therapy for tuberculosis were not included. Up to three specimens per patient were included in the study. The great majority of specimens were induced sputum samples taken with an Ultra-Neb 99 nebulizer (DeVilbiss, Somerset, Pa.) with 0.45% NaCl solution and a 50-ml sterile conical tube collection kit (Sage Products, Crystal Lake, Ill.). The remaining specimens were sputum samples collected into the same kit. Specimens were held and transported at 4°C, received by the laboratory within 2 h, and processed by a 15-min treatment with a final concentration of 0.25% *N*-acetyl-L-cysteine–1% sodium hydroxide, centrifugation at 3,000 × *g* for 20 min, and the addition of 1.5 ml of 0.2% bovine serum albumin (BBL, Cockeysville, Md.), 45.5 U of penicillin G per ml, and 9% wide range indicator (LaMotte Chemical Company, Chestertown, Md.) to the final pellet, followed by titration to pH 6.8 to 7.2 with 0.5 N HCl (21). For each specimen, one Lowenstein-Jensen agar slant and one selective 7H11 agar slant (BBL) were inoculated with 0.1 ml of specimen, a Bactec 12B vial (BBL) was inoculated with 0.5 ml of specimen, and a smear was made for

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TABLE 1. Initial comparison of Abbott LCR and fluorochrome stain to culture for *M. tuberculosis* (n = 493)

Test and result	No. of culture results		Sensitivity (%)	Specificity (%)
	Positive	Negative		
Abbott LCR				
Positive	25	8	74	98
Negative	9	451		
Fluorochrome stain				
Positive	13	8	38	98
Negative	21	451		

fluorochrome staining. The remainder of the sample was refrigerated at 4°C until LCR testing and then frozen at −70°C for retesting if necessary. Fluorochrome staining was performed by standard procedures (21). Tube cultures were examined weekly for a total of 6 weeks. Bactec cultures were tested every day for 7 days and biweekly for 5 additional weeks. Positive cultures were quantitated, and acid-fast isolates were identified by standard biochemical techniques (21), DNA-RNA hybridization (Accu-Probe; Gen-Probe Diagnostics, San Diego, Calif.), or high-performance liquid chromatography (8).

LCR assay. A 0.5-ml aliquot of the refrigerated sediments from processed clinical specimens was tested within 3 days of processing with the LCx MTB Assay. The assay amplifies DNA coding for protein b, which is present in *M. tuberculosis* complex organisms (27). The assay consists of three major steps: specimen processing, amplification, and detection. Specimen processing was carried out by adding 0.5 ml of decontaminated specimen to an LCx specimen preparation tube, vortexing for 5 s, and centrifuging at 1,500 × *g* for 10 min. The supernatant was discarded, and 1.0 ml of resuspension buffer was added to the pellet, followed by vortexing for 5 s and centrifugation at 1,500 × *g* for 10 min. The supernatant was discarded, and 0.5 ml of resuspension buffer was added to the pellet. The sample was vortexed for 5 s, incubated at 95°C for 20 min in an LCx covered dry bath, and cooled for 10 min at room temperature, followed by an automated lysis cycle in the LCx Lysor. The sample was cooled at room temperature for 5 min and centrifuged for 2 min in an Abbott Microfuge. Amplification and detection were carried out in a room separate from that where specimen processing was carried out. The amplification step was carried out by adding 100 μl of processed specimen to an LCx amplification vial containing 90 μl of LCR mixture (DNA ligase, DNA polymerase, DNA triphosphates, and two pairs of hapten-labeled oligonucleotide probes) and amplifying in an LCx thermal cycler. Thirty-seven cycles of 1 s at 94°C, 1 s at 64°C, and 40 s at 69°C were utilized. The automated detection step was carried out in an LCx analyzer by transferring 18 patient vials and 6 control vials per run into the instrument and running as per the manufacturer's instructions. The sample preparation steps resulted in no concentration or dilution of bacteria in the sample. Thus, the effective sample size for amplification was 100 μl. Control values met the package insert specifications for all runs.

Discrepant analysis. Samples that were culture positive and LCR negative for *M. tuberculosis* were tested for the presence of inhibitors of the LCR by rerunning the test on an aliquot of the sample with 25 genome equivalents of *M. tuberculosis* DNA added after the sample preparation step. *M. tuberculosis* isolates cultured from specimens with false-negative LCR results were tested by processing approximately 1 loopful of the isolate suspended in saline and testing by the normal procedure. Gen-Probe *Mycobacterium tuberculosis* detection test (MTD) (Gen-Probe Diagnostics) and Amplicor *Mycobacterium tuberculosis* Test (Roche Diagnostics, Branchburg, N.J.) were performed on discrepant specimens in accordance with the manufacturer's directions on the package insert. ATS (American Thoracic Society) class was utilized to determine if a patient had an active case of tuberculosis.

