Evaluation of MODS assay for rapid detection of *Mycobacterium tuberculosis* resistance to second-line drugs in a tertiary care tuberculosis hospital in China

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**SUMMARY**

In this study we evaluated the performance of microscopic observation drug susceptibility (MODS) assay for rapid detection of *Mycobacterium tuberculosis* resistance to second-line drugs. 246 multidrug-resistant *M. tuberculosis* clinical isolates were used to compare MODS with the agar proportion method for rapid detection of resistance to 8 second-line drugs: ofloxacin, amikacin, kanamycin, capreomycin, ethionamide, cycloserine, ciprofloxacin and para-aminosalicylic acid. The sensitivity of the MODS for different drugs ranged from 88.1% to 100%, whereas the specificity ranged from 92.3% to 100%. Results for MODS assay were obtained in a median time of 7 days (range 5–18). Thus MODS assay could be used as a fast, reliable and inexpensive method for detection of *M. tuberculosis* resistance to second-line drugs in resource-limited settings.

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1. Introduction

Tuberculosis (TB) continues to be a global health problem due to the emergence and spread of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) worldwide. [1] According to the World Health Organization (WHO), 84 countries had reported at least one case of XDR-TB by 2012 [2]. In China, which has the highest burden of MDR-TB in the world, ~8% of MDR cases are XDR-TB, most of which result from primary transmission [3,4]. Timely detection of resistance to second-line drugs is of key importance to optimize treatment and to direct infection control measures to block transmission of MDR-TB [1].

The WHO developed guidelines for drug susceptibility testing (DST) for first- and second-line drugs on Löwenstein-Jensen (LJ) medium or Middlebrook agar using the proportion method. However, DST of *Mycobacterium tuberculosis* against second-line drugs is difficult and not well standardized. Conventional phenotypic drug susceptibility tests pose serious delays to the detection of resistance. Currently many second-line drugs susceptibility testing methods have been developed, including both commercial assays such as Genotype MTBDRsl assay, Sensititre® MYCOTB plate and in house assays such as high-resolution (or triplex real-time PCR) melting curve analysis and pyrosequencing [5–8]. However, these methods are beyond the reach of laboratories in most developing countries including China, due to high cost and the need for complex infrastructure facilities. Accurate, timely, and affordable DST for detect second-line drugs resistance in resource-limited settings is urgently needed.

The microscopic observation drug susceptibility (MODS) assay is a relatively low cost and simple liquid culture method that relies on the microscopic detection of cording growth characteristic of *M. tuberculosis* [9]. The MODS assay has been also reported to reliably identify *M. tuberculosis* isolates with resistance to isoniazid and to rifampin [9–14]. Our previous study has shown that MODS can offer a rapid and simple method for the rapid detection of pyrazinamide resistance in resource-limited settings [15]. There are currently only a few studies evaluating the use of the MODS assay for the detection of resistance to second-line drugs [16,17].
The objective of this study was to assess the accuracy of the MODS assay for detecting resistance of \textit{M. tuberculosis} to ofloxacin (OFX), amikacin (AMK), kanamycin (KM), capreomycin (CAP), ethionamide (ETH), cycloserine (CYP), ciprofloxacin (CIP) and para-aminosalicylic acid (PAS) in clinical strains. MODS results were compared with those obtained with the agar proportion method (APM).

2. Materials and methods

2.1. Study setting and clinical isolates

Jiangxi Province is a resource-limited and high TB burden area in the southeast part of China; here, the prevalence of bacteriologically positive pulmonary TB was estimated at 203.6 cases per 100,000 inhabitants in 2000 [18]. This study was conducted at the Province TB Reference Laboratory, Jiangxi Chest Hospital located in the capital of Jiangxi Province and serving as the sole specialized tertiary care TB hospital in the province. A total of 246 MDR-TB isolates were randomly chosen from cultures isolated between 2006 and 2011 at the Province TB Reference Laboratory of Jiangxi Chest Hospital. All isolates were freshly subcultured on Middlebrook 7H10 (M7H10) agar before being tested by the different methods.

2.2. Antimicrobial agents

Drugs used were OFX, KM, CYP, PAS and ETH (Sigma–Aldrich, St. Louis, MO); AMK, CAP and CIP (MP Biomedicals, Solon, OH). KM, AMK, CAP, CIP and CYP were dissolved in sterile distilled water. OFX was dissolved in 0.1 N NaOH, ETH was dissolved in dimethyl sulfoxide (DMSO), and PAS was dissolved in ethanol. All stock solutions were filter-sterilized and stored in single-use aliquots at −80 °C.

