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Screening of Respiratory Tract Specimens for the Presence of *Mycobacterium tuberculosis* by Using the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test

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A prospective 2-month trial involving 617 respiratory tract specimens was conducted to compare sensitivity, specificity, and predictive values of the newly developed Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test kit (AMTDT; Gen-Probe, Inc., San Diego, Calif.) for the rapid detection of *Mycobacterium tuberculosis* and of fluorescent acid-fast staining versus combined BACTEC 12B and solid-medium cultures as the "gold standard." A total of 590 specimens were culture and AMTDT negative. Twenty-one (3.4%) cultures yielded *M. tuberculosis*. Of these, 15 (71.4%) were detected by AMTDT, whereas 6 (28.6%) were missed. *M. tuberculosis* did not grow in six (28.6%) of AMTDT-positive specimens derived from three patients under treatment for tuberculosis. AMTDT exhibited a sensitivity, a specificity, a negative predictive value, and a positive predictive value of 71.4, 99, 99, and 71.4%, respectively. In comparison, the same values for fluorescent microscopy were 66.7, 98.3, 98.8, and 58.3%, respectively. AMTDT was easy to perform and highly specific. However, a screening test would require an improved sensitivity and, when feasible, the implementation of an internal amplification control.

Laboratory diagnosis of mycobacterial infections by conventional techniques is cumbersome and often requires weeks. Although molecular techniques already have an important impact on the clinical mycobacteriology laboratory (5, 10, 24–26), DNA amplification by PCR for the detection of *Mycobacterium tuberculosis* from clinical specimens remains of limited value to the clinical laboratory (2, 15, 18). The newly developed Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test kit (AMTDT; Gen-Probe, Inc., San Diego, Calif.) for the rapid detection of *M. tuberculosis* in clinical specimens is based on the isothermal amplification of target rRNA by DNA intermediates. Detection of the amplification product is achieved by using an acridinium-ester labelled DNA probe that targets the same genomic region as the probe of the AccuProbe culture confirmation kit (Gen-Probe, Inc.) (6, 12). The reported sensitivities of AMTDT range from 79.8 to 100%, resulting in negative predictive values above 96% (3, 8, 11, 17, 29). In addition, AMTDT is reported to fit the work flow of a routine laboratory and potentially reduces the time for the microbiological diagnosis of tuberculosis to 1 day (8).

We have prospectively screened 621 respiratory tract specimens for the presence of *M. tuberculosis* to evaluate the possibility of eliminating AMTDT-negative specimens from being cultured. This would allow the rapid diagnosis of pulmonary tuberculosis and reduce culture work load by more than 95%.

MATERIALS AND METHODS

Preevaluation specimens. Sediments of 11 microscopy-positive respiratory tract specimens stored at −20°C were used for preevaluation. Cultures revealed *M. tuberculosis* in seven specimens, *M. avium* complex (MAC) in two specimens, and another nontuberculous mycobacterium (NTM) that was known to render a false-positive *M. tuberculosis* identification by the AccuProbe culture confirmation kit in one specimen. One specimen remained culture negative.

Clinical specimens. The study included all respiratory tract specimens—sputa, tracheobronchial secretions, and bronchoalveolar washings (BAL)—received by the mycobacteriology laboratory in a 2-month period. The specimens were homogenized, decontaminated with sodium dodecyl sulfate-sodium hydroxide, and neutralized, and the sediments were resuspended in 1 ml of phosphate-buffered saline (22). One hundred microliters of this sediment was screened for the presence of acid-fast bacilli by fluorescence microscopy (1+ to 4+ score) (9). One BACTEC 12B vial containing 4 ml of 7H12 medium (16) supplemented with 0.1 ml of PANTA PLUS (Becton Dickinson Diagnostic Instrument Systems, Sparks, Mass.), and one glycerol-free Löwenstein-Jensen slant supplemented with sodium pyruvate were inoculated with 0.5 and 0.2 ml of the resuspended sediment, respectively. Slants were incubated at 37°C in ambient air for 8 weeks and inspected for growth weekly. BACTEC 12B vials were incubated at 37°C, and growth index readings were recorded, according to the instructions of the manufacturer, using the BACTEC 460 TB instrument (Johnston Laboratories, Inc., Towson, Md.) for 6 weeks. Mycobacteria were identified to the species level by PCR-restriction fragment length polymorphism analysis (26) and by conventional methods (9). Cultures of *M. tuberculosis* isolates missed by AMTDT were checked by using the AccuProbe *M. tuberculosis* complex culture confirmation kit (Gen-Probe, Inc.) according to the instructions of the manufacturer.

