Evaluation of a modified direct agar proportion method for testing susceptibility of *Mycobacterium tuberculosis* from MGIT samples

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**Background/Purpose:** The emergence of resistance to anti-tuberculosis (TB) drugs has become an obstacle to effective TB control. Thus, there is an urgent need to identify patients and initiate adequate treatment for drug-resistant cases in a timely manner. The BACTEC MGIT 960 system is well known for its rapid culturing time, and is in widespread use in Taiwan. In this study, we evaluated the possibility of replacing the traditional indirect agar proportion method with a modified direct agar proportion method (MDAPM), as a technique for rapid testing the drug susceptibility of *Mycobacterium tuberculosis* without additional cost.

**Methods:** In this study, 432 positive MGIT 960 samples that were identified as *M. tuberculosis* complex using the MeDiPro *M. tuberculosis* Antigen Rapid Test or the Cobas Amplicor MTB test were evaluated. Each sample was tested separately by the MDAPM and indirect agar proportion method, between July 2008 and December 2008, to compare the consistency and total turnaround time.

**Results:** Four first-line anti-TB drugs—rifampin, isoniazid, ethambutol, and streptomycin—were tested. For the MDAPM and indirect agar proportion method, the respective consistencies for each drug were 99.31%, 98.38%, 98.38%, and 97.22%. Our results also indicated that the MDAPM leads to an average saving in working time of 2 weeks, compared with the traditional indirect agar proportion method.
Introduction

Tuberculosis (TB) is a contagious disease and continues to be a significant problem in many parts of the world. In Taiwan, morbidity and mortality rates for TB have remained high in recent years. In addition to the high health burden of TB on society, the emergence of resistance to anti-TB drugs has become a major public health problem and an obstacle to effective global TB control. Meanwhile, the spread of multidrug-resistant isolates that are resistant to the two most important first-line anti-TB drugs—isoniazid and rifampin—could also have serious epidemiological consequences and implications for disease control programs. This is particularly worrisome as there is no effective cure for some multidrug-resistant TB (MDR-TB) isolates.

Drug-resistant TB is more difficult to treat than drug-susceptible TB. In Taiwan, the prevalence of MDR-TB among new TB patients and previously treated TB patients was 1% and 6.2%, respectively, as reported by the Taiwan Centers for Disease Control. In our previous surveillance study, we also found that cases of drug-resistant Mycobacterium tuberculosis were rapidly increasing in our hospital. Therefore, approaches that result in a shorter turnaround time for mycobacterial isolation are crucial for rapid identification of patients with active TB. In addition, further determination of drug susceptibility of M. tuberculosis complex (MTBC) isolates, for quick initiation of appropriate antibiotic therapy, would facilitate earlier treatment and prevention of TB transmission.

Recently, with the aim of shortening the time needed for detection of MTBC, the fully automated and nonradiometric BACTEC MGIT 960 system has been widely adopted in Taiwan. Compared with the conventional solid medium, the BACTEC MGIT system was found to have advantages in terms of increasing the recovery of mycobacteria and shortening the turnaround time.

Differentiation of MTBC from nontuberculous mycobacteria (NTM) is important from a clinical viewpoint. Early and reliable identification of MTBC facilitates timely and appropriate treatment initiation, while avoiding unnecessary treatment in cases of environmental NTM. In recent years, major advances in the understanding of the genetic structure of mycobacteria have been achieved. As a result, several genetic probes and nucleic acid amplification methods have been developed and are available as commercial kits for direct detection and identification of MTBC in clinical specimens. These methods facilitate reductions in diagnostic time and are being increasingly applied in medical laboratories that perform TB diagnosis. For example, the Cobas Amplicor M. tuberculosis (MTB) test (Roche Diagnostics, Basel, Switzerland) is designed to amplify a 584-bp segment of the 16S-rRNA gene using a polymerase chain reaction. This test enables the rapid and accurate identification of MTBC in positive BACTEC MGIT 960 cultures, as indicated by our previous study. Another culture confirmation test that uses lateral flow immunochromatographic assay to detect MTBC-specific antigen—the MeDiPro M. tuberculosis Antigen Rapid Test—provides high sensitivity and specificity for the differentiation of MTBC from NTM in positive BACTEC MGIT 960 cultures (described in our previous study). In the present study, we performed the Cobas Amplicor MTB test and the MeDiPro M. tuberculosis Antigen Rapid Test for the rapid identification of MTBC.

