Practical Strategies for Performance Optimization of the Enhanced Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test

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The enhanced Gen-Probe Amplified Mycobacterium Tuberculosis Direct (MTD) test was evaluated using a combined set of 338 acid-fast smear-positive and smear-negative, respiratory and nonrespiratory clinical specimens received by the Massachusetts State Tuberculosis Laboratory from September 1999 through March 2002. Microbiological culture was used as the reference method; therefore, the sensitivity and specificity of the MTD test were calculated for culture-positive specimens only. The initial assessment indicated that the overall sensitivity, specificity, and positive and negative predictive values of the MTD test for all specimens grouped together were 62, 98, 99, and 68%, respectively. A detailed discrepancy analysis revealed that two major factors causing negative MTD results in specimens that were culture positive for M. tuberculosis complex were patient treatment with antituberculosis drugs prior to testing and the presence of inhibitory substances in the specimen. Based on these findings, a protocol for optimizing MTD test performance in this setting is proposed in which (i) specimens from patients taking antituberculosis medications are excluded from testing and (ii) all initially MTD-negative or MTD-equivocal specimens are subjected to testing for inhibitors. If this strategy was followed, the MTD test sensitivity would be at least 91%, a significant improvement over the initial sensitivity of 62%. Accordingly, the negative predictive value would increase from 68 to 91%.

Tuberculosis (TB) remains a public health problem in the United States, despite a continual decline in incidence during the past several years. Rapid detection of Mycobacterium tuberculosis plays a key role in TB control. For many years, the microbiological diagnosis of TB infection has been determined by acid-fast bacillus (AFB) smear microscopy or by culturing for mycobacteria. Both of these methods have well-known impediments: lack of specificity and low sensitivity for AFB smears and excessively long incubation time for culture (6, 10). Nucleic acid amplification (NAA) methods are sensitive and specific, and they allow for detection of mycobacterial DNA or RNA directly from the specimens before the culture results are available.

One of the NAA tests, the enhanced Amplified Mycobacterium Tuberculosis Direct (MTD) test (Gen-Probe Inc., San Diego, Calif.), uses transcription-mediated amplification and hybridization procedures to qualitatively detect M. tuberculosis complex (MTBC) rRNA. Since the introduction of the MTD test into diagnostic mycobacteriology, many publications have confirmed its high sensitivity and specificity. Compared to culture, the sensitivity of the MTD test ranged from 65 to 97% in different studies, whereas its specificity was always high (3–5, 8, 9). It is well recognized that variability in laboratory methods, TB prevalence, and prevalence of other mycobacterial diseases in a specific local area may all have a substantial impact on the predictive values of the MTD test. Consequently, it is important to collect information on the performance of the MTD test in local settings and to provide this information to clinicians.

MTD testing was integrated into the standard operations of the Massachusetts State TB Laboratory in 1997, shortly after its approval by the Food and Drug Administration (FDA). Tests were initially performed on a weekly basis (or more frequently upon urgent request) with smear-positive specimens from new patients; later, after FDA approval, tests were also performed on smear-negative specimens (if requested). In September 1999, the FDA approved an enhanced version of the MTD test which was simpler to perform, required less processing time, and used a larger sample volume to increase the sensitivity. The present report summarizes results obtained using this enhanced MTD test to evaluate respiratory and nonrespiratory specimens during the period from September 1999 through March 2002. We also describe our strategy for optimizing MTD test performance in this setting.

MATERIALS AND METHODS

Clinical specimens. All AFB smear-positive human respiratory specimens submitted from new patients between September 1999 and March 2002 to the Massachusetts State TB Laboratory for detection of mycobacteria were subjected to MTD testing and mycobacterial culture. A subset of AFB smear-negative specimens and nonrespiratory specimens was also tested, by special request of the ordering physician. Specimens tested prior to September 1999, when the manufacturer changed the test format, were excluded from consideration in this study. All specimens were processed within 24 h of receipt in the laboratory.

