Detection of *Mycobacterium tuberculosis* in Clinical Specimens Other than Sputum by a Specific DNA Probe with Amplification of the Ribosomal RNA

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Abstract

Objective: To evaluate the Gen-Probe® Amplified Mycobacterium tuberculosis Direct Test (MTD) for detection of *Mycobacterium tuberculosis* in clinical specimens other than sputum, especially cerebrospinal fluid (CSF) and pleural fluid (PF) specimens.

Design: Six CSF, 17 PF and 23 bronchoalveolar lavage fluid (BALF) specimens that were submitted to our clinical laboratory for detection of mycobacteria were subjected to MTD and conventional smear and culture examinations.

Results: Of 6 smear-negative CSF specimens, two were positive in MTD and culture examination. In PF specimens, two of 15 smear- and culture-negative specimens were positive in MTD, while one smear-negative but culture-positive specimen was MTD negative. Three of 20 smear- and culture-negative BALF specimens were positive in MTD. Three BALF specimens in which mycobacteria other than the *M. tuberculosis* complex detected by culture were negative in MTD.

Conclusion: MTD is very useful for rapid detection of *M. tuberculosis* in clinical specimens other than sputum as well as sputum, and should be especially valuable for rapid diagnosis of tuberculous meningitis which requires prompt initiation of appropriate anti-tuberculosis drug treatment.

Introduction

Detection of *Mycobacterium tuberculosis* in clinical specimens is most important in making a diagnosis of tuberculosis. Therefore a rapid and sensitive method for detection of *M. tuberculosis* has been strongly desired, especially for diagnosis of tuberculous meningitis which requires prompt initiation of anti-tuberculosis drug treatment, tuberculosis complicating an immunocompromised host, and pulmonary tuberculosis which is difficult to differentiate from lung cancer.

A new rapid *M. tuberculosis* detection method, Gen-Probe® Amplified Mycobacterium Tuberculosis Direct Test (MTD), which provides direct detection of *M. tuberculosis* in clinical specimens without prior culturing, was recently developed by Gen-Probe Inc. (San Diego, Calif., USA). In this test, *M. tuberculosis* ribosomal RNA (rRNA) is amplified, and the amplification products (RNA) are hybridized with an *M. tuberculosis*-specific DNA probe to produce an RNA-DNA hybrid which is determined by hybridization protection assay. The sensitivity and specificity of MTD in detection of...
*M. tuberculosis* have been studied by several research groups, and its usefulness has been verified in sputum specimens\(^{1-5}\). We have reported two cases of tuberculous meningitis rapidly diagnosed by MTD\(^{6}\). There are few data, however, on pleural fluid (PF) specimens and bronchoalveolar lavage fluid (BALF) specimens.

In the present study, MTD and conventional smear and culture examinations were performed on CSF, PF and BALF specimens, and the sensitivity and specificity of detection of *M. tuberculosis* by MTD were compared with those obtained by culture on Ogawa egg medium or in liquid medium (MB-Check).

### Materials and Methods

**Clinical specimens:**
Six CSF, 17 PF and 23 BALF specimens were used. They were submitted in a blind fashion to the Clinical Laboratory of National Higashi-Saitama Hospital for detection of acid-fast bacteria.

**Processing of specimens, and smear and culture examinations:**
All specimens were decontaminated by the N-acetyl-L-cysteine-NaOH (NALC-NaOH) method. One to two milliliters of the specimen was mixed with an equal volume of NALC-NaOH solution (2% NaOH, 1.45% sodium citrate, 0.5% NALC) by applying on a mixer for 20 sec, and the mixture was allowed to stand for 15 min at room temperature. Phosphate buffer (0.067 M, pH 6.8) was added to the mixture to make a final volume of 10 ml, and after being mixed well, the mixture was centrifuged at 3,000 \(\times\) g for 15 min. After the supematant fluid was decanted carefully, the resulting sediment was suspended in 1 ml of the same buffer, and 0.1 ml quantities of the suspension were inoculated onto two Ogawa egg medium slants and 0.2 ml into an MB-Check bottle (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan). One-tenth milliliter of the suspension was spread on a glass slide and stained with Auramine for fluorescence microscopic examination. The remaining portion of each processed specimen was stored at \(-20^\circ\)C until used for MTD.

The inoculated cultures were checked for growth of mycobacteria for 8 wk, and mycobacteria grown in the cultures were identified by AccuProbe (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) or the DDH Mycobacteria (Kyokuto Pharmaceutical Co., Ltd., Tokyo, Japan) test.

