MOLECULAR DETECTION AND IDENTIFICATION OF *Mycobacterium Tuberculosis* COMPLEX AND FOUR CLINICALLY IMPORTANT NONTUBERCULOUS MYCOBACTERIA IN SMEAR NEGATIVE CLINICAL SAMPLES BY GENOTYPE MYCOBACTERIA DIRECT TEST

Running Title: Detection of mycobacteria in smear negative samples

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**Key words:** GenoType Mycobacteria Direct, *Mycobacterium tuberculosis*, nontuberculous mycobacteria, smear negative, molecular detection

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ABSTRACT

Although the sensitivity and specificity of nucleic acid amplification assays are high in smear positive samples, the sensitivity in smear negative and extrapulmonary samples still remains to be investigated in the diagnosis of suspicious tuberculosis cases. This study evaluates the performance of GenoType Mycobacteria Direct (GTMD) test for rapid molecular detection and identification of *Mycobacterium tuberculosis* complex and four clinically important nontuberculous mycobacteria (*M. avium*, *M. intracellulare*, *M. kansasii* and *M. malmoense*) in smear negative samples. A total of 1570 samples (1103 bronchial aspiration, 127 sputum and 340 extrapulmonary samples) were analyzed. When we evaluated the performance criteria in combination with the culture positive samples and/or clinical outcome of the patients, the overall sensitivity, specificity, positive and negative predictive values were found as 62.4, 99.5, 95.9% and 93.9% whereas 63.2, 99.4, 95.7 and 92.8% for pulmonary samples and 52.9, 100, 100 and 97.6% for extrapulmonary samples, respectively. Among the culture positive samples which had detectable mycobacterium species by the GTMD test, three samples were identified as *M. intracellulare* and one sample as *M. avium*. However, five *M. intracellulare* and a *M. kansasii* could not be identified by the molecular test and found as negative. GTMD test has been a reliable, practical and easy tool for rapid diagnosis of smear negative pulmonary and extrapulmonary TB to take effective precautions and initiate an appropriate treatment. However, low sensitivity level should be taken into consideration to make a decision of differentiation in suspicious patients until taking the culture result as negative and coming to a true picture of clinical outcome.
INTRODUCTION

Acid-fast smear examination and culture (liquid and solid based media, automated and semi-automated systems) are conventional techniques for microbiological detection of mycobacteria causing tuberculosis (TB) (14, 24). However, the sensitivity of smear has been variable (range of 20–80%) (1). In some smear negative cases, TB diagnosis might have been difficult to differentiate from a number of clinical pictures. Therefore, invasive medical procedures are necessary for sampling in patients from whom a qualified sputum sample cannot be obtained or in case of an extrapulmonary TB which requires histopathological, cytopathological and microbiological examination of tissue specimens and body fluids. Nucleic acid amplification (NAA) techniques have been used for early detection of causative mycobacteria in clinical samples and also to support clinical and radiological diagnosis in patients with presumptive TB infection (3, 19, 33). Although the high specificity values obtained both in smear positive and smear negative samples, the sensitivities of molecular assays are rather less in smear negative samples than the values found in smear positive samples and reported within a wide range between 50% and 80% in previous studies (7, 8). Recently, a combined system of Nucleic Acid Sequence Based Amplification (NASBA) (a registered trademark of BioMerieux) technique (6) and reverse hybridization method based GenoType Mycobacteria Direct (GTMD) (Hain Lifescience GmbH, Nehren, Germany) test has been used in routine practice to obtain better sensitivity and specificity and achieve early diagnosis. In several studies which compared GTMD with culture as the reference method and/or other NAA tests, the study populations for smear negative samples have not been sufficient for an accurate evaluation of this method in routine hospital conditions (9, 15, 20, 25, 28). In this study, it was intended to evaluate the
performance of the GTMD test for direct detection of *Mycobacterium tuberculosis* (MTB) complex and four clinically important nontuberculous mycobacteria (NTM) (*Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii*, *Mycobacterium malmoense*) in smear negative samples obtained from patients with suspicious TB infection. Among these nontuberculous mycobacterium species, *M. avium*, *M. intracellulare* and *M. kansasii* have been reported as the common agents causing disease worldwide and also in Aegean Region of Turkey, while *M. malmoense* has rarely been reported in most geographic regions of the world except northern Europe (2, 10).