RESULTS

The clinical performance of the test was determined by comparing the Abbott LCR results and the fluorochrome AFB stain results to those of standard culture for 493 specimens collected prospectively from 205 patients. Thirty-four (6.9%) of the specimens were culture positive for *M. tuberculosis*. The overall results presented in Table 1 indicate that, compared to culture, the Abbott LCR sensitivity was 74% and the specificity was 98%, while fluorochrome stain had a sensitivity of 38% and a specificity of 98%. As indicated in Table 2, the sensitivity for the Abbott LCR varied with the concentration of *M. tuberculosis* in the specimens as determined by semiquantitative culture and with whether the specimen was smear positive or negative. Sixty-two percent of the culture-positive specimens were smear negative, and 47% had <500 CFU of *M. tuberculosis*/ml. The Abbott LCR had a sensitivity of 100% for smear-positive specimens, and for all specimens with ≥500 CFU of *M. tuberculosis* per ml. Sensitivity for smear-negative specimens was 57%, and the sensitivity for specimens with <500 CFU/ml was 44%. Quantitation of the amount of *M. tuberculosis* in a sample by AFB smear and semiquantitative culture were highly correlated. Of the 16 specimens with ≥500 CFU/ml, 13 were also AFB smear positive and 3 were AFB smear negative, while all 16 specimens with <500 CFU/ml were AFB smear negative.

Acid-fast bacteria other than *M. tuberculosis* were isolated from 49 specimens, including 26 from the *M. avium* group, 10 *M. gordonae*, 5 M. fortuitum, 2 *M. chelonae*, and 1 *M. kansasii*. Five mycobacteria other than *M. tuberculosis* were isolated in very low concentrations that are not considered clinically significant; these were not identified. None of these specimens were positive by Abbott LCR. Nine specimens were Abbott LCR negative and *M. tuberculosis* culture positive. These were all specimens with low concentrations of *M. tuberculosis*. Eight were AFB smear negative and had between 10 and 50 CFU of *M. tuberculosis* per ml, while one specimen was smear negative and had 250 CFU/ml. None of the specimens had an inhibitor present, and all *M. tuberculosis* isolates from these specimens were positive in retests of the Abbott LCR.

The eight specimens that were Abbott LCR positive and culture negative were resolved with patient ATS class, clinical history, and repeat testing. Five specimens were from patients who were ATS class 3 (current tuberculosis disease). Four of these were on antimicrobial therapy for tuberculosis when the specimen was collected and had previous or subsequent specimens from which *M. tuberculosis* was isolated. Repeat LCR testing, Roche Amplicor PCR, and Gen-Probe MTD testing were also positive for all five specimens. These five specimens were all considered LCR true positives. Three specimens were from ATS class 4 patients (previous tuberculosis disease) who were not on antimicrobial therapy when the specimen was taken. Even though two of these three specimens were positive by either Gen-Probe MTD or Roche Amplicor PCR, all three were considered Abbott LCR false positives.

With the results from the discrepant analysis, the results from Table 1 were recalculated; they are presented in Table 3. The final Abbott LCR sensitivity was 77%, and specificity was 99%, while the fluorochrome smear sensitivity and specificity were 33 and 98%, respectively. Positive and negative predictive

TABLE 2. Abbott LCR sensitivity by fluorochrome smear result and semiquantitative culture for 34 culture-positive specimens

Test and result	No. of specimens	% Culture-positive specimens	No. LCR positive	LCR sensitivity (%)
AFB smear				
Positive	13	38	13	100
Negative	21	62	12	57
Semiquantitative culture (CFU/ml)				
1–<500	16	47	7	44
≥500–<2,000	1	3	1	100
≥2,000	15	44	15	100
Bactec only[a]	2	6	2	100

[a] Positive on Bactec 460 with 12B medium only, no growth on solid medium.

TABLE 3. Resolved results: LCR and fluorochrome stain

Test and result	Final result		Sensitivity (%)	Specificity (%)	Predictive value (%)	
	No. positive	No. negative			Positive	Negative
Abbott LCR						
Positive	30	3	77	99	91	98
Negative	9	451				
Fluorochrome stain						
Positive	13	8[a]	33	98	62	94
Negative	26	446				

[a] All eight specimens were culture positive for *M. avium* complex.

values were 91 and 98% for Abbott LCx MTB Assay and 62 and 94% for fluorochrome smear.