**Procedure of MTD:**
The MTD (Gen-Probe) was performed according to the manufacturer’s directions. In brief, 50 \(\mu\)l of the processed specimen was placed in a lysing tube containing glass beads and specimen dilution buffer (200 \(\mu\)l) and ultrasonicated at 20 kHz for 15 min at room temperature. The lysate (50 \(\mu\)l), which contains free rRNA to serve as a template for *in vitro* replication, was transferred to a polypropylene tube which contained 25 \(\mu\)l of amplification reagent, including specific primers to hybridize with *M. tuberculosis* rRNA at defined sites, and 200 \(\mu\)l of oil reagent overlying the contents of the tube. The reaction mixture was heated at 95\(^\circ\)C for 15 min, and after the tube was cooled at 42\(^\circ\)C for 5 min, 25 \(\mu\)l of an enzyme reagent containing reverse transcriptase, RNA polymerase and nucleoside triphosphatase was added. The mixture was incubated at 42\(^\circ\)C for 2 hr to allow synthesis of cDNA of the target rRNA followed by synthesis of the cDNA duplex and finally synthesis of a large number of RNA transcripts having the target sequence. After that, 20 \(\mu\)l of termination reagent was added to the tube, and incubation at 42\(^\circ\)C was continued for 10 min.

Then, 100 \(\mu\)l of chemiluminescent acridinium ester-labeled *M. tuberculosis* complex-specific DNA probe reagent was added to the tube, which was then incubated at 60\(^\circ\)C for 15 min to allow hybridization. After addition of selection reagent (200 \(\mu\)l), the tube was vortexed and incubated for 10 more minutes. After the tube was cooled at room temperature for at least 5 min, the result was read in a luminometer. Samples producing signals greater than or equal to the cutoff value of 30,000

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relative light units (RLU) were considered positive, and signals less than this cutoff value were considered negative.

Results

Detection of *M. tuberculosis* in CSF by MTD:

As shown in Table 1, two of 6 smear-negative CSF specimens were MTD positive and in the cultures growth of *M. tuberculosis* appeared about 4 wk later. Thus, the two patients whose CSF specimens were MTD positive were rapidly diagnosed as having tuberculous meningitis, and prompt initiation of appropriate therapy against tuberculous meningitis and miliary tuberculosis resulted in good prognosis of the diseases. The other 4 patients, whose CSF specimens were MTD negative and culture negative, were later diagnosed as having multiple sclerosis (2 patients) or conventional bacterial meningitis (2 patients) according to their clinical records.

Detection of *M. tuberculosis* in PF by MTD:

MTD was compared with conventional smear and culture examinations for specificity and sensitivity of detection of *M. tuberculosis* in PF specimens (Table 2). Among 17 PF specimens, one was positive in MTD as well as in smear and culture examinations, one was negative in MTD and smear examination but positive in culture, and two of 15 smear- and culture-negative specimens were positive in MTD.

The PF specimen which was culture positive but considered to be MTD negative showed a signal of 22,381 RLU in MTD, which is close to the cutoff value of 30,000 RLU. The two patients whose PF specimens were MTD positive but smear- and culture-negative were diagnosed as having tuberculous pleuritis, since one patient’s PF specimen contained 63.41 IU of adenosine deaminase (ADA) per liter and the other patient occasionally expectorated culture-negative but smear-positive sputum.

By referring to the clinical records of the patients later, it was found that among 13 PF specimens, which were negative in MTD and in smear and culture examinations, 9 specimens were obtained from 3 patients with lung cancer, 2 patients with bacterial pleuritis, 1 patient with heart failure, 1 patient with pancreatitis or 1 patient with chylothorax (2 specimens), and the remaining 4 specimens were from patients clinically diagnosed as having tuberculous pleuritis.

Detection of *M. tuberculosis* in BALF by MTD:

As shown in Table 3, among 23 BALF specimens, two smear- and culture-positive specimens and one smear-negative but culture-positive specimen was MTD negative, and three of 20 smear- and

Table 1 Comparison of MTD with conventional smear and culture examinations in sensitivity of detection of *M. tuberculosis* in cerebrospinal fluid specimens

<table>
<thead>
<tr>
<th>Results of conventional examinations</th>
<th>Identification</th>
<th>MTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear negative</td>
<td>Culture positive</td>
<td>M. tuberculosis</td>
</tr>
<tr>
<td></td>
<td>on Ogawa medium</td>
<td>complex</td>
</tr>
<tr>
<td></td>
<td>and/or in MB-Check</td>
<td>2</td>
</tr>
<tr>
<td>Smear negative</td>
<td>Culture negative</td>
<td>0</td>
</tr>
</tbody>
</table>

