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Diagnosis of Extrapulmonary Tuberculosis by Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test

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A total of 294 specimens collected from nonrespiratory sites of 268 patients were tested for direct detection of *Mycobacterium tuberculosis* complex by the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test (AMTD). The specimens included ascitic, pleural, pericardial, and synovial fluids, abscess aspirates, and tissue and lymph node biopsy samples, as well as gastric aspirates and cerebrospinal fluid samples. All samples were processed by the *N*-acetyl-L-cysteine-sodium hydroxide decontamination procedure prior to testing. Twenty samples showed acid-fast bacilli on auramine staining, and 48 samples were positive by AMTD, 9 of which were negative for *M. tuberculosis* complex by culture. After reviewing the patients' clinical charts to resolve discrepancies, the test result of one cerebrospinal fluid sample was considered to be false positive by AMTD. The overall sensitivity, specificity, positive predictive value, and negative predictive value were 83.9, 99.6, 97.9, and 96.3%, respectively. No significant differences were found when AMTD results obtained with specimens of nonrespiratory origin were compared with assay results obtained with samples of respiratory origin ($P > 0.05$). In conclusion, our results demonstrate that AMTD performs equally well with all types of specimens.

Over the past decade, diagnostic tools for the detection of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), have considerably improved. The introduction of liquid-based culture media has significantly reduced the time required to detect mycobacterial growth (2, 8, 10), which remains indispensable for definitive species assignment and susceptibility testing of patient isolates. More recently, commercially available standardized nucleic acid-based amplification techniques were shown to yield reliable results within 5 to 7 h of sample processing (1, 4–7, 9). Several clinical studies encompassing more than 5,000 respiratory samples have demonstrated high sensitivities and specificities of these assay systems, generally ranging from 80 to 90 and from 95 to 99%, respectively (12, 13, 16, 18, 19). Nevertheless, these techniques do not allow differentiation among the different species belonging to the *M. tuberculosis* complex (i.e., *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, and *M. africanum*). Thus, culture and subsequent biochemical tests are still necessary to specifically identify *M. tuberculosis.*

The clinical diagnosis of extrapulmonary TB can be particularly challenging, partly because this manifestation of TB disease occurs infrequently in industrialized countries. Diagnostic microbiology services are often called upon by attending clinicians to assist in the rapid diagnosis of extrapulmonary TB, but samples such as pleural exudates and cerebrospinal fluids (CSFs) are known to contain only few mycobacteria, resulting in a low sensitivity of acid-fast staining techniques.

Commercial nucleic acid amplification kits are licensed for use with respiratory specimens only, and experience with specimens of nonrespiratory origin is still limited. While some investigators have reported preliminary sensitivities and specificities similar to those obtained when respiratory samples are tested (7, 18), it has been demonstrated that, at least with some specimens like pleural exudates, sensitivity may be as low as 20% (18). For the most part, sample sizes have been too small to draw definitive conclusions as to the usefulness of these techniques in the diagnosis of extrapulmonary tuberculosis.

The purpose of this study was to investigate whether the Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test (AMTD) was suitable for a wide range of specimens other than respiratory secretions to establish early diagnosis of extrapulmonary tuberculosis. Our data clearly show that sensitivities and specificities of the assay applied to these specimens are at least equal to those obtained when samples from the respiratory tract are tested and that improving sample processing to remove inhibitors might further increase the usefulness of the assay.

MATERIALS AND METHODS

Specimens. From May 1994 to December 1995, 261 respiratory specimens (108 sputa, 11 bronchial aspirates, and 142 bronchoalveolar lavages) and 294 specimens other than respiratory secretions (ascitic, pleural, pericardial and synovial fluids, abscess aspirates, and tissue and lymph node biopsy samples, as well as gastric aspirates and CSF samples) were obtained from patients admitted to the Benjamin Franklin University Hospital of the Free University of Berlin, to the Virchow-Hospital of the Humboldt University of Berlin, or to a number of municipal hospitals in the Berlin metropolitan area. Of the 294 extrabronchial specimens, there were 79 body fluids and aspirates (including 35 pleural effusions, 6 pericardial effusions, 8 ascitic fluids, 6 synovial fluids, and 24 aspirates of pus or miscellaneous cystic tumors), 90 tissue specimens (including 29 lymph node samples, 23 bone, intervertebral disc, or marrow biopsy samples, 9 lung biopsy samples, 6 liver biopsy samples, and 23 miscellaneous biopsy samples from the skin, pleura, synovia, kidney, pericardium, uterus, epidydimis, and pharynx), 51 CSF samples, 59 gastric aspirates, and 15 miscellaneous specimens (urine, blood, and stool). For most patients, there was a high suspicion of tuberculosis. Upon receipt, specimens were kept at 4°C prior to processing (two processings per day).