**MATERIALS AND METHODS**

**Clinical samples**

A total of 1570 samples [1230 pulmonary (1103 bronchial aspiration and 127 sputum), and 340 extrapulmonary (210 urine, 29 pleural fluid, 57 gastric lavage, 18 cerebrospinal fluid (CSF), 8 biopsy and 18 various sterile body fluids or pus)] recovered from 1462 patients between June 2006 and September 2008 were analyzed in the Microbiology Laboratory of Izmir Training and Research Hospital for Chest Diseases and Chest Surgery which is a regional reference hospital for TB patients at the Aegean Coast of Turkey (West Anatolian Region). The patients included in the study did not receive an anti-tuberculous treatment within the last 12 months and were evaluated as having suspicious TB infection. Bronchial aspiration procedure was applied to the patients in whom a positive smear of acid-fast bacilli could not be obtained from sputum or qualified sputum could not be collected. All the clinical specimens were subjected to direct smear microscopy by a standard Kinyoun’s cold staining method (17) and evaluated by an experienced microbiologist.
Additionally, two separate direct smears from morning sputum were prepared for each patient at the following days in order to increase the sensitivity of smear examination for these samples. Standard *M. tuberculosis* H37Rv (ATCC 27294) strain was used for quality control in staining process. Mycobacterial cultivation, identification and molecular detection were applied to each sample as follows:

**Mycobacterial cultivation and identification**

Specimens other than sterile specimens, which contained normal bacterial flora such as sputum and nonsterile specimens were digested and decontaminated with the N-acetyl-L-cysteine–sodium hydroxide method (17) by using a commercial decontamination kit; Mycoprosafe (Salubris AS, Istanbul, Turkey). Mycobacterial cultivation was performed by BACTEC MGIT 960 system (BD Biosciences, Sparks, MD, USA) according to the recommendations of the manufacturer as described elsewhere (30) and in Lowenstein-Jensen slants (Salubris AS, Istanbul, Turkey). An acid-fast smear preparation by Kinyoun staining was also applied to each processed specimen. Differentiation of MTB and NTM were performed by conventional methods (17) and the BACTEC 460 p-nitro-α-acetylamino-β-hydroxypropiophenone (NAP) test (BD Biosciences, Sparks, MD, USA). Additionally, commercially available PCR based reverse hybridization (Line Probe Assay=LiPA) kits [GenoType Mycobacterium CM and AS (additional species) (Hain Lifescience GmbH, Nehren, Germany)] were used for further identification of atypical mycobacteria in species level (2).

**Molecular detection**

Rapid molecular detection and identification for each sample was performed with the GenoType Mycobacteria Direct (GTMD) test Ver. 4.0 (Hain Lifescience GmbH,
Nehren, Germany) according to the manufacturer’s recommendations as described elsewhere (9). The whole procedure was divided into three steps: RNA isolation from decontaminated patient specimen using a Magnetic Beads Capture method, amplification based on the NASBA technique and reverse hybridization. A master mix containing 15 µL of primer/nucleotide mix and 10 µL isolated RNA in a 0.5 mL screw cap tube was prepared for each reaction per sample. The amplification program and the hybridization procedure were carried out in the TwinCubator (Hain Lifescience, Nehren, Germany) with hybridization block. Hybridization procedure included chemical denaturation of amplification products, hybridization of single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate and an AP mediated staining reaction. Band patterns which occurred as a result of hybridization were evaluated visually by an evaluation sheet provided by the manufacturer.

**Evaluation and interpretation of results**

Results were evaluated visually on a reading chart according to the hybridization bands corresponding to the patterns of MTB and other mycobacteria on the strips. Interpretation and evaluation of test results and quality controls were applied as recommended by the manufacturer. Thus, a conjugate control band and an internal amplification control (IAC) band were observed for quality control and validation of the assay. The other five bands determined the reaction zones corresponding to specific probes belonging to MTB complex and four mycobacteria (\(M. avium\), \(M. intracellulare\), \(M. kansasii\) and \(M. malmoense\)). The molecular assays with the discrepant results according to the culture and which were considered as false positive or had cross-contamination were repeated using frozen aliquots of the
samples. The same result which was taken twice (either negative or positive) repeatedly was accepted as the final result in these discrepant assays. Specimens showing IAC inhibition by the GTMD test were also retested with the dilutions of 1/100 and 1/1000, if necessary.