Specimen processing and culture. The entire amount of specimen received (approximately 0.5 to 5 ml) was decontaminated with 1% (final concentration) sodium hydroxide-N-acetylcyesteine and concentrated by centrifugation at 3,000 × g for 20 min, according to standard procedures (7). After centrifugation, the sediment was resuspended in 1.5 ml of phosphate buffer. Approximately 0.2 ml of the sediment was used to prepare an AFB smear for microscopy. An additional 0.3 ml of the sediment was placed into each of the three tubes with different media. In some cases with a highly positive AFB smear, an additional portion of the sediment could also be used for direct susceptibility testing. The remainder of the sediment was frozen at −70°C and reserved for MTD testing. Cultures were grown at 37°C and examined weekly. Isolates of mycobacteria...
were identified by DNA probes (AccuProbe; Gen-Probe, Inc.) or by conventional biochemical tests, according to standard protocol (7).

MTD test. Sediment for MTD testing and for inoculation of nutrient media were drawn from the same processed sputum sample. MTD testing was performed according to the manufacturer’s protocol, which requires the use of 450 μl of sediment for each reaction. Results were interpreted, as specified by the manufacturer, as follows. With an initial test, a result of >500,000 relative light units (RLU) was scored positive, <30,000 RLU was considered negative, and 30,000 to 500,000 RLU was considered equivocal. Upon retesting a specimen with an equivocal initial result, a result of ≥30,000 RLU was regarded positive, and <30,000 RLU was regarded negative.

Resolution of discrepancies. MTD test results were compared to the “gold standard” of mycobacterial cultures positive for MTBC or mycobacteria other than M. tuberculosis complex (MOTT). Specimens with negative (sterile) cultures were excluded from this analysis.

For specimens with an equivocal MTD test result, and for specimens with a negative MTD test result and a positive culture for MTBC, the ordering physicians were contacted retrospectively to determine whether the patient had been on anti-TB drug therapy for an extended period of time prior to MTD testing. These data were not always available at the time of testing. Treated patients were excluded from subsequent analysis, because testing of samples from such patients is not recommended by the MTD test kit manufacturer due to frequent false-negative results. This additional medication information was not sought for MOTT culture-positive specimens with negative MTD tests, since the focus of this study was on specimens with discordant MTD test and culture results.

For all other specimens with equivocal or false-negative MTD test results, a second smear-positive specimen from the same patient was tested, if available, provided that culture results of the first and second specimens were the same and that the two specimens were collected within 2 months of each other. If a patient had an initial specimen that was MTD test negative and a second specimen that was MTD test positive, that patient was considered to have a positive MTD test result. This definition of a positive MTD test result was chosen because M. tuberculosis may not be present in every specimen obtained from a patient with active TB disease, and the question of ultimate clinical importance is whether any specimen for a given patient yields a positive MTD test result. Repeat MTD testing of original specimens was not possible, since an additional 450 μl of sediment was not available for any of the specimens.

In cases in which no additional culture-positive specimens from a patient were available, MTBC culture-positive specimens with negative low equivocal MTD test results (<100,000 RLU) were analyzed for the presence of inhibitors. (This protocol is now modified so that all smear-positive specimens with negative MTD test results are automatically analyzed for the presence of inhibitors.) No smear-negative specimens could be retested, because follow-up testing for these specimens was not anticipated and the sediments had not been reserved. Specimens with discordant MTD test and culture results were not subjected to any additional testing.

Protocol for inhibitor detection. The conventional, manufacturer-recommended protocol for detection of MTD test reaction inhibitors (described in detail elsewhere [1]) requires a larger quantity of additional processed sputum sediment than is usually available in our laboratory. As such, a simple alternate protocol for detection of inhibitors or interfering substances was developed during the course of this study. This protocol also has the marked advantage of being able to distinguish M. tuberculosis from MOTT, even when inhibitors are present. Specifically, 450 μl of buffer was added to 50 μl of the original specimen sediment, which dilutes inhibitors to a degree to which they no longer have an impact on the MTD test reaction but leaves MTBC concentrations high enough for detection by the MTD test. Of this 1:10 diluted sample, 450 μl was transferred to the amplification tube and tested along with positive and negative controls in accordance with the manufacturer’s instructions. Interpretation of the results was as follows: for values of ≤50,000 RLU, the specimen is positive for MTBC and contains substances that inhibit amplification; for values of <50,000 RLU, the specimen is negative for MTBC.

