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DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* BY MOLECULAR METHODS

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Molecular methods for identifying *Mycobacterium tuberculosis* in culture have been routinely used in the clinical laboratory for many years. Nucleic acid probes are now commonly used to specifically identify *M. tuberculosis* grown from both solid media and liquid media such as BACTEC (Becton Dickinson Diagnostic Instrument Systems, Sparks, Maryland) broth. The Gen-Probe AccuProbe Mycobacterium Tuberculosis Complex Culture Identification Test demonstrates a sensitivity and specificity of 99.2% and 99.9%, respectively, prior to discrepant resolution (Gen-Probe AMTD, package insert). Evans et al[14] have described the use of the AccuProbe tests directly from BACTEC. They concluded that the identification of *M. tuberculosis* occurred more rapidly with AccuProbe than with conventional identification methods, but that a sensitive and rapid method for detecting *M. tuberculosis* directly from clinical specimens was required. The sensitivity of nucleic acid probes for the identification of *M. tuberculosis* using the AccuProbe acridinium ester technology[34] is approximately 500,000 to 1 million organisms (unpublished data), which explains the need for more sensitive methods. Probes are available for *M. tuberculosis, M. avium, M. intracellulare, M. gordonae, M. kansasii,* and *M. avium* complex.

High-pressure liquid chromatography (HPLC) is also used to identify mycobacteria and can identify any known species. This method has

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been used routinely in the clinical laboratory with isolates from solid media as described by Butler and Kilburn.[6] More recently, Jost et al[20] have applied this method to growth from BACTEC medium and to clinical specimens. They reported 99.0% sensitivity, relative to culture, with HPLC applied to BACTEC growth; however, sensitivity determined directly from clinical specimens was only 56.8%. The specificity of both applications of HPLC was 100%. The sensitivity and specificity reported for HPLC from the BACTEC medium is adequate for use in the clinical laboratory as a tool for diagnosing tuberculosis; however, HPLC applied to clinical specimens lacks adequate sensitivity.

Because molecular methods such as hybridization with nucleic acid probes and HPLC are not sufficiently sensitive for use directly from clinical specimens and require culturing the organism, several weeks to 2 months or more are still needed to detect and identify *M. tuberculosis*. Thus, several groups have developed nucleic acid target amplification technologies for more rapid diagnosis of tuberculosis.

Various nucleic acid target amplification technologies have been applied to detection of *M. tuberculosis*.[1–5, 7–19, 21–44] These include polymerase chain reaction (PCR) and transcription-mediated amplification (TMA) as well as strand displacement amplification (SDA) and ligase chain reaction (LCR).[34, 38, 39] All have been used with patient specimens; however, because the latter two methods have not been routinely used in the clinical laboratory to date, this review will focus on PCR and TMA. The primary amplification targets for PCR are the IS6110 insertion sequence and the 16S rRNA gene. TMA's target is the 16S rRNA.

TMA is a transcription-based target amplification system whose primary product is RNA. The amplification can use either RNA or DNA as a target. For detecting mycobacteria, the preferred target is rRNA. Ribosomal RNA is present at about 2000 copies per mycobacterial cell, which provides an increase in sensitivity compared with targets such as IS6110, which are present in 1 to 10 copies per cell.[13] As shown in Figure 1, two primers are utilized for TMA. The first primer contains a promoter sequence for RNA polymerase as well as a region complementary to the 16S rRNA target sequence. Reverse transcriptase extends this primer to make a cDNA copy of the rRNA target. RNase H activity, associated with reverse transcriptase, then makes the cDNA available to the second primer. The second primer is complementary to a portion of the cDNA copy. Reverse transcriptase then extends this primer, resulting in a double-stranded DNA intermediate. A part of the DNA intermediate contains a double-stranded promoter region that is recognized by RNA polymerase. The RNA polymerase generates multiple copies of RNA. This RNA is available for amplification using the same primers and enzymes. A billion-fold amplification is achieved using TMA with *M. tuberculosis* rRNA as a target in less than 2 hours.