RESULTS

GTMD test results in accordance with the culture and clinical evaluation

All the specimens tested were smear negative with Kinyoun acid-fast staining. Ratio of positive and negative results of culture and GTMD according to the sample type has been shown in Table 1. GTMD tests of 93 (5.9%) samples were repeated because of discrepancy. After evaluation of GTMD test results, 34 (2.2%) samples were culture negative and GTMD positive, whereas 86 (5.5%) samples were culture positive and GTMD negative (Table 2). GTMD negative and culture positive samples were also confirmed as TB by clinical evaluation. Among GTMD positive and culture negative samples, 28 (82.4%) samples were recovered from the patients who were evaluated as having TB infection and applied anti-TB treatment according to the clinical data and/or positive mycobacterial cultures taken from the other separate samples. Totally, amplification band was not observed in 74 samples and evaluated as inhibition. In these samples, 72 and 2 samples gave valid results with the dilutions of 1/100 and 1/1000, respectively. Representative image of mycobacterial band patterns on the strips of GTMD assay has been shown in Fig. 1.

Performance evaluation of GTMD test

When we evaluated the performance criteria in combination with the other culture positive samples and/or clinical outcome of the patients in discrepant results, the
overall sensitivity, specificity, positive and negative predictive values were 62.4, 99.5
95.9 and 93.9% whereas the values were 63.2, 99.4, 95.7 and 92.8% for pulmonary
samples and 52.9, 100, 100 and 97.6% for extrapulmonary samples. Sensitivity,
specificity, positive and negative predictive values for GTMD test in smear negative
clinical samples when compared with the culture and with the culture in combination
with the clinical diagnosis have been shown in Table 3.

Nontuberculosis mycobacterial test results
Among all samples, a total of eight nontuberculous mycobacterium species [M.
intracellulare (n=8), M. abscessus (n=2), M. avium (n=1), M. kansasii (n=1), M.
scrofulaceum (n=1), M. szulgai/intermedium (n=1) and two Mycobacterium spp.]
were isolated from the culture media of 16 pulmonary samples. Three
mycobacterium species isolated in 10 of these 16 pulmonary samples [M.
intracellulare (n=8), M. avium (n=1) and M. kansasii (n=1)] were detectable by the
GTMD test. However, GTMD test detected and identified M. intracellulare in three of
eight samples and a M. avium accurately, while M. intracellulare in the remaining five
samples and a M. kansasii could not be detected by the GTMD test and found as
negative. M. malmoense was not isolated from any of the clinical specimens included
in this study.

DISCUSSION
In general, conventional laboratory methods, including microscopic
examination by acid-fast staining and/or culture in solid and liquid media, automated
and semi-automated systems have been used in microbiological detection of
mycobacteria in microbiological diagnosis of active TB and other mycobacterial
infections. In smear positive cases, the patients almost always have classical TB symptoms and especially in high incidence settings, clinicians generally do not need an additional fast molecular method for detection of TB except for the conventional culture techniques and liquid based automated systems which have a reporting time as early as one week. On the other hand, acid-fast staining methods are known to have low sensitivity levels caused by technical and conditional variations such as duration of examination, experience of microbiologist, sample type, immune status of patient, stage of infection and application procedure. The sensitivity level of smear microscopy can change according to the acid-fast staining method as well (12, 16, 26). However, in our study, 89.7% (n=1103) of pulmonary specimens were bronchial aspirations which were thought to be as a criteria to increase the sensitivity of microscopic examination. In previous studies, bronchial aspiration by fiberoptic bronchoscopy was found to be a useful procedure for a definitive diagnosis in pulmonary TB by increasing smear and culture sensitivity (5, 23). It is known that the sensitivity level of microscopic examination is very low in extrapulmonary samples (4, 21, 22) and in general, invasive medical procedures which are difficult to repeat are necessary for sampling. In patients who could not have a certain diagnosis but showing symptoms of active TB infection, the usage of NAA tests has been recommended due to a testing and an interpretation algorithm (3). If smear and NAA test results are negative, clinical evaluation for a certain diagnosis is necessary to start anti-tuberculous treatment before resolution of the culture and additional test results. Recent studies showed that reliable molecular methods used in routine practice have led to make a decision about initiation of therapy for 20%-50% of TB cases or to reduce nonindicated TB treatment (11, 29).
In previous studies, the sensitivity and the specificity levels of the GTMD test were reported within the ranges of 92-97% and 90-100%, respectively (9, 15, 20, 28). The evaluation of discrepant results in accordance with the other positive cultures and clinical outcome increased the sensitivity and the specificity levels (9, 15). In this study, the evaluation of culture and clinical findings made a low level of increase in the sensitivity and specificity values, but a high level of increase was observed in positive predictive value (Table 3). Sensitivity of GTMD test was evaluated as low as the other studies using different NAA methods in smear negative samples whilst the specificity was high. However, when we compare with the previous studies investigating the diagnostic performance of GTMD, the present study differs from the others which have reported high sensitivity values (>90%) previously. This difference might have occurred by the presence of smear positive samples in the study population which has led to an increase in sensitivity levels. Additionally, to make a decision regarding the sensitivity level of GTMD test in a few number of smear negative patients, even if they were confirmed clinically, may lead to a misinterpretation in performance evaluation. It was recommended by Syre et. al. (28) that a larger study with more smear-negative, culture positive samples would be required to evaluate the GTMD test performance on smear negative samples. The present study has completed the absence of data regarding the efficient use of GTMD test in smear negative samples so far.