Test performance and statistical analysis. The sensitivity of the MTD test was defined as the probability of having a positive MTD test result given that the specimen being tested was culture positive for M. tuberculosis. The specificity was defined as the probability of having a negative MTD test result given that the specimen being tested was culture positive for MOTT. Specimens with equivocal MTD test results were excluded from the numerators in these calculations but were included in the denominators. Positive predictive value (PPV) was defined as the probability that a specimen will actually be culture positive for M. tuberculosis given a positive MTD test result. Negative predictive value (NPV) was defined as the probability that a specimen will actually be culture positive for MOTT given a negative MTD test result. Chi-square and Fisher’s exact tests were performed using Epi-Info 2002 (2nd revision; Centers for Disease Control and Prevention, Atlanta, Ga., 2003).

RESULTS

Between September 1999 and March 2002, a total of 338 specimens from 290 individual patients were cultured and subjected to MTD testing (Table 1). A total of 151 specimens grew MTBC, 106 grew MOTT (including, by decreasing prevalence, Mycobacterium avium complex; M. kansasi, M. abscessus, M. xenopi, M. chelonae, M. gordonae, and M. marinum), and 81 specimens remained culture negative after 90 days of incubation. These culture-negative specimens were excluded from subsequent analysis.

Assuming that in a clinical laboratory the end point for assessment of test performance is quality patient diagnosis, we present the study data based on the number of patients and not on the number of specimens. Initial results of MTD testing for respiratory and nonrespiratory specimens isolated from 221 patients are presented in Table 2. For respiratory specimens, the MTD test correctly identified 73 of 120 MTBC culture-positive specimens (sensitivity, 61%) and 78 of 80 MOTT culture-positive specimens (specificity, 98%). PPV and NPV were 99 and 67%, respectively. For nonrespiratory specimens, the MTD test correctly identified 8 of 10 MTBC culture-positive specimens (sensitivity, 80%) and 11 of 11 MOTT culture-positive specimens (specificity, 100%). PPV and NPV were 100 and 85%, respectively. The sensitivities, specificities, and predictive values were not significantly different between respiratory and nonrespiratory specimens and were therefore subsequently calculated for both groups combined.

Further analysis was done for the 9 specimens with equivocal MTD test results and for the 41 specimens with discordant MTD test and culture results (Table 3). Twenty-three specimens (2 nonrespiratory and 21 respiratory) were excluded from the analysis because they were collected from patients who were on anti-TB drug therapy. For seven MTBC culture-positive patients (three with negative and four with equivocal MTD test results), additional specimens from the same patients were tested and were all found to be MTD test positive.

### Table 1. MTD test and culture results for all specimens, September 1999 to March 2002

<table>
<thead>
<tr>
<th>Organism or culture result</th>
<th>No. of specimens (no. of patients) with indicated MTD test result</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTBC</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>50 (41)</td>
</tr>
<tr>
<td>MOTT</td>
<td>103 (89)</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>4 (3)</td>
</tr>
<tr>
<td>M. chelonae</td>
<td>1 (1)</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>1 (1)</td>
</tr>
<tr>
<td>M. kansasi</td>
<td>5 (4)</td>
</tr>
<tr>
<td>M. avium complex</td>
<td>84 (72)</td>
</tr>
<tr>
<td>M. marinum</td>
<td>1 (1)</td>
</tr>
<tr>
<td>M. species</td>
<td>3 (3)</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>4 (4)</td>
</tr>
</tbody>
</table>

Culture negative          | 71 (61)  | 9 (7)    | 1         | 81 (69)   |

Total                     | 224 (191)| 103 (89)| 11 (10)   | 338 (290) |
A second specimen was also available for the MTD test-equivocal, MOTT culture-positive patient; this second specimen was MTD test positive. This false-positive MTD test result was further confirmed by failure to detect MTBC with the AccuProbe test (Gen-Probe, Inc.). Nine of the MTD test-negative specimens were subjected to inhibitor testing with our modified protocol, and all were found to be MTD test positive. The initial test results for the remaining 10 specimens (9 MTD test negative and 1 equivocal) could be neither confirmed nor disproved, due to insufficient quantity of the original specimen and lack of another specimen. Six of these 10 specimens were AFB smear negative.