In addition, a rapid and reliable drug susceptibility testing is also crucial because of the increasing incidence of drug-resistant MTBC isolates. The traditional indirect agar proportion method using the source of the inoculum made from the primary isolation medium usually takes from 3 weeks to 4 weeks to obtain susceptibility results. This method may therefore carry potential dangers for patients, health workers, and the community at large owing to a prolonged detection period. Thus, rapid methods for TB diagnosis, followed by prompt evaluations for determination of drug susceptibility, are urgently required for the timely initiation of appropriate antibiotic therapies. As M. tuberculosis grows more rapidly in liquid medium than on solid medium, and because direct susceptibility testing can be done immediately without prior subculture of M. tuberculosis on solid medium, the time required to identify susceptibility patterns is significantly reduced. We therefore evaluated the modified direct agar proportion method (MDAPM) as a method for achieving faster turnaround time for drug susceptibility test of MTBC.

Methods

Patients

In this study, 432 BACTEC MGIT 960 samples, identified as MTBC by the Cobas Amplicor MTB test or the MeDiPro M. tuberculosis Antigen Rapid Test, were examined. Each sample was tested separately between July 2008 and December 2008 using the MDAPM and indirect agar proportion methods in order to compare the consistency and the total turnaround time of these two methods.

Specimen processing

We processed specimens with standard N-acetyl-L-cystein-NaOH procedures, and these decontaminated specimens were inoculated into two media: an MGIT culture tube (Becton, Dickinson, and Company, Sparks, MD, USA) and a
Löwenstein–Jensen slant medium (L-J; Becton, Dickinson, and Company).19

Culture systems

Solid media method
The L-J slant medium was inoculated with three drops of decontaminated specimen. Each L-J slant medium was incubated at 37°C in a 10% CO2 atmosphere for 8 weeks or until mycobacterial colonies were observed. All positive cultures or suspicious colonies on L-J slant medium were checked by Ziehl–Neelsen staining to confirm the presence of acid-fast bacilli (AFB). If mycobacteria were detected on L-J slant medium, they were identified by conventional biochemical tests.19

BACTEC MGIT 960 culture systems
Five hundred microliters of each decontaminated specimen was inoculated into an MGIT culture tube containing both 10% OADC (oleic acid, albumin, dextrose, and catalase; Becton, Dickinson, and Company) and 0.8 mL of PANTA antimicrobial supplement (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Becton, Dickinson, and Company) according to the manufacturer’s instructions.17 The MGIT culture tube contained a fluorescent sensor to detect the oxygen concentration in the culture medium. The level of fluorescence corresponded to the amount of O2 consumed by the organisms in the inoculant. In turn, this was proportional to the numbers of bacteria present. When a certain level of fluorescence was reached, the instrument indicated a positive result for the culture.20

After inoculating each tube with 0.5 mL of the processed specimen, we incubated the tubes in the BACTEC MGIT 960 Culture System at 37°C. The instrument automatically read the MGIT tubes every hour, for a period of 6 weeks or until a positive result was detected. The AFB in the MGIT positive samples were examined by Ziehl–Neelsen staining, followed by the Cobas Amplicor MTB test or MeDiPro M. tuberculosis Antigen Rapid Test for rapid identification of MTBC. The positive MGIT cultures were then further cultured onto the L-J slant medium for species identification by conventional methods.2

Identifying mycobacteria

Conventional methods
Conventional methods for identifying mycobacteria were based on colony morphology, colony pigmentation, rate of growth on the L-J slant medium, and results of biochemical tests (such as the niacin test, nitrate reduction test, Tween 80 hydrolysis at 7 days and 14 days, urease, 5% NaCl tolerance, and arylsulfatase at 3 days and 14 days).19 MTBC could be readily identified by its rough, nonpigmented pattern, a positive niacin test, and a positive nitrate reduction test.