Detection of *M. tuberculosis* complex rRNA in respiratory tract specimens. Detection of rRNA was performed, according to the instructions of the manufacturer, by using the Gen-Probe AMTDT kit. All runs included a positive and negative amplification and hybridization control, respectively. Fifty microliters of decontaminated specimen was transferred into lysing tubes containing glass beads and 200 μl of Specimen Dilution Buffer. The tubes were vortexed briefly and sonicated

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for 15 min in a water bath sonicator (Bandelin Sonorex RK 100, Instrumenten-Gesellschaft AG, Zürich, Switzerland) at room temperature. Fifty microliters of the lysates was added to the bottom of amplification tubes containing 25 μl of Amplification Reagent and 200 μl of Oil Reagent. The tubes were incubated at 95°C for 15 min and 42°C for 5 min in a heating block (Gen-Probe, Inc.). Twenty-five microliters of the Enzyme Mix were added, and the tubes were shaken to mix and incubated at 42°C for 2 h. Twenty microliters of Termination Reagent was added, and the tubes were incubated at 42°C for 10 min. Then, 100 μl of Mycobacterium Tuberculosis Complex Probe Reagent was added to every tube. After incubation at 60°C for 15 min, 300 μl of Selection Reagent was added, and the tubes were vortexed and incubated at 60°C for 10 min. The mixture was allowed to cool to room temperature, and chemiluminescence was read after addition of Detection Reagent in a LEADER 50 luminometer (Gen-Probe, Inc.). Results were recorded in relative light units (RLU).

Detection of incomplete hybridization. To demonstrate incomplete hybridization, the selection step was increased by 5 min. *M. tuberculosis* H37rv ATCC 27294 was included as the control.

Detection of *M. tuberculosis* by PCR. A sputum sample from a tuberculosis patient, whose infection was missed by AMTDT, was decontaminated as described above. The sediment was then prepared for PCR by mechanical disintegration as described previously (25), and 2.5 μl of the supernatant was used for the amplification of IS*6110*, the gene encoding for the 65-kDa heat shock protein and human β-globin by PCR (7, 21, 26).

RESULTS

Preevaluation samples. The seven sediments containing *M. tuberculosis* were positive (cutoff, 30,000 RLU) by AMTDT (range, 1,029,914 to 3,067,152 RLU). In contrast, the two MAC-containing sediments and the culture-negative one were negative (range, 5,317 to 5,851 RLU).

The AMTDT chemiluminescence signal of the specimen containing the NTM that had previously rendered a false-positive *M. tuberculosis* identification by the AccuProbe culture confirmation test was 1,803,608 RLU. This strain was recovered on two different occasions from sputum from a 33-year-old female AIDS patient with fever and persistent cough. Evaluation of the gene encoding for the 65-kDa heat shock protein by PCR-restriction fragment length polymorphism analysis of this slowly growing, nonchromogenic mycobacterium revealed a new pattern distinct from those of *M. tuberculosis* and the *M. terrae* complex (26). Partial sequencing of the gene encoding for the 16S rRNA (signature positions 129 to 209) (20) disclosed a sequence similar to that of *M. tuberculosis* except for substitutions of cytosine to thymine at positions 156 and 190. Conventional tests (9) showed growth at 42°C and heat-stable catalase and arylsufatase activity. Negative reactions for niacin, nitrate reduction, semiquantitative catalase, Tween hydrolysis, tellurite reduction, and pyrazinamidase activity further confirmed the nontuberculous nature of this mycobacterial isolate. Definite identification to the species level has not been established. The AMTDT chemiluminescence signal of this strain was reduced from 1,803,608 RLU to 51,836 RLU by prolongation of the selection step from 10 to 15 min. The respective values for the *M. tuberculosis* H37rv reference strain were 3,175,997 RLU and 2,797,052 RLU.

Detection of *M. tuberculosis* complex rRNA in respiratory tract specimens. The cultures of 4 (0.6%) of 621 respiratory tract specimens were contaminated and were excluded from further evaluation. The remaining 617 specimens obtained from 304 patients included 510 sputum samples, 55 tracheobronchial secretion samples, and 52 BAL. Cultures revealed 21 *M. tuberculosis* isolates (Table 1) and 7 NTM isolates (Table 2). The pretest probability of *M. tuberculosis* detection by culture was 3.4%.

AMTDT was easy to perform, and no major technical problems occurred. Carryover contamination was avoided by adherence to the manufacturer's protocol, including careful bleach decontamination of work surfaces. The minimum, maximum, and median values for the 596 AMTDT-negative specimens (cutoff, 30,000 RLU) were 2,248, 28,130, and 4,539 RLU, respectively. The respective values for the 21 AMTDT-positive specimens were 36,534, 3,199,637, and 2,510,418 RLU. The three AMTDT-positive readings below 100,000 RLU were from treated tuberculosis patients and were negative by culture (Table 3).