In summary, of the original 50 discrepant or equivocal specimens, 23 false negatives could be attributed to patient use of anti-TB medications, 10 could not undergo confirmatory testing, and 16 were correctly recategorized after additional testing. Only one specimen had a confirmed false-positive MTD test result. If a protocol was followed in which (i) specimens from patients taking anti-TB medications were excluded and (ii) all initially MTD test-negative or -equivocal specimens (regardless of smear result) were subjected either to retesting or to inhibitor testing, then the MTD test sensitivity would be at least 91%, a significant improvement over the initial sensitivity of 62% (P value, 0.0001), while the specificity (98%) and PPV (98%) would remain essentially unchanged.

**DISCUSSION**

As previously reported by the Centers for Disease Control and Prevention, the sensitivity and specificity of the MTD test may differ among laboratories “as a result of unrecognized procedural differences and differences in cross-contamination rates” (2), as with other NAA tests. Although there have been several reports evaluating the MTD test in ideal, controlled settings (4, 5, 9), few studies have examined the performance of the test under routine testing conditions, such as those occurring in a TB diagnostic laboratory with a large specimen flow (3, 8). The results of MTD test evaluation at the Massachusetts State TB Laboratory presented in this report demonstrate how real-life sources of error in MTD testing (namely, inadvertent testing of specimens from patients taking anti-TB medications and having inadequate amounts of specimens for confirmatory testing) can have a significant impact on test performance. Clinicians should be aware that actual test performance may fall short of manufacturer expectations, and they should be familiar with how well the MTD test performs at their local laboratories. Likewise, laboratory workers should be aware of, and develop strategies to avoid, potential pitfalls that may adversely affect test performance.

One of the chief causes of false-negative MTD test results...
and low test sensitivity in our laboratory was the presence of MTD test reaction inhibitors in the tested specimens. Several reports have shown that respiratory specimens may contain inhibitory substances that can affect the activity of the MTD test enzymes and, ultimately, reduce the sensitivity of the test. The detection of such inhibitors can substantially improve the MTD test performance (1, 5). One widely used protocol for inhibitor detection (the protocol recommended by the MTD test manufacturer) (1) requires an additional 450-μl aliquot of sediment. In reality, this additional aliquot is unavailable most of the time, since the total volume of the sediment usually does not exceed 1.2 ml, and this volume must be distributed among the AFB smear, the tubes with growth media, and the first round of MTD testing. In contrast, the modified protocol that we have described above offers two important advantages. First, only a small quantity (50 μl) of processed sputum specimen is required for this assay. Second, any inhibitors that may be present are diluted to a degree to which they no longer have an impact on the MTD test reaction, while MTBC concentrations remain high enough for detection by the MTD test. As such, positive findings unambiguously indicate the presence of MTBC in the sample. Furthermore, the modified protocol may be carried out with the materials already provided in the MTD test kits. The use of this approach allowed us to successfully identify nine out of nine cases of MTD test reaction inhibition and to change the MTD test results from negative to positive. Depending on individual laboratory situations (e.g., regularity of MTD testing, percentage of specimens with suspected inhibitors, etc.), it may be more efficient to perform MTD testing on diluted and undiluted sample pairs simultaneously, thereby saving costs on labor, reducing the turnaround time, and improving the effectiveness of MTD testing.

Also contributing to the initially low sensitivity of the MTD test was the fact that almost half of the false-negative specimens were taken from patients treated with anti-TB drugs prior to testing. The MTD kit package insert states that the test may produce false-negative results if the patient has been on TB treatment for at least 7 days prior to specimen collection. The Massachusetts State TB Laboratory has made an effort to make known to the specimen providers the need for this information, and the laboratory requests that providers complete a special section of the test requisition form regarding such treatment. For a substantial number of specimens, however, this section is left blank when the specimen is submitted, and the MTD test is performed prior to confirmation of the patient’s medication status. The reporting of negative MTD test findings in such cases, especially for AFB smear-positive specimens, becomes cumbersome and requires lengthy explanations. Therefore, better communication between the laboratory and the surrounding medical community is needed and may substantially improve the reliability and usefulness of the MTD test.