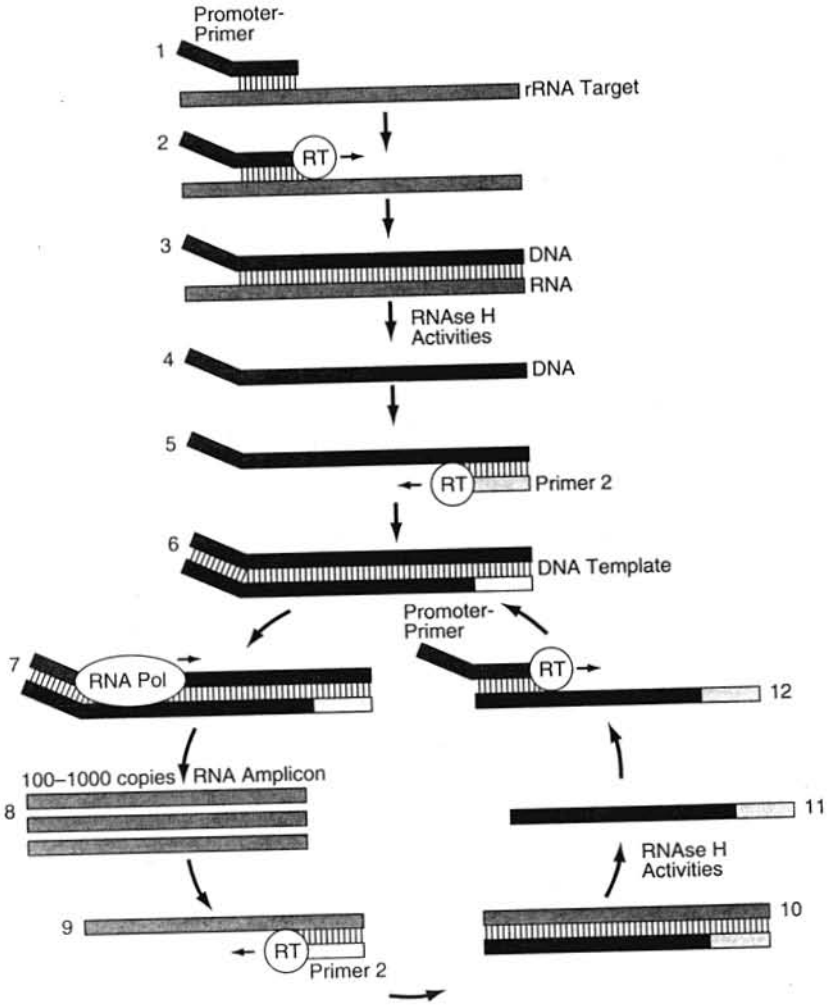

Figure 1. Transcription-mediated amplification. Two primers are used for TMA (see text for further details). RT = reverse transcriptase; Pol = RNA polymerase. Black bar = DNA extension product of RT; Dark tone bar = promoter primer; Medium tone bar = RNA amplicon; Light tone bar = primer; Vertical lines = base-pairing.

PCR uses two primers, one enzyme, and repeated thermal cycling between denaturation, reannealing, and extension steps. Each cycle of PCR begins with denaturation of the double-stranded DNA amplicon followed by primer annealing to target and extension of PCR products by *Taq* polymerase. Each cycle theoretically doubles the number of

target nucleic acid molecules; therefore, after 20 cycles a million-fold amplification is achieved, and after 30 cycles, a billion-fold.

PCR has been applied to direct detection of *M. tuberculosis* using a number of different target nucleic acids. Owing to the lack of standardization of reagents and protocols with "home brew" PCR protocols, there have been inconsistent results in the clinical performance from laboratory to laboratory, and sensitivity and specificity have varied greatly.[27] More recently, the literature has indicated that results with manufactured kit reagents are more consistent. Specificities of PCR have been in the high range (exceeding 95%), whereas sensitivities, compared with culture, have ranged between 67% and 100%.[2, 3, 8, 11, 22, 24, 25, 33, 42] This variation in sensitivity is probably due to multiple factors including sampling error and specimen inhibition of the PCR. Amplification-based assays from Gen-Probe and Roche Molecular Systems have shown comparable levels of sensitivity and specificity in clinical evaluations.[1, 18, 37, 44]

TMA has been combined with the Hybridization Protection Assay (HPA) for amplicon detection to develop a single-tube clinical diagnostic test for the detection of *M. tuberculosis* in respiratory sediments. The test has recently been approved by the US Food and Drug Administration (FDA) for use in acid-fast bacilli (AFB) smear-positive specimens. The Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (AMTD) was evaluated in a six-site clinical trial in the United States. The results of AFB smear, culture, and AMTD were evaluated for 6079 specimens from 2609 patients. Of the total specimens tested, 198 specimens were AFB smear-positive from 78 patients not on therapy for tuberculosis. The sensitivity of the AMTD test for these samples was 95.5% (147 of 154) and the specificity was 100% (44 of 44).