*The 4 patients whose CSF specimens were negative in MTD as well as in smear and culture examinations were later found to be diagnosed as having multiple sclerosis (2 cases) or conventional bacterial meningitis (2 cases) by referring to their clinical records.*
Table 2: Comparison of MTD with conventional smear and culture examinations in sensitivity of detection of *M. tuberculosis* in pleural fluid specimens

<table>
<thead>
<tr>
<th>Results of conventional examinations</th>
<th>Identification</th>
<th>MTD</th>
</tr>
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<tbody>
<tr>
<td>Smear positive</td>
<td>Culture positive</td>
<td><em>M. tuberculosis</em> complex</td>
</tr>
<tr>
<td>on Ogawa medium</td>
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<tr>
<td>and/or in MB-Check</td>
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<td>Culture positive</td>
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<tr>
<td>on Ogawa medium</td>
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<td>and/or in MB-Check</td>
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<tr>
<td>Smear negative</td>
<td>Culture negative</td>
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</tbody>
</table>

*Signals in MTD were 22,381 RLU being close to cutoff value of 30,000 RLU.
**By referring to the clinical records of the patients later, it was found that among these 13 PF specimens, 9 specimens were from 3 patients with lung cancer, 2 patients with bacterial pleuritis, 1 patient with heart failure, 1 patient with pancreatitis or 1 patient with chylothorax (2 specimens), and the remaining 4 specimens were from patients clinically diagnosed as having tuberculous pleuritis.

Table 3: Comparison of MTD with conventional smear and culture examinations in sensitivity of detection of *M. tuberculosis* in bronchoalveolar lavage fluid specimens

<table>
<thead>
<tr>
<th>Results of conventional examinations</th>
<th>Identification</th>
<th>MTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smear positive</td>
<td>Culture positive</td>
<td>MOTT*</td>
</tr>
<tr>
<td>on Ogawa medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and/or in MB-Check</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smear negative</td>
<td>Culture positive</td>
<td>MOTT</td>
</tr>
<tr>
<td>on Ogawa medium</td>
<td></td>
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<tr>
<td>and/or in MB-Check</td>
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<tr>
<td>Smear negative</td>
<td>Culture negative</td>
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</table>

*MOTT, mycobacteria other than *M. tuberculosis*.
**The two BALF specimens were obtained from one patient at different times.
***Referring to the clinical records of the patients later, it was found that among these 17 BALF specimens, 14 specimens were from 7 patients with lung cancer or 7 patients with pneumonia and the remaining 3 specimens were from patients clinically diagnosed as having pulmonary tuberculosis.

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culture-negative specimens were MTD positive.

Mycobacteria appearing in the cultures of three MTD-negative BALF specimens were subjected to AccuProbe or the DDH Mycobacteria test and all were identified as mycobacteria other than the M. tuberculosis complex (MOTT). Among three patients whose BALF specimens were positive in MTD but negative in smear and culture examinations, two were found to occasionally expectorate culture-positive sputum, and one patient was found by transbronchial lung biopsy (TBLB) to have tuberculous pathological changes, i.e., infiltration of epithelioid cells including Langhan's giant cells and granulomatous lesions, in the lung. Therefore these three patients were considered to have pulmonary tuberculosis.

By referring to the clinical records of the patients later, it was found that among 17 BALF specimens, which were negative in MTD and smear and culture examinations, 14 specimens were from 7 patients with lung cancer or 7 patients with pneumonia and the remaining 3 specimens were from patients clinically diagnosed as having pulmonary tuberculosis.

Discussion

Since the rate of M. tuberculosis detection in CSF of tuberculous meningitis patients and PF of tuberculous pleuritis is low, the diagnosis in most of these cases has been based on characteristics of the CSF or PF, including cellular components and biochemical data, and the clinical course such as symptomatic responses to anti-tuberculosis drugs.