Decontamination procedure. Specimens of nonrespiratory origin judged likely to be bacterially contaminated (because of their opaque appearance) and all respiratory specimens were decontaminated by using the *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) protocol (10, 11). Briefly, an equal volume of digestant (3% NaOH, 1.45% sodium citrate, 0.5% NALC [Sigma, Deisenhofen, Germany]) was added to up to 10 ml of a specimen. After vortexing, the mixture was shaken for 20 min, neutralized by adding sterile distilled water to a final volume of 50 ml, and centrifuged at 3,500 × *g* for 20 min. The supernatant was discarded, and the sediment was resuspended in 2 ml of distilled water. Half of

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TABLE 1. Results of AMTD and culture of respiratory specimens and clinical diagnosis of TB

Test and result	No. of assays		
	Positive	Negative	Total
Detection of *M. tuberculosis* complex by AMTD[a]		Culture	
Positive	39	11	50
Negative	8	203	211
Total	47	214	261
Diagnosis of TB by AMTD[b]		Clinical diagnosis	
Positive	47	3	50
Negative	8	203	211
Total	55	206	261
Diagnosis of TB by culture[c]		Clinical diagnosis	
Positive	47	0	47
Negative	8	206	214
Total	55	206	261

[a] Sensitivity, 83.0%; specificity, 94.9%; PPV, 78.0%; NPV, 96.2%.
[b] Sensitivity, 85.5%; specificity, 98.5%; PPV, 94.0%; NPV, 96.2%.
[c] Sensitivity, 85.5%; specificity, 100%; PPV, 100%; NPV 96.3%.

the sediment was frozen at −70°C until AMTD was performed, and the other half was inoculated onto culture media and used for acid-fast staining. Tissue biopsy samples were homogenized by using a sterile plastic pistil fitting into conically shaped Eppendorf tubes (Eppendorf, Hamburg, Germany) before NALC-NaOH processing.

For specimens of nonrespiratory origin judged likely not to be contaminated (because of their transparent appearance), the procedure was modified as follows in order not to compromise the sensitivity of the culture. First, the samples were divided into two equal aliquots, one of which was inoculated onto culture media and used for acid-fast staining. The other aliquot was frozen at −70°C and processed immediately prior to AMTD by the NALC procedure outlined above.

Culture. Equal aliquots (approximately 300 μl) of the processed sediment were inoculated onto two solid slants, Löwenstein-Jensen and Stonebrink (an egg-based medium containing pyruvic acid and malachite green) (17), and incubated at 37°C for 10 weeks in a humidified atmosphere. In addition, 300 μl of the sediment was cultivated in the Septi-Chek biphasic culture system (Becton Dickinson, Heidelberg, Germany), supplemented with an antimicrobial mixture (polymixin B, azlocillin, nalidixic acid, trimethoprim, amphotericin B [PANTA], Becton Dickinson). Solid and liquid cultures were inspected twice weekly for turbidity and growth on the slants for 10 weeks.

Microscopy. Smears were stained with auramine. Positive slides were confirmed by a Ziehl-Neelsen stain (10).

Identification of mycobacteria. Routine biochemical methods and the Accuprobe culture confirmation kits (Gen-Probe, Biermann GmbH, Bad Nauheim, Germany) were employed for identification of isolates.