The Cobas Amplicor MTB test
The Cobas Amplicor MTB test was performed according to the manufacturer’s instructions. The procedure consists of four steps: (1) specimen preparation, (2) target amplification by polymerase chain reaction, (3) hybridization of amplified products to oligonucleotide probes, and (4) detection of the probe-bound amplified products. The whole process, with the exception of sample preparation, was automated by the Cobas Amplicor instrument.

One hundred microliters of each decontaminated sample was mixed with 500 µL sputum specimen wash solution (RW) and centrifuged at 13,000g for 10 minutes. The supernatant was discharged, 100 µL of lysis reagent (RL) was added, and the tubes were incubated at 60°C for 45 minutes. After incubation, 100 µL of neutralization reagent (RN) was added and 50 µL of the mixture was transferred to an amplification tube containing 50 µL of master mix, which included primers for MTBC, nucleotides, the internal control, and DNA polymerase. Carryover contamination was prevented by the incorporation of dUTP (2’-deoxyuridine-5’-triphosphate) in place of dTTP (thymidine 5’-triphosphate) in the amplification reaction, and utilization of uracil-N-glycosylase (AmpErase) to cleave any amplicon carried over from previous reactions.

Amplification was accomplished with the built-in thermocycler. The internal control of amplification in the Cobas Amplicor MTB test contains a fragment of plasmid DNA with primer-binding regions identical to those of the MTBC target sequences. A unique probe-binding region differentiates the internal control from the target amplicon. The internal control was introduced into each amplification reaction and coamplified with the possible target DNA in the tested specimen. If a negative result for the internal control was obtained owing to the presence of inhibitory substances, the test was repeated and analyzed using both undiluted and 10-fold diluted samples in the neutralization solution.

After amplification, the amplified nucleotide sequences of MTBC and the internal control were automatically detected with target-specific DNA probes, and measurements were obtained with the built-in spectrophotometer at an absorbance of 660 nm (A660). MTB-negative and MTB-positive controls were included in each run.

Specimens with an absorbance greater than 0.35 were considered positive, regardless of the internal control result, whereas those with an absorbance less than 0.35 combined with an internal control absorbance greater than 0.35 were considered negative. However, specimens with an absorbance less than 0.35 combined with an internal control absorbance less than 0.35 were regarded as inconclusive.17

The MeDiPro M. tuberculosis antigen rapid test
The MeDiPro M. tuberculosis Antigen Rapid Test (Formosa Biomedical Technology Corporation, Taiwan) was performed according to the manufacturer’s instructions. This test depends on contacting the sample on a lateral flow nitrocellulose membrane, which is coated with the primary anti-TB antibody. The antigens bind specifically to gold-conjugated capture antibody.18 In brief, approximately 150 µL positive liquid culture fluid was placed into the loading sample zone, and the result was read after 20 minutes. The appearance of two colored bands, one in the test zone and the other in the control zone, indicated a positive result. The presence of one colored band in the control zone and no colored band in the test zone indicated a negative result. If no colored band was detected in the control zone, the result was considered invalid, and a repeat test of the sample with a new kit was deemed necessary.
Drug susceptibility testing

The agar proportion method is currently being used by most laboratories that perform mycobacterial susceptibility testing in Taiwan,19,20 MTBC suspension was inoculated onto Middlebrook 7H10 agar that contained anti-TB drugs; agar that did not contain any drug was also used for control experiments. The drug concentrations in the medium were either 0.2 μg/mL or 1.0 μg/mL for isoniazid (INH), 1.0 μg/mL for rifampin (RIF), either 5 μg/mL or 10 μg/mL for ethambutol (EMB), and either 2.0 μg/mL or 10 μg/mL for streptomycin (SM). These inoculated culture media were incubated at 37°C for 3 weeks. For a test to be considered valid, between 50 and 150 individual colonies that were countable had to be obtained on the drug-free medium. The numbers of colonies observed on the drug-containing medium were then compared with the numbers on the drug-free medium. The proportion of bacilli resistant to a given drug was determined and expressed as a percentage of the total population tested. This proportion was set at 1%, because the therapeutic agent was no longer effective when more than 1% of the mycobacterial population was resistant to the critical concentration of one of the tested drugs.