Table 1. REPORTED CLINICAL SENSITIVITY AND SPECIFICITY OF THE GEN-PROBE AMPLIFIED MYCOBACTERIUM TUBERCULOSIS DIRECT (AMTD) TEST IN SEVERAL LABORATORIES

		AMTD	
Reference	No. Specimens	Sensitivity (%)	Specificity (%)
Abe et al[1]	135	91.9	100
Bodmer et al[4]	617	71.4	99
Bradley et al[5]	844	93.6	97.8
Ehlers et al[12]	71	96.2	97.7
Jonas et al[19]	758	82.4	99.4
LaRocco et al[23]	760	95	98
Miller et al[24]	750	91	98.5
Pfyffer et al[28]	938	97.8	97.1
Pfyffer et al[30]	1117	86.6	96.4
Vlaspolder et al[36]	412	98.4	98.9
Welch et al[41]	339	100	99.7

Table 2. REPORTED CLINICAL SENSITIVITY AND SPECIFICITY OF AMPLICOR/PCR FOR *M. TUBERCULOSIS* IN SEVERAL LABORATORIES

		PCR	
Reference	No. Specimens	Sensitivity (%)	Specificity (%)
Beavis et al[2]	532	95	96
Bergmann et al[3]	956	79.4	99.6
D'Amato et al[8]	985	66.7	99.6
DeVallois et al[11]	784	90.9	100
Kirschner et al[22]	729	84.5	99.5
Moore et al[25]	1009	85	99.6
Schirm et al[33]	504	70.4	>98
Wobeser et al[42]	1480	79	99

Elsewhere in the world the test has been evaluated with a wide variety of samples for the diagnosis of tuberculosis. Sensitivities and specificities of AMTD and PCR on AFB smear-positive and -negative respiratory specimens have ranged from 71% to 100%. Tables 1 to 3 summarize these data. Researchers have reported that the test allows the reliable and rapid detection of tuberculosis in patients suspected of disease.

Vlaspolder et al[36] have applied the AMTD test to 138 nonrespiratory specimens. They report sensitivity, specificity, and positive and negative predictive values of 100%, 95.0%, 85.0%, and 100%, respectively, on the samples they tested, with the exception of pleural exudates, which were analyzed separately. One of five culture-positive samples and two culture-negative samples from pleural exudates were detected by AMTD. Vlaspolder et al[36] suggest the possibility of using alternative specimen-processing procedures to improve the performance of AMTD with pleural exudates, which may have inhibitors not present in respiratory samples.

Pfyffer et al[30] performed the largest study to date on nonrespiratory specimens. They tested 322 samples for *M. tuberculosis* using the ampli-

Table 3. COMPARISON OF GEN-PROBE AMTD AND AMPLICOR/PCR PERFORMANCE IN SEVERAL LABORATORIES

		AMTD		PCR	
Reference	No. Specimens	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Ichiyama et al[18]	422	100	99.3	97.8	98.9
Miller et al[24]	750	91	98.5	91	92.3
Vuorinen et al[37]	256	86.2	100	82.8	100
Zolnir-Dove et al[44]	281	94.0	94.4	96.0	94.8

fied AMTD test. Sensitivity and specificity compared with culture were 93.1% and 97.7%, respectively. Of special interest is their adaptation of the AMTD test for use with cerebrospinal fluid. They used a 500 μ sample instead of the 50 μ recommended for respiratory specimens, pretreated the sample with sodium dodecyl sulfate (SDS), and increased the amplification time to 3 hours. Six cases of tuberculous meningitis were diagnosed by AMTD. Five of the six cases were detected by culture; the sixth was culture-negative, but the patient had disseminated tuberculosis documented by culture-positive specimens from other sites in the body. Pfyffer et al[30] also tested 65 pleural fluids, of which two were positive by AMTD and culture, three were positive by AMTD only, and two were positive by culture only. Again it was demonstrated that pretreatment of the sample with SDS improved the results. SDS pretreatment might improve the performance of the test by eliminating the potential inhibitors present in pleural fluids.

Folgueira et al[15] describe the use of PCR for the rapid diagnosis of *M. tuberculosis* bacteremia by PCR. *M. tuberculosis* DNA was present in peripheral blood mononuclear cell samples from 9 of 11 HIV-infected patients and in 7 of 21 non–HIV infected patients with different forms of tuberculosis. All samples from HIV-infected patients with extrapulmonary tuberculosis were PCR-positive, as were 5 of 13 samples from non–HIV infected patients. Samples from 12 control patients with positive PPD tests were PCR-negative. These researchers concluded that only patients with active tuberculosis will be PCR-positive, and that PCR applied to blood samples will aid in the rapid diagnosis of tuberculosis, especially in HIV-infected patients.