AMTD. AMTD reagents (Gen-Probe) were reconstituted and stored as aliquots to increase storage life, as previously described (16). The AMTD protocol was followed according to the manufacturer's instructions. In brief, 50 μl of a decontaminated sample was added to 200 μl of specimen dilution buffer in a lysing tube, and the mixture was sonicated for 15 min in a water bath sonicator at room temperature. For amplification, 25 μl of reconstituted amplification reagent was placed in a reaction tube and covered with 200 μl of mineral oil. Fifty microliters of lysate was transferred to the amplification tube, incubated at 95°C for 15 min, and cooled at 42°C for 5 min. An enzyme reagent mix (25 μl) was added, and the mixture was incubated at 42°C for 2 h. To terminate amplification, 20 μl of a termination reagent was added to each tube, and the mixtures were kept at 42°C for another 10 min. For detection, the reconstituted acridinium ester-labeled probe (100 μl) was added to the tubes, and they were incubated at 60°C for 15 min; then the selection reagent (300 μl) was added, and the mixtures were reincubated at 60°C for 10 min. All temperature-controlled incubation steps were carried out in heating blocks. Prior to being read in a luminometer (LEADER 50, Gen-Probe) the tubes were cooled at room temperature for 5 min. All runs included MTD amplification-positive and -negative controls and hybridization-positive and -negative controls. The cutoff value was set at 30,000 relative light units (RLU). Samples with values of >30,000 RLU were considered positive; samples with values <30,000 RLU were considered negative. To control for potential inhibition of amplification, samples of extrapulmonary origin were run in duplicate. The duplicate vial contained 5 μl (i.e., 1/10) of the amplification-positive control provided by the manufacturer.

Patients' clinical data. In those cases in which discrepant results for the AMTD and culture were obtained, the clinical charts of the patients were evaluated. Clinical assessment included the patient's history, symptoms, chest X-ray, laboratory results, and follow-up observation as well as the results obtained with additional specimens from the patient that were sent to the mycobacteriology laboratory.

Statistical analyses. Data were analyzed statistically by the chi-square test.

RESULTS

Detection of *M. tuberculosis* complex in respiratory specimens. During the period of May 1994 through December 1995, a total of 261 respiratory specimens from 242 patients were analyzed in our laboratory, enabling us to compare sensitivities and specificities of the assay applied to specimens obtained from both respiratory and nonrespiratory sites. Sixteen respiratory samples were positive upon auramine staining, and 50 samples tested positive in the AMTD, of which only 47 grew *M. tuberculosis* in culture (Table 1). After resolution of discrepant results by reviewing patients' charts, three test results were regarded as false positives. The calculated sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the AMTD thus were 85.5, 98.5, 94, and 96.2%, respectively, which is in good accordance with results published by other investigators (1, 9, 12, 14, 16, 18, 19).

Detection of *M. tuberculosis* complex in specimens other than respiratory secretions. During the study period, a total of 294 samples obtained from extrabronchial sites of 268 patients were assayed by AMTD. Twenty samples were positive by auramine staining, and 48 samples tested positive by AMTD, of which 39 grew *M. tuberculosis* complex upon culture (Table 2). After resolution of discrepant results, only one test result was regarded to be false positive by AMTD. Twenty-two samples showed inhibition of amplification because the same sample tested in parallel but spiked with the positive control RNA did not give a positive signal. When samples showing inhibition were counted as negative test results, the calculated sensitivity (83.9%) was similar to that obtained for respiratory specimens (83.0%) and, in particular, was almost identical to the calculated sensitivity of culture (85.7%) (Table 2). When samples showing evidence of inhibitory substances were excluded from

TABLE 2. Results of AMTD and culture of extrabronchial specimens and clinical diagnosis of TB

Test and result	No. of assays[a]		
	Positive	Negative	Total
Detection of *M. tuberculosis* complex by AMTD[b]		Culture	
Positive	39 (0)	9 (0)	48 (0)
Negative	9 (3)	235 (19)	246 (22)
Total	48 (3)	246 (19)	294 (22)
Diagnosis of TB by AMTD[c]		Clinical diagnosis	
Positive	47 (0)	1 (0)	48 (0)
Negative	9 (3)	237 (19)	246 (22)
Total	56 (3)	238 (19)	294 (22)
Diagnosis of TB by culture[d]		Clinical diagnosis	
Positive	48	0	48
Negative	8	238	246
Total	56	238	294