Traditional indirect agar proportion method

The drug susceptibility test was carried out according to the instructions of the Clinical and Laboratory Standards Institute.41 The inoculum source for the traditional indirect agar proportion method came from L-J slant medium that was identified as MTBC. For sample inoculation, suspensions of MTBC equivalent to a McFarland 1.0 concentration were prepared. From this preparation, serial dilutions corresponding to two levels—1:10^2 (level 1) and 1:10^4 (level 2)—of the McFarland 1.0 suspension were prepared. The drug-containing and drug-free control Middlebrook 7H10 agars were inoculated with three drops of either of these 1:10^2- or 1:10^4-diluted suspensions.

MDAPM

The source of the inoculum for the MDAPM came from positive MGIT samples. Once the presence of AFB was confirmed by Ziehl–Neelsen staining in positive MGIT samples, the Cobas Amplicor MTB test or the MedDiPro M. tuberculosis Antigen Rapid Test was performed for rapid identification of MTBC. If MTBC was identified in positive MGIT samples, the MDAPM for drug susceptibility testing of MTBC was performed.

Each positive MGIT sample was tested with the MDAPM within 24 hours of the instrument having indicated a positive signal. For the inoculation procedure, the positive MGIT sample suspension was used (which corresponded to level 1) for MDAPM testing; the 1:10^2-diluted suspension was also tested (which corresponded to level 2).

The drug-containing and drug-free control Middlebrook 7H10 agars were inoculated with three drops of either the original or the 1:10^2-diluted positive MGIT sample (which corresponded to level 1 or level 2, respectively).

Results

The objectives of this study were twofold: (1) to compare the turnaround time for obtaining drug susceptibility results by the MDAPM and the traditional indirect agar proportion method, and (2) with regard to drug susceptibility, to compare the MDAPM with the traditional indirect agar proportion method (for the same isolates of MTBC) for RIF, INH, EMB, and SM.

The mean turnaround time by the MDAPM was 36.0 days, which was shorter than the mean of 49.9 days by the traditional indirect agar proportion method (Table 1). The results indicated that the MDAPM reduced working time by 2 weeks on average, compared with the traditional indirect agar proportion method. In addition, a high level of concordance was observed between the MDAPM and the traditional indirect agar proportion method, based on drug susceptibility testing data. For four first-line anti-TB drugs—RIF, INH, EMB, and SM—the tests showed respective consistencies of 99.31%, 98.38%, 98.38%, and 97.22% (Table 2).

Discussion

The accurate and prompt detection of drug resistance is a key priority in TB disease control programs, as this enables the initiation of appropriate treatment and also supports surveillance and monitoring of drug resistance. Drug susceptibility testing based on growth inhibition in drug-free and drug-containing media, with subsequent macroscopic observation, is technically demanding; moreover, this approach is associated with a long turnaround time when the traditional indirect agar proportion method is used. Although the time usually required to obtain drug susceptibility test results (more than 3 weeks) may be considered disadvantageous by physicians for the purpose of case management, it remains the most popular routine test in medical laboratories that perform mycobacterial susceptibility testing in Taiwan.19 Nevertheless, the global resurgence of TB infection and the increase in MDR-TB strains have led to a concomitant increase in the demand for obtaining drug susceptibility test results at an earlier stage. The effectiveness of automated drug susceptibility test methods for testing of mycobacterial susceptibility to first-line anti-TB drugs has been evaluated extensively.42 However, these methods are technically complex, expensive, and are not in common use in most medical laboratories in Taiwan. For the detection of drug-resistant strains, there is a need for the development and implementation of new drug susceptibility test methods.

<table>
<thead>
<tr>
<th>DST method</th>
<th>TAT (d)</th>
<th>% Reported by days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Range</td>
<td>30</td>
</tr>
<tr>
<td>MDAPM</td>
<td>36.0</td>
<td>20–60</td>
</tr>
<tr>
<td>IDAPM</td>
<td>49.9</td>
<td>27–71</td>
</tr>
</tbody>
</table>

- **a** The turnaround time shown in days starting from date of specimen processing to date that drug susceptibility test report was obtained.
- **b** The cumulative percentages of drug susceptibility test results.
that have a high level of accuracy while still being cost-effective, sustainable, and accessible.