False-negative MTD test results, from both of the major sources discussed above, can be minimized through implementation of our proposed test strategy, which improves MTD test sensitivity and NPV significantly. The strategy we describe is easily implemented, requiring very little effort and expense beyond those already associated with current practice; the only additional costs would be those associated with contacting physicians about patient medication use and with performing our modified test for MTD test inhibitors. In fact, the MTD test strategy we propose here has already been readily integrated into routine practice in our own laboratory and has been easy and inexpensive to maintain.

There were several limitations in our analyses that need to be considered. First, this analysis retrospectively explored reasons for discrepancies between MTD test and culture results. Retesting was done after culture results were known, on the basis of the culture result. Specifically, only specimens that were false negative by the MTD test were subjected to additional testing. It is possible that if all MTD test-negative specimens had been tested, as would have occurred in a nonstudy setting, additional false-positive MTD test results would have been observed, particularly among specimens with high MOTT bacterial loads (6). However, the false-positive rate is low (approximately 1 to 2% in our laboratory) and would not have a significant impact on our calculations of sensitivity, specificity, and predictive values (calculations not shown). Similarly, efforts were made to confirm the anti-TB medication status only for MTD test false-negative specimens. It is possible that some of the MTD test true-negative (MOTT culture-positive) patients were also taking anti-TB medications and should have been excluded from the test performance calculations shown in Table 4. However, even if one hypothesizes an extreme case in

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Organism identified by culture</th>
<th>No. of specimens with indicated MTD test result</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
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<tbody>
<tr>
<td>Initial</td>
<td>M. tuberculosis</td>
<td>No column data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOTT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Proposed</td>
<td>M. tuberculosis</td>
<td>No column data</td>
<td></td>
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<tr>
<td>MOTT</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Total</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

*The proposed protocol included exclusion of patients receiving anti-TB medications and retesting of specimens initially MTD test negative or equivocal.

*b In our study, we made no attempts to repeat MTD testing on most of these 89 true negatives. If these had been retested according to our proposed protocol, additional false positives may have occurred. However, with a false-positive rate of 2%, no more than two additional false positives would be expected, and the calculated values in this table would not change significantly.
which half of the MTD test-negative patients were excluded due to their being on anti-TB medications, there still would not be any significant changes in the results of our test performance calculations presented in Table 4 (calculations not shown).

A second limitation was that cultures positive for either MTBC or MOTT were used as the gold standards in evaluation of the MTD tests and that specimens with negative cultures were excluded from analysis. Arguably, a better gold standard might have been a combination of culture results and clinical characteristics. To explore this possibility, we performed a separate analysis of the 69 patients with culture-negative results by comparing MTD test results to the gold standard of clinical TB diagnosis (data not shown). These results were difficult to interpret. Specifically, we found no false-positive MTD test results but found that 9 out of 15 patients with clinically diagnosed TB had apparently false-negative MTD test results. However, four of these nine were taking anti-TB medications, and the other five had smear-negative culture-negative tissue biopsy specimens that very well may not have contained any M. tuberculosis bacteria. Based on our findings, we concluded that for evaluation of MTD test performance in patients with extrapulmonary TB, positive bacteriological culture results would be a better gold standard than clinical diagnosis alone.

In summary, the MTD test performs very well when used for routine clinical diagnosis. To ensure optimal test performance, efforts should be focused on developing strategies to minimize false-negative outcomes. In this report, we demonstrated how rigorous confirmation of patient medication status, combined with additional testing of initially MTD test-negative specimens, can substantially increase MTD test sensitivity and NPV without compromising high specificity and PPV. Our modified protocol for the detection of MTD test reaction inhibitors is simple, practical, and produces reliable MTD test results even when reaction inhibitors are present.

ACKNOWLEDGMENTS

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REFERENCES