[a] Numbers in parentheses indicate the number of assays revealing inhibition of amplification.
[b] Sensitivity, 81.3%; specificity, 96.3%; PPV, 81.3%; NPV, 96.3%. Sensitivity and NPV increased to 86.7 and 97.3%, respectively, when samples showing evidence of inhibition of amplification were excluded.
[c] Sensitivity, 83.9%; specificity, 99.6%; PPV, 97.9%; NPV, 96.3%. Sensitivity and NPV increased to 88.7 and 97.3%, respectively, when samples showing evidence of inhibition of amplification were excluded.
[d] Sensitivity, 85.7%; specificity, 100%; PPV, 100%; NPV, 97.3%.

TABLE 3. Results of AMTD of body fluids and aspirates and clinical diagnosis of TB

Test and result	Clinical diagnosis[a]		
	Positive	Negative	Total
Diagnosis of TB in body fluids and aspirates, including pleural fluids, by AMTD[b]			
Positive	11 (1)	0 (0)	11 (1)
Negative	1 (1)	67 (7)	68 (8)
Total	12 (2)	67 (7)	79 (9)
Diagnosis of TB in pleural fluids by AMTD[c]			
Positive	3 (0)	0 (0)	3 (0)
Negative	0 (0)	32 (3)	32 (3)
Total	3 (0)	32 (3)	35 (3)

[a] Number of specimens. Numbers in parentheses indicate the number of assays revealing inhibition of amplification.
[b] Sensitivity, 91.6%; specificity, 100%; PPV, 100%; NPV, 98.5%. Sensitivity and NPV increased to 100% when samples showing evidence of inhibition of amplification were excluded.
[c] Sensitivity, specificity, PPV, and NPV were all 100%.

TABLE 4. Results of AMTD of tissue specimens and clinical diagnosis of TB

Test and result	Clinical diagnosis[a]		
	Positive	Negative	Total
Diagnosis of TB in tissue specimens, including lymph node biopsy samples, by AMTD[b]			
Positive	23 (0)	0 (0)	23 (0)
Negative	4 (3)	63 (1)	67 (4)
Total	27 (3)	63 (1)	90 (4)
Diagnosis of TB by AMTD of lymph node biopsy samples[c]			
Positive	13 (0)	0 (0)	13 (0)
Negative	1 (1)	15 (1)	16 (2)
Total	14 (1)	15 (1)	29 (2)

[a] Number of specimens. Numbers in parentheses indicate the number of assays revealing inhibition of amplification.
[b] Sensitivity, 85.2%; specificity, 100%; PPV, 100%; NPV, 98.4%. Sensitivity and NPV increased to 95.8 and 98.4%, respectively, when samples showing evidence of inhibition of amplification were excluded.
[c] Sensitivity, 92.8%; specificity, 100%; PPV, 100%; NPV, 93.8%. Sensitivity and NPV increased to 100% when samples showing evidence of inhibition of amplification were excluded.

the calculation of the AMTD's performance characteristics, the sensitivity rose to 88.7% and the negative predictive value increased to 97.3%.

(i) Body fluids and aspirates. Of 79 body fluids and aspirates, which included 8 ascites, 6 pericardial effusions, 6 synovial fluids, 24 abscess drainages and wound secretions, and 35 pleural fluids, 11 of 12 were positive by AMTD as compared with only 1 of 12 positive by auramine staining (Table 3). No false-positive AMTD reactions were recorded. Inhibition of amplification occurred in nine samples, including the one for which the test result was counted as false negative for the purpose of calculating assay sensitivity.

Of 35 pleural exudates examined, 3 tested positive, and no diagnosis was missed (sensitivity, specificity, PPV, and NPV were all 100%). Inhibition of amplification was observed in three of these samples.