In order to significantly improve the turnaround time for case management, the detection of growth inhibition at the earliest possible stage is of paramount importance. It is well known that MTBC grows more rapidly in liquid medium than on solid medium, in consequence of direct susceptibility testing, can be done immediately without prior subculture of M. tuberculosis on solid medium, which means that the time needed to identify susceptibility patterns can be reduced. In this study, we evaluated a new susceptibility test (based on modification of the direct agar proportion method), the MDAPM, which uses positive MGIT samples as the inoculum source. In addition, we evaluated the level of concordance for the MDAPM and the traditional indirect agar proportion method (for the same isolates of MTBC) for the assessment of mycobacterial susceptibility to the drugs RIF, INH, EMB, and SM, when the inoculum source came from positive L-J slant medium. Compared with the traditional indirect agar proportion method (used as a standard), our results indicated respective consistencies between the methods indicates that the drug susceptibility test results generated by the MDAPM and the traditional indirect agar proportion are reliable with regard to resistance and susceptibility.

Despite the high level of concordance, we noted that with the MDAPM, six (1.63%) INH-susceptible isolates were misidentified as resistant (of 368 true INH-susceptible isolates), two (0.5%) RIF-susceptible isolates were misidentified as resistant (of 402 true RIF-susceptible isolates), seven (1.65%) EMB-susceptible isolates were misidentified as resistant (of 424 true EMB-susceptible isolates), and three (0.8%) SM-susceptible isolates were misidentified as resistant (of 373 true SM-susceptible isolates). By contrast, with the MDAPM, one (1.56%) INH-resistant isolate was misidentified as susceptible (of 46 true INH-resistant isolates), nine (15.25%) SM-resistant isolates were misidentified as susceptible (of 59 true SM-resistant isolates). These results are all summarized in Table 2. The reasons for the discrepancies when using the MDAPM and the traditional indirect agar proportion method were not clear. However, the lack of standardization of the inoculum and representative of the M. tuberculosis population in MGIT samples were the possible main reasons. Although the discrepancies were few, they would need to be addressed in future investigations.

The mean turnaround time for the MDAPM was 36.0 days, which was lower than that for the traditional indirect agar proportion method (49.9 days; Table 1). Furthermore, the MDAPM for the testing of drug susceptibility of MTBC can be performed at no additional cost. This suggests that MDAPM can provide an accurate and rapid method for testing mycobacterial susceptibility to the drugs RIF, INH, EMB, and SM. In conclusion, the MDAPM significantly reduces the turnaround time without affecting diagnostic accuracy, in addition to offering an efficient and cost-effective diagnostic procedure. This would be a potential benefit for TB diagnostic strategies in medical laboratories equipped with the MGIT 960 system.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

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References


Table 2. Assessment of concordance for the modified direct agar proportion method (MDAPM) and the indirect agar proportion method (IDAPM) for drug susceptibility test

<table>
<thead>
<tr>
<th>Antibiotic (µg/mL)</th>
<th>MDAPM</th>
<th>IDAPM</th>
<th>Overall agreementa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>INH (0.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>63</td>
<td>6</td>
<td>98.38</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>362</td>
<td></td>
</tr>
<tr>
<td>RIF (1.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>29</td>
<td>2</td>
<td>99.31</td>
</tr>
<tr>
<td>S</td>
<td>1</td>
<td>400</td>
<td></td>
</tr>
<tr>
<td>EMB (5.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>8</td>
<td>7</td>
<td>98.38</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>417</td>
<td></td>
</tr>
<tr>
<td>SM (2.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>50</td>
<td>3</td>
<td>97.22</td>
</tr>
<tr>
<td>S</td>
<td>9</td>
<td>370</td>
<td></td>
</tr>
</tbody>
</table>

a Overall agreement (%) calculated based on: [(number of true resistant isolates + number of true susceptible isolates)/total number of Mycobacterium tuberculosis isolates (n = 432)] × 100.