Patient population selected for NAA testing in TB can be variable according to the clinical findings, stage of the disease (i.e. anti-TB treatment), the incidence of mycobacteria in that region and the experience of the laboratory. Each TB control or treatment program should evaluate the overall costs and benefits of NAA testing in deciding the value and optimal use of the test in their setting. As the incidence of
causative NTM has been reported to be too low (<1%) in our region (2), it has been considered that a molecular detection test with a low sensitivity may not be necessary for identification of NTM directly from clinical samples. Thus, the present study has shown that molecular identification of NTM from culture in BACTEC 960 vial which would be much more sensitive would be sufficient for effective diagnosis in our setting. Although the present study and the current data (25) have determined a low performance of GTMD for detection and identification of four NTM directly from clinical specimens, the performance of GTMD test for atypical mycobacteria still needs to be evaluated with larger number of sample population.

Patients with smear-negative status are capable of transmitting MTB and smear negative cases appear responsible for at least one sixth of culture positive episodes of TB transmission (1, 13, 31). Diagnostic delay related with the patient or health provider leads to poor outcomes for individual patients and to increased spread of TB within the community. It was demonstrated that patients with extra-pulmonary or smear negative disease have significantly been more likely to be hospitalized and have experienced treatment delay (32). Smear-negative TB often requires assessing the response to antibiotic treatment as well as reviewing radiological investigations (27). The clinical variables (i.e. HIV infection, lymphadenopathy, cavitary lung lesion, history of contact with TB, persistent cough, weight loss) couldn’t be used ubiquitously because of the different epidemiological characteristics of each population and are not always the predictors of the treatment outcome (18). Improved diagnostic tools are necessary to introduce a curative resolution for smear negative TB patients at the proper time and also to protect the patients without TB from inappropriate, potentially toxic, treatment. Nonetheless, starting empirical treatment or waiting for the culture results and continuing or
stopping empirical treatment if a final culture showed negative results have still been
a conflict for the clinicians.

GTMD test was reliable, rapid (results were available in 5 hours), practical and
easy to apply in pulmonary and extrapulmonary TB in routine hospital conditions. The
findings have supported that a positive GTMD test in smear negative patients would
help to take effective precautions to prevent transmission among population and
initiate anti-TB treatment against a possible TB infection. However, low sensitivity
level should be taken into account to make a decision of differentiation in suspicious
patients. It would be better follow these patients and take precautions against until
taking the culture result as negative and coming to a true picture of clinical outcome.
New diagnostic tools for early detection of MTB complex and nontuberculous
mycobacteria in smear negative TB patients and in extrapulmonary cases are
needed to be investigated in further studies.

REFERENCES
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   C. L. Daley, and P. M. Small. 1999. Transmission of Mycobacterium TB from
   patients smear-negative for acid-fast bacilli. Lancet 353:444-449.
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   the use of nucleic acid amplification tests in the diagnosis of TB. M.M.W.R.
   58:7-10.