The situation regarding tuberculous meningitis is urgent, since delay of the diagnosis that results in delay of initiation of appropriate treatment will threaten the patient's life or bring about serious sequelae. The fear of such dangers leads to a "carpet-bombing", blind administration of combination of an antibiotic, anti-tuberculosis drug, fungicide or virucide without knowing the etiology, which can result in serious side effects. Therefore, a rapid, highly sensitive and highly specific method of detecting M. tuberculosis in the CSF has been earnestly desired. In this study, though the number of cases was small, two smear-negative CSF specimens showed positive results in MTD, and thus these two patients were rapidly diagnosed as having tuberculous meningitis by MTD. It is worth noting that these patients achieved a favorable outcome in response to rapid initiation of an appropriate treatment for tuberculous meningitis. The two CSF specimens yielded growth of M. tuberculosis in cultures about 4 weeks later. Thus, if the initiation of the treatment for tuberculous meningitis had been suspended until the positive culture results were obtained, these two patients must have had a sad fate.

Elevation of the ADA value in PF has been used for diagnosing tuberculous pleuritis, and this slightly increased the reliability of diagnosis in the absence of detection of M. tuberculosis in PF. The ADA value, however, only reflects the response of host cells such as T-cells and cannot directly prove infection by M. tuberculosis. A highly sensitive, highly specific and rapid method for detection of M. tuberculosis in PF has been anticipated. In the present study, of the 15 smear- and culture-negative PF specimens, two specimens from two patients who were suspected of having tuberculous pleuritis based on the ADA value in PF or the positive results in smear examination of sputum were positive in MTD. Furthermore, one PF specimen, which was culture positive for M. tuberculosis but considered to be MTD negative, showed a signal of 22,381 RLU in MTD close to the cutoff value of 30,000 RLU. These results suggest that MTD is not only a rapid method for detecting M. tuberculosis in PF but its sensitivity and specificity are higher than smear and culture examination as well. Although the RLU value of the MTD-negative, culture-positive specimen described above suggested the necessity to re-examine the specimen by MTD and to reassess the cutoff value in MTD, the specimen was not retested by MTD.
It is said that the spread of bronchoscopy improved the rate of detection of *M. tuberculosis* in pulmonary tuberculosis, and consequently the accuracy of the diagnosis of pulmonary tuberculosis. However, in the 23 BALF specimens investigated *M. tuberculosis* was not detected by conventional smear and culture examinations, and only MOTT was detected in three specimens by culture examination. A possible reason for this is that in our hospital, bronchoscopy is seldom performed in cases which were diagnosed as pulmonary tuberculosis either by the result of conventional smear and culture examinations, X-ray- or computed tomography-examination of the chest, or from clinical features.

Of the 20 BALF specimens which were negative in conventional smear and culture examinations, specimens from three patients were positive in MTD, and the three patients were later verified as having pulmonary tuberculosis based on the results of sputum culture or TBLB. Thus, these results indicate that MTD is a rapid and sensitive method for detecting *M. tuberculosis* in BALF also. On the other hand, three BALF specimens in which MOTT was detected by a culture examination were MTD negative. This results, together with the findings that CSF, PF and BALF specimens from patients with diseases other than tuberculosis were all MTD negative, demonstrated that MTD is specific for the *M. tuberculosis* complex.

In this study, CSF, PF, and BALF specimens were processed by the NALC-NaOH method in the same way as that used for sputum specimens and then tested by MTD. It is to be noted that the results of MTD of CSF, PF and BALF specimens processed in such a way were quite satisfactory in rapidity, sensitivity and specificity of detecting *M. tuberculosis*.

**Acknowledgment**

We thank Chugai Pharmaceutical Co. Ltd. for providing kit the used in this study.

**References**

核酸（rRNA）増幅を応用した結核菌直接検出法による
喀痰以外の臨床検体中の結核菌検出成績

国立療養所東埼玉病院内科
大角 光彦 豊田 丈夫 川城 丈夫 青柳 昭雄

要 旨
結核菌の ribosomal RNA (rRNA) を増幅し、
検体より直接結核菌を検出する Gen-Probe®
Amplified Mycobacterium Tuberculosis Direct
Test (MTD) が開発され、喀痰については有用性
が高く評価され、既に広く実施されている。我々
は胸水17検体、膿汁6検体、気管支肺胞洗浄液
(BALF) 23検体について従来の塗抹培養検査と
MTD を施行し、その成績を比較検討した。

培養で結核菌陽性で、MTD が陰性であったも
のは胸水で1検体存在した。従来法では結核菌が
検出されなかった胸水の2例、BALF の3例が
MTD 陽性を示し、その臨床像と合わせて結核症
の確定診断が可能であった。また MTD 陽性で
あった膿汁の2検体は、いずれも塗抹陰性で、培
養で約4週間後に結核菌が検出されており、
MTD により結核性膿腫炎の迅速な診断が可能で
あった。