A total of 590 specimens were culture and AMTDT negative. Fifteen (71.4%) of the specimens that were positive for *M.*

TABLE 1. Culture-positive specimens that yielded *M. tuberculosis*

Patient	Specimen	RLU	Microscopy result[a]	Culture score[b]	Time delay[c]
1	Sputum	3,259	Negative	B	31
2	Sputum	3,943	Negative	B	27
3	TBS[d]	8,310	Negative	<1+	21
4	Sputum	7,626	Negative	B	18
	Sputum	4,384	Negative	B	41
5	BAL	3,100,231	Negative	B	11
6	Sputum	259,187	1+	1+	NA[d]
7	Sputum	1,378,121	1+	B	14
	Sputum	700,574	1+	<1+	16
8	BAL	3,199,637	2+	1+	10
	Sputum	6,512	Negative	B	31
9	Sputum	500,452	2+	B	7
	Sputum	3,097,645	2+	B	11
	Sputum	2,976,977	2+	B	10
	Sputum	1,194,668	1+	B	14
	Sputum	2,772,952	3+	B	16
	Sputum	2,881,713	3+	B	11
10	Sputum	2,383,169	3+	4+	7
11	Sputum	3,055,476	4+	2+	4
12	Sputum	3,082,030	4+	<1+	20
	Sputum	2,510,418	4+	3+	20

[a] Semiquantitative reporting according to reference 9. 1+, 1 to 9 AFB per 10 fields; 2+, 1 to 9 AFB per field; 3+, 10 to 90 AFB per field; 4+, >90 AFB per field. Fluorochrome staining was at 250× magnification.

[b] Modified according to reference 9: B, growth on BACTEC only; <1+, <50 colonies on slant; 1+, 50 to 100 colonies on slant; 2+, 100 to 200 colonies on slant; 3+, almost confluent growth on slant; 4+, confluent growth on slant.

[c] Time delay (days) until first positive (growth index ≥ 100) BACTEC reading. NA, Not available.

[d] TBS, tracheobronchial secretion.

TABLE 2. Culture-positive specimens that yielded NTMs[a]

Patient	Specimen	RLU	Species
13	BAL	3,411	NTM
14	Sputum	4,744	*M. kansasii*
15	Sputum	15,676	MAC
16	Sputum	4,719	NTM
17	Sputum	4,597	MAC
	Sputum	3,962	MAC
18	Sputum	5,329	*M. xenopi*

[a] Microscopy results were negative for all specimens.

TABLE 3. AMTDT-positive but culture-negative sputum specimens

Patient	RLU of specimen	Microscopy result[a]	Comment
7	121,132	Negative	Cavernous tuberculosis, under treatment.
	36,534	Negative	
12	84,229	4+	Tuberculosis, under treatment.
	2,753,016	3+	
	3,081,766	3+	
19	78,021	1+	Tuberculosis, under treatment.

[a] Semiquantitative reporting according to reference 9. For details, see Table 1, footnote *a*.

tuberculosis by culture were detected by the AMTDT, whereas six (28.6%) were missed. In these, culture confirmation using the AccuProbe *M. tuberculosis* complex probe yielded positive results ranging from 577,525 to 693,127 RLU, excluding hybridization failures due to an aberrant target region. Tuberculosis was suspected in three of the patients (Table 1, patients 1, 2, and 3). In addition, two specimens from patient 4, an AIDS patient presenting with pulmonary infiltrates and persistent cough, and a follow-up specimen from a tuberculosis patient receiving antituberculous medication (Table 1, patient 8) were AMTDT negative but culture positive. Cultures of five specimens (Table 1, patients 1, 2, 4, and 8) were positive by BACTEC only. However, patient 2 had two follow-up specimens that yielded growth on both BACTEC and solid media, and patient 8 had a prior positive culture. PCR demonstrated the presence of *M. tuberculosis* in the specimen from patient 4. Therefore, cross-contamination in the BACTEC system (28) was unlikely to have occurred in these specimens.

M. tuberculosis did not grow in six (28.6%) of the AMTDT-positive specimens (Table 3). These specimens were from three patients receiving antituberculous medication for culture-proven tuberculosis. These results were not considered false positive.

Comparison of the initial fluorescence microscopy with culture results revealed growth of *M. tuberculosis* in 14 (58.3%) of the 24 AFB-positive specimens. One specimen (4.2%) grew MAC, and nine (37.5%) remained culture negative. Four of these were from treated tuberculosis patients (Table 3), and three were from a patient treated for infection due to MAC. Thus, 22 (91.7%) were considered true positives and 2 (8.3%) were considered false positives.