(ii) Tissue and lymph node biopsy samples. Of 90 tissue specimens, which included biopsy samples of liver, bone, pleura, lung, peritoneum, soft tissue, and lymph nodes, 23 tested positive in the AMTD as compared with only 10 showing acid-fast bacilli upon auramine staining (Table 4). No false positives were recorded, but four false negatives occurred, predominantly due to amplification inhibition, which occurred in three out of these four samples. A total of four samples showed inhibition of amplification.

Of the 29 specimens from lymph nodes that were examined, only 5 were shown to be positive by auramine fluorochrome staining, whereas 13 samples tested positive by AMTD. Again, no false-positive results were recorded, but diagnosis was missed on one occasion; this sample showed inhibition of amplification. Altogether, two samples were demonstrated to contain inhibitors of amplification.

(iii) CSF samples. Of 51 CSF specimens examined, one was shown to contain acid-fast bacilli by auramine staining and five were positive by AMTD (Table 5). After analysis of patients' charts, one sample was considered to have a false-positive test result. In two cases, the diagnosis was missed by AMTD, although in this series, no inhibition of amplification was observed.

(iv) Gastric aspirates. In the absence of sputum expectoration, gastric aspirates often are submitted to establish diagnosis of pulmonary TB. Of 59 gastric aspirates tested, 8 were positive by AMTD (Table 6), whereas none revealed acid-fast bacilli in the auramine stain; in three cases, diagnosis was missed by AMTD, although no inhibition of amplification was found in these samples. Overall, seven samples showed evidence of substances inhibitory of nucleic acid amplification.

(v) Other samples. A total of 15 miscellaneous samples (4 urine, 9 blood, and 2 stool) were also run in the AMTD. One stool sample was positive by AMTD and culture, and inhibition occurred twice with blood samples.

DISCUSSION

It has been our experience since introducing AMTD for rapid detection of *M. tuberculosis* complex into the laboratory that clinicians have repeatedly requested the testing of samples other than respiratory secretions to accelerate the diagnosis of extrapulmonary tuberculosis. As commercial kits are only licensed for testing respiratory samples processed by NALC-NaOH decontamination, we decided to investigate the suitability of the AMTD for specimens of nonrespiratory origin in a prospective study.

We had previously reported on sample inhibition of amplification, particularly when CSF and pleural fluids were examined (7). Since the NALC-NaOH protocol as used for decontamination of respiratory secretions seems to result in the nonspecific removal of many inhibitory substances (6a, 15), we decided to also subject all samples from extrapulmonary sites

TABLE 5. Results of AMTD of cerebrospinal fluids and clinical diagnosis of TB

AMTD result[a]	Clinical diagnosis[b]		
	Positive	Negative	Total
Positive	4	1	5
Negative	2	44	46
Total	6	45	51

[a] Sensitivity, 66.7%; specificity, 97.8%; PPV, 80.0%; NPV, 95.7%.
[b] Number of specimens.

TABLE 6. Results of AMTD of gastric aspirates and clinical diagnosis of TB

AMTD result[a]	Clinical diagnosis[b]		
	Positive	Negative	Total
Positive	8 (0)	0 (0)	8 (0)
Negative	3 (0)	48 (7)	51 (7)
Total	11 (0)	48 (7)	59 (7)

[a] Sensitivity, 72.7%; specificity, 100%; PPV, 100%; NPV, 94.1%. Numbers in parentheses indicate number of assays revealing inhibition of amplification. NPV decreased to 93.2% when samples showing evidence of inhibition of amplification were excluded.
[b] Number of specimens.

to this protocol. In addition, 1/10 of the positive control supplied by the manufacturers was added to a duplicate sample assayed in parallel in order to test for inhibitors of amplification.

In this series of 294 samples obtained from extrabronchial sites, the overall sensitivities and specificities were shown to be as high as those reported by other investigators, including ourselves, for respiratory secretions (3, 7, 9, 12, 14). In particular, diagnosis was first established by AMTD for 11 body fluids and aspirates, 23 tissue specimens, and 4 CSF samples, all of which are notoriously low in bacterial numbers. For example, the sensitivity of auramine staining in our study was highest for lymph node samples (38.5%), and the use of AMTD with lymph node samples resulted in a remarkable increase of diagnostic sensitivity to 92.8%.