TABLE 1. Ratio of positive and negative results of culture and GTMD according to the sample type

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Culture&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GTMD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
</tr>
<tr>
<td>Bronchial aspiration</td>
<td>161 (14.6)</td>
<td>942 (85.4)</td>
</tr>
<tr>
<td>(n=1103)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum (n=127)</td>
<td>26 (20.5)</td>
<td>101 (79.5)</td>
</tr>
<tr>
<td>Urine (n=210)</td>
<td>6 (2.9)</td>
<td>204 (97.1)</td>
</tr>
<tr>
<td>Pleural fluid (n=29)</td>
<td>1 (3.4)</td>
<td>28 (96.6)</td>
</tr>
<tr>
<td>Gastric lavage (n=57)</td>
<td>6 (10.5)</td>
<td>51 (89.5)</td>
</tr>
<tr>
<td>CSF&lt;sup&gt;b&lt;/sup&gt; (n=18)</td>
<td>0</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Biopsy (n=8)</td>
<td>0</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Other samples&lt;sup&gt;c&lt;/sup&gt; (n=18)</td>
<td>1 (5.6)</td>
<td>17 (94.4)</td>
</tr>
<tr>
<td>Total (n=1570)</td>
<td>201 (12.8)</td>
<td>1369 (87.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Culture was done by BACTEC 960 system and in Lowenstein Jensen medium.

<sup>b</sup>CSF, cerebrospinal fluid

<sup>c</sup>Sterile body fluids (e.g. pericardial fluid, wound, ascites)
TABLE 2. Distribution of result patterns determined by GTMD test and culture among smear negative clinical samples

<table>
<thead>
<tr>
<th>Result Patterns</th>
<th>GTMD and culture positive (%)</th>
<th>GTMD and culture negative (%)</th>
<th>GTMD negative and culture positive (%)</th>
<th>GTMD positive and culture negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Type</td>
<td>Gallium-67 and In-111</td>
<td>Positive (%)</td>
<td>Negative (%)</td>
<td>Positive (%)</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>Gallium-67 and In-111</td>
<td>109 (8.9)</td>
<td>1012 (82.3)</td>
<td>78 (6.3)</td>
</tr>
<tr>
<td>(n=1230)</td>
<td>(8.9)</td>
<td>(82.3)</td>
<td>(6.3)</td>
<td>(2.5)</td>
</tr>
<tr>
<td>Extrapulmonary</td>
<td>Gallium-67 and In-111</td>
<td>6 (1.8)</td>
<td>323 (95.0)</td>
<td>8 (2.3)</td>
</tr>
<tr>
<td>(n=340)</td>
<td>(1.8)</td>
<td>(95.0)</td>
<td>(2.3)</td>
<td>(0.9)</td>
</tr>
<tr>
<td>Total (n=1570)</td>
<td>Gallium-67 and In-111</td>
<td>115 (7.3)</td>
<td>1335 (85.0)</td>
<td>86 (5.5)</td>
</tr>
<tr>
<td></td>
<td>(7.3)</td>
<td>(85.0)</td>
<td>(5.5)</td>
<td>(2.2)</td>
</tr>
</tbody>
</table>

*GTMD negative and culture positive samples were also confirmed as TB by clinical evaluation.*

*Among GTMD positive and culture negative samples, 28 (82.4%) samples were recovered from the patients who were evaluated as having TB infection and applied anti-TB treatment according to the clinical data and/or positive mycobacterial cultures taken from the other separate samples.*
TABLE 3. Sensitivity, specificity, positive and negative predictive values for GTMD test in smear negative clinical samples$^a$

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>58.3</td>
<td>63.2</td>
<td>97.0</td>
<td>99.4</td>
</tr>
<tr>
<td>Extrapulmonary</td>
<td>42.9</td>
<td>52.9</td>
<td>99.1</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>57.2</td>
<td>62.4</td>
<td>97.5</td>
<td>99.5</td>
</tr>
</tbody>
</table>

$^a$ Column A indicates the sensitivity, specificity, positive and negative predictive values compared with the culture, whereas column B indicates the values compared with the culture in combination with the clinical diagnosis.
FIG. 1. Representative image of mycobacterial band patterns on the strips of GTMD assay.

Lane 1: Scale for alignment, Lane 2: *M. intracellulare* positive,
Lane 3: *M. avium* positive, Lane 4: MTB complex positive,
Lane 5: Negative, Lane 6: Scale for alignment.