In summary, in our setting the sensitivity and the specificity of the AMTDT were 71.4 and 99%, respectively (Table 4). Given a pretest probability of 3.4% of *M. tuberculosis* isolates in respiratory tract specimens, the negative and positive predictive values were 99 and 71.4%, respectively. The same values for direct fluorochrome stain were 66.7, 98.3, 98.8, and 58.3%, respectively (Table 4).

TABLE 4. Sensitivity, specificity, and predictive values for fluorescence microscopy and the AMTDT versus culture as the gold standard

Method	% Sensitivity	% Specificity	Predictive value (%)	
			Positive	Negative
AMTDT	71.4	99	99	71.4
Microscopy	66.7	98.3	98.8	58.3

DISCUSSION

The implementation of AMTDT to routine work in our laboratory proved to be uncomplicated. The assay was easy to perform, and no major technical problems occurred. However, in the absence of a definitive amplification product inactivation protocol the long-term prospective use of AMTDT may lead to an accumulation of amplification products in the laboratory. Therefore, false positives remain a long-term risk of using AMTDT.

The 617 specimens included in the 2-month study period represented about one-sixth (17%) of the respiratory tract specimens processed at our institution in the previous year. The rate of positive *M. tuberculosis* cultures during the study period was 3.4%. In comparison to combined liquid- and solid-medium cultures, which today are considered "gold standard" procedures (27), AMTDT exhibited a sensitivity, specificity, negative predictive value, and positive predictive value of 71.4, 99, 99, and 71.4%, respectively. The same values for fluorescence microscopy were 66.7, 98.3, 98.8, and 58.3%, respectively.

This sensitivity is considerably lower than those reported by others (3, 8, 11, 17, 29). However, although AMTDT is reported to detect less than one organism (analytical sensitivity), it failed to detect 46% of culture-positive specimens that contained less than an estimated 100 CFU/ml (8). Therefore, the low sensitivity in our hands reflects the high proportion specimens showing only a low degree of positivity (6 of 21) included in the study, as well as the sensitivity of our culture technique. In favor of this view are the facts that only AFB-negative specimens were missed and that five of the six specimens not detected by AMTDT were positive in the BACTEC 12B medium only. The corresponding detection times in the BACTEC system ranged from 18 to 41 days, again indicating a low bacterial load. This finding is compatible with the higher sensitivity of BACTEC as compared with solid-medium cultures (1). As cross-contamination by the BACTEC system (28) can be ruled out, we feel that this could be an additional factor responsible for the lower sensitivity of AMTDT in our investigation. However, the lack of a generally accepted standard procedure for mycobacterial cultures when evaluating culture-independent assays renders the direct comparison of different studies very difficult, because an improved sensitivity of the respective gold standard procedure in a laboratory necessarily lowers the sensitivity of the evaluated assay.

In our hands, AMTDT was unreliable in detecting specimens with a low degree of positivity and therefore does not fulfill the criteria for a screening tool. Because AMTDT-negative specimens cannot be eliminated from being cultured—this would reduce the culture-dependent work load by more than 95%—the implementation of AMTDT as a screening tool would increase the work load and the expenses of the mycobacteriology laboratory considerably without significant benefit.

In a comparison study using culture as the reference technique, a discrepant analysis of the initial false-positive results is needed because of the nucleic acid amplification techniques' ability to detect noncultivable organisms. The final performance of AMTDT for specificity was 100% because all AMTDT-positive but culture-negative specimens were from patients with culture-proven tuberculosis undergoing treatment and thus were considered true positives. However, the detection of nonviable organisms by AMTDT may limit its value for the rapid assessment of treatment efficacy.

Several reports describe false-positive *M. tuberculosis* iden-

tification by the AccuProbe *M. tuberculosis* complex test despite its excellent specificity (13, 14, 19, 30). Because amplification product detection of the AMTDT relies on the same genomic target, we expect that increasing application of AMTDT will reduce the assay's specificity. With our NTM isolate, prolongation of the selection step revealed incomplete hybridization and improved specificity.

Because of its high degree of specificity, the AMTDT could be a confirmatory tool for the presence of *M. tuberculosis* in acid-fast bacillus-positive specimens. However, the absence of an internal amplification control may prevent the detection of the presence of enzyme-inhibiting activity in clinical specimens, and thus a negative AMTDT result does not necessarily rule out tuberculosis (8).

In summary, this assay represents a first step towards an easy-to-perform and highly specific test for the rapid diagnosis of pulmonary tuberculosis. However, to become a general purpose screening test, it would require (i) an improved sensitivity for the reliable detection of specimens containing low numbers of *M. tuberculosis* and, when feasible, an internal amplification control to detect enzyme inhibiting activity of clinical specimens, (ii) an automated format, and (iii) reasonable costs, because tuberculosis has always been a disease of the poor (4, 23).

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