Several researchers have questioned the relevance of a positive nucleic acid target amplification result on specimens from patients on therapy when culture is negative for *M. tuberculosis*.[7, 16, 21, 26, 43] It has been shown that viability of *M. smegmatis* can be assessed by amplification of rRNA using nucleic acid sequence-based amplification (another transcription based amplification system).[35] *M. smegmatis* that had been treated with antituberculosis drugs and shown to be nonviable were still detected by PCR, and not by NASBA. The results of the study by Van Der Vliet et al[35] imply that the decay of rRNA occurs more rapidly after cell death than does the degradation of genomic DNA. It should, therefore, be possible to determine the viability of *M. tuberculosis* by using another amplification method such as TMA to detect rRNA. A study was undertaken with the public health laboratory in Santa Ana, California (Orange County) to evaluate the persistence of rRNA in successfully treated patients.[26] Patients whose samples were AFB smear-positive within the first three samples submitted were enrolled in the study. All specimens submitted for analysis while patients were under care were included in the study and were tested by AFB smear, routine culture methods, and

AMTD. Results of smear and culture were unknown by the person performing the AMTD test. The AFB smear result was the last positive test result in 4% of cases, culture was last in 22%, and the AMTD test was last in 52% of cases. Fifty-six percent of patients had a period of shedding noncultivable organisms that were detected by AMTD. The noncultivable period lasted from 7 to 245 days. All three tests became reproducibly negative and remained negative during follow-up of up to 1 year. These data suggest that with time (and more experience), TMA may be applied to determine whether therapy is efficacious.

Schluger et al[32] evaluated the clinical utility of IS6110 PCR for the diagnosis of tuberculosis and concluded it is not useful in a routine setting based on its lack of specificity for diagnosis of active TB. As discussed previously, however, Moore et al believe that nucleic acid amplification tests will play a role in the routine diagnosis of tuberculosis. Kennedy et al[21] also believe that ". . . PCR is a promising method for assessing treatment response in pulmonary tuberculosis," and Yuen et al[43] agreed with this assessment of clinical utility of PCR. Hellyer et al[16] have concluded that the detection of *M. tuberculosis* DNA by target amplification will not be useful in therapeutic monitoring based on the inability of PCR to differentiate between live and dead organisms. This area is being further explored as researchers become more familiar with the power of nucleic acid amplification tests.

Nucleic acid amplification techniques such as TMA and PCR have been shown to be effective in the detection of *M. tuberculosis* from a variety of specimens. Preliminary data suggest that nucleic acid amplification methods that detect rRNA can be used on samples from patients undergoing therapy for tuberculosis. Much more work remains to be done in this area. Clearly, TMA and PCR aid in the rapid detection of tuberculosis in patients suspected of having the disease.

Obviously these methods are currently being used to detect tuberculosis. Nucleic acid amplification tests already aid in patient management and in making public health decisions. Improvements in sensitivity will render these tests even more useful than they are today. Ultimately the tests will be automated for greater ease of use. It is clear that improved detection and monitoring of tuberculosis will help in the fight to eradicate this disease and effectively manage the infected patient.

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SIMULTANEOUS MULTIANALYTE NUCLEIC ACID DETECTION FOR GASTROINTESTINAL BACTERIAL PATHOGENS USING GENESTAR TECHNOLOGY

Linxian Wu, PhD, Jana Coombs, BS, Sharon Malmstrom, BS, and Michael Glass, PhD

Acute diarrheal illnesses are very common among young children and infants.[5, 11] In developing countries, approximately 4 to 5 million deaths each year have been reported as the result of gastroenteritis.[5] In developed countries, gastrointestinal (GI) bacterial infections are also a major cause of morbidity and mortality.[5] Five bacterial species have been determined to play a significant role in bacterial gastroenteritis: *Salmonella* spp., *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC), *Campylobacter* spp., enterohemorrhagic *E. coli* (EHEC), especially 0157:H7, and *Yersinia enterocolitica*. Differentiation of these bacterial species is critical for physicians to select an adequate treatment regimen. The wrong treatment regimen may worsen the illness or even result in death.

Classical culture techniques are typically used in clinical laboratories to identify and differentiate the GI bacterial pathogens.[11] Such methods usually take a minimum of 18 hours to several days. It is evident that rapid identification methods are needed for clinical diagnosis of GI bacterial pathogens.

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