DISCUSSION

The Abbott LCx MTB Assay is the third commercial NAAT for the detection of *M. tuberculosis* to be developed and widely evaluated. Results of this study and two other recent articles (3, 28) indicate that the sensitivity and specificity of this test is similar to those of both Gen-Probe MTD and the Roche Amplicor *M. tuberculosis* Test. In contrast to these two tests, the detection steps in the Abbott test are automated in an LCx analyzer, saving some technologist time. The maximum run size of the LCx analyzer is 24. Because 6 controls are required for each run, only 18 patient specimens can be tested at a time. Since the LCx thermocycler holds 48 samples, larger runs may be started and amplified, and the specimens can be queued up to be run in the LCx. In our laboratory, a run of 24 specimens and controls can be completed in 6 h. Since processing a group of respiratory specimens requires 2 h, it is possible to incorporate this assay into the normal work flow, with the ability to report results after one 8-h shift.

The final sensitivity of 77% reported is lower than the 90 to 95% reported in two recent studies (3, 28). However, it is close to the 85 and 83% sensitivities reported from our laboratory for Roche Amplicor (24) and Gen-Probe MTD (19), respectively. The differences between LCx sensitivity for our laboratory and that seen by other researchers is most likely due to the low percentage of smear-positive specimens in this study. Only 38% of culture-positive specimens were acid-fast smear positive in this study, while in the two previous studies 80 to 87% were smear positive (3, 27). Since we have demonstrated that the test is more sensitive for smear-positive specimens, the lower percentage of smear-positive specimens most likely led to the lower sensitivity reported here. This also could have affected the sensitivity of the LCR test reported here compared to those of Roche Amplicor and Gen-Probe MTD, studied in this laboratory previously. During those earlier studies, 51 to 56% of culture-positive specimens were smear positive, a percentage substantially higher than the 38% for this report (19, 24). Thus, until a direct comparison of these three tests is carried out, the sensitivities of the tests should be presumed to be approximately equal.

In this study, no false-negative results could be traced to inhibitors. This lack of inhibitors is even lower than the rate of 1.2% previously reported for Roche Amplicor Test (24). All false-negative samples had low concentrations of *M. tuberculosis* present. They were all smear negative and had <250 CFU of *M. tuberculosis*/ml. The effective sample size for this test, 100 μl, is greater than the effective sample sizes of 25 μl for Roche Amplicor and 10 μl for Gen-Probe MTD. However, it is much less than the total inoculum of 700 μl for culture techniques (100 μl inoculated onto two solid medium tubes and 500 μl inoculated into a Bactec 12B vial). Forty-seven percent of positive samples in this study contained <500 CFU of *M. tuberculosis* per ml. A small sample size compared to standard techniques may be one reason the LCR as well as the Roche and Gen-Probe tests lack sensitivity for specimens containing low concentrations of *M. tuberculosis*—those that are either smear negative or have <500 CFU of *M. tuberculosis*/ml.

Initial specificity of the LCR test was 98% (Table 1). Discrepant analysis of the eight LCR-positive, culture-negative specimens indicated that five were from patients with active tuberculosis and four of these five were from patients on antibiotic therapy for tuberculosis when the specimens were taken. The remaining three specimens were from patients who were classified as ATS class 4 (previous tuberculosis disease). Two of these three specimens were also positive by either the Roche Amplicor Test or Gen-Probe MTD. NAAT-positive, culture-negative specimens from patients on antimicrobial therapy have been shown to be due to the presence of *M. tuberculosis* in a noncultivable state (20, 25) and are commonly observed in most comparison studies. Because of this, patients on antimicrobial therapy should not be utilized in studies comparing performance of NAAT to culture. In this study, the clinic personnel identified specimens as either diagnostic (screening for presence of *M. tuberculosis*) or follow-up (testing patients with known tuberculosis infections who are on therapy for treatment efficacy). This classification was not always accurate, leading to the inclusion of a few patients on therapy in this study. If these patients had not been included, the final sensitivity of LCR would be equal to the initial sensitivity of 74% instead of to the value of 77% based on the discrepant analysis. The final LCR specificity was 99%, compared to an AFB smear sensitivity of 33% and a specificity of 98%. Most interesting is the positive predictive value of smear, 62%, compared to that of LCR, 91%. The low-positive predictive value of smear was due to eight smear-positive specimens from the *M. avium* group. None of these smear-false-positive specimens were positive when tested by LCR, reinforcing the fact that smear results, while indicating an infection due to *Mycobacterium*, are often not helpful in determining which species is causing the infection. An LCR or another NAAT of smear-positive specimens rapidly identifies specimens which contain *M. tuberculosis* so that patient treatment and public health efforts can be confidently undertaken.

The sensitivity and specificity of the LCR test presented here indicate that the sensitivity is not high enough to replace culture for detection of *M. tuberculosis*. Even if the sensitivity was higher, culture is needed to provide an isolate for antimicrobial testing. The greatest use of this test will be to rapidly confirm *M. tuberculosis* in specimens which are smear positive. This use has the potential for reducing the amount of time needed to confirm smear-positive *M. tuberculosis* infections to 48 h. In our public health setting, this has proved to be very advantageous to both the clinicians treating the patient and the public health personnel carrying out contact screening.

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