Twenty-two samples (7.5%) showed evidence of substances inhibitory of amplification. In fact, the majority of false-negative results could be shown to be due to inhibition of amplification. If these results are excluded from analysis, the sensitivity of the amplification assay for detection of *M. tuberculosis* complex rose to 100% in the case of body fluids and aspirates and to 95.8% in the case of tissue biopsy samples. Although there was a clear tendency towards a higher sensitivity if inhibitors could be successfully removed (83.9 versus 88.7% for all specimens), this finding did not attain statistical significance in our study ($P > 0.05$). However, the number of positive specimens inhibited for amplification was rather small in this series. Moreover, when reporting individual assay results to the attending clinicians, we continue to feel more comfortable if we can exclude inhibition of amplification as a possible cause of a negative test result. However, the duplicate format is expensive and prone to cross-contamination. Therefore, the reliable removal of inhibitory substances remains a major goal. We have tried a number of protocols, including commercially available nucleic acid purification procedures employing DNA and RNA extraction columns as well as capture resins, but results have been inconsistent thus far and we have been unable to define a procedure that will reproducibly result in removal of all inhibitors. Using the NALC-NaOH decontamination protocol, we were able to reduce inhibition by approximately 80% compared with a smaller previous series (data not shown); the mode of action of this procedure is unknown but presumably reflects the dilution of inhibitory substances in the repeated washing steps associated with processing of the samples in this way.

In this series of extrabronchial specimens, there was only one false-positive test result. The CSF sample originated from an AIDS patient on a four-drug regimen to treat disseminated *M. avium* infection who developed neurological symptoms and inflammatory signs indicative of chronic meningitis. Cryptococcal infection was excluded, and after the AMTD result was communicated to the treating physician, the patient received a four-drug standard therapy for *M. tuberculosis*. Although the patient initially improved, he died within 2 months. Autopsy was denied, and no unequivocal proof could be obtained (the culture remaining negative) that his neurological symptoms were due to *M. tuberculosis* infection. It was therefore decided to score this test result as false positive.

Although the overall sample number of CSFs was too low to allow for statistical comparison to other body fluids, there were two false negatives in this series, leading to the low sensitivity of 66.7%. It is possible that the AMTD is less sensitive with this type of specimen. Alternatively, the few bacteria present in the sample may have been unevenly distributed between the aliquot subjected to culture and the aliquot reserved for AMTD, explaining the discrepant test result.

It was previously reported that AMTD showed a low sensitivity with pleural fluid samples (18). We did not encounter this problem in our series, but there were only three positive samples in our study and the overall number of specimens tested was considerably lower than in the study cited (35 versus 80 samples). It is also possible that pretreatment with NALC-NaOH as performed with our specimens may have removed inhibitors, thereby resulting in a sensitivity of 100%.

Because of the small size of our sample, we are at present unable to reliably calculate the sensitivity and specificity of the assay when applied to samples such as blood, urine, and stool. We feel, however, that the performance of the AMTD with these samples was encouraging in that only two samples showed inhibition. It may therefore be possible to apply this sensitive technique to samples that are easy to obtain.

We have examined a few specimens obtained from seven patients undergoing therapy. In three of them treatment was started less than 5 days before specimen collection (2, 2, and 4 days prior). Specimens of these patients showed positive results in both AMTD and culture. In four patients treatment duration before specimen collection (1 bronchoalveolar lavage, 2 sputa, and 2 gastric aspirates) was at least 14 days (14, 15, 17, and 21 days). These specimens showed positive results in AMTD but did not subsequently yield growth of *M. tuberculosis.*

On the other hand, we have also observed that AMTD results become negative after initiation of therapy, and this seemed to correlate with the time the patient had been on therapy. The number of specimens studied, however, is too small to address the question whether AMTD might also be useful for monitoring the response to therapy.

In conclusion, our study demonstrates the efficacy of AMTD in establishing the diagnosis of tuberculosis when samples other than respiratory secretions are assayed. The use of nucleic acid-based amplification techniques in the diagnosis of extrapulmonary tuberculosis may allow significantly earlier initiation of specific treatment.

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