2.3. DST by the agar proportion method

DST was performed on M7H10 agar according to standard procedures [19]. Briefly, a 1.0 McFarland suspension was diluted 10-fold serially in sterile distilled water, and dilutions of \(10^{-2}\) and \(10^{-4}\) were inoculated onto M7H10 agar with and without drug and incubated at 35 °C. Critical concentrations established by WHO [20] were directly used and the MIC of other drugs was determined both on M7H10 agar. MIC was defined as the lowest concentration of drug that inhibited more than 99% survival of susceptible \textit{M. tuberculosis} strain, H37Rv. Results were read 21 days after inoculation of media. At the critical concentration of each drug, the bacterial growth was measured and the percentage of resistance was calculated, whereby \(\geq 1\%\) is defined as a resistance. Staff participating in the study were blinded to the original phenotypical DST results.

2.4. MODS assay

The MODS assay was performed as described by Caviedes et al. [9] and Bwanga et al. [14], with minor modifications. A part of the inoculum, adjusted to have turbidity equal to the turbidity of a tube of 1.0 McFarland standard, was diluted 1:100 in distilled water. Middlebrook 7H9 medium with 10% oxalic acid, albumin dextrose and catalase (OADC) enrichment was prepared as previously described. Cultures were prepared in 24 well tissue culture plates. Each well of a 24-well plate contained 800 \(\mu\)l of MODS liquid medium, 100 \(\mu\)l of the 1:100 bacterial inoculum and 100 \(\mu\)l of drugs diluted solutions, giving a final volume of 1 ml. To determine the critical concentration of drug in MODS testing, the MIC of all tested drugs was established by aforementioned method in M7H9 medium. The various drugs and their critical concentrations used were OFX (2 \(\mu\)g/ml), KM (5 \(\mu\)g/ml), AMK (4 \(\mu\)g/ml) CIP (1.25 \(\mu\)g/ml), CYP (40 \(\mu\)g/ml) ETH (5 \(\mu\)g/ml) and PAS (10 \(\mu\)g/ml) [6,16,17]. A sterility control with only MODS liquid medium and a growth control well with MODS liquid medium plus bacteria were included. Plates were sealed with tape and ziplock bags and incubated at 37 °C. Mycobacterial growth was observed daily with an inverted light microscope at \(\times 40\) magnification from the 3rd to the 15th day of incubation. After 15 days of incubation, observation was limited to twice a week. A strain was considered susceptible if cord-like structures were observed in the drug-free well but not in the drug-containing well. A strain was considered resistant if cord-like structures were observed in both the control and drug-containing wells.

2.5. Quality control and resolution of discrepancies

The \textit{M. tuberculosis} H37Rv reference strain (ATCC 27294) was included as a quality control. If the quality control strain showed unexpected results, all tests of that batch had to be repeated. If discordant results were obtained between the MODS and the APM, MODS and APM tests were repeated. If the repeated results were identical to the original results, the corresponding results were used for the data analysis. If discordant results were obtained between the original and repeated results, corresponding tests were repeated again and the results presented in 2 tests were used for the data analysis. The discrepancy between MODS and APM reference method was classified as very major errors (PM result was resistant and MODS was susceptible), major errors (PM result was susceptible and MODS was resistant), or minor errors (an intermediate result was obtained by only one of the methods) [21]. If discrepant results were observed after repetition, DNA was amplified and sequenced for known drug-resistance determining regions.

2.6. PCR and sequencing

For discordant second-line drugs resistance results between MODS and APM the known drug-resistance relevant genes \textit{gyrA}/\textit{gyrB} (OFX and CIP), \textit{rrs} (KM, AMK and CAP) and \textit{tlyA} (CAP) were sequenced, as previously described [18,22]. Genomic DNA was extracted from samples using the CTAB (cetyltrimethylammonium bromide)-NaCl method as described previously [23]. Sequencing of the amplicons was carried out at Beijing Genomics Institute (Shenzhen, China). The sequences generated with the program were compared to the respective wild-type sequences by using clone manager software.

2.7. Statistical analysis

In our study, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of MODS assay for detection of resistance to individual drugs were calculated by using APM results as the reference standard. Data were analyzed by statistical software SPSS 17.0.

3. Results

A total of 246 MDR-TB isolates of \textit{M. tuberculosis} were tested using MODS, all isolates gave interpretable results. No contamination was observed in the negative controls (sterility control). A total of 32/246 (13.0%) isolates met the criteria for classification as XDR-TB (MDR-TB as well as additional resistance to any fluoroquinolone and second line injectable drugs such as KM, AMK, CAP) using the...
APM. The agreement was respectively 98%, 96.3%, 98.8%, 97.2%, 96.3%, 91.5%, 93.9 and 97.1% for OFX, KM, AMK, CAP, PAS, ETH, CYC and CIP. DST results of the MODS assay as compared to the APM are shown in Table 1. All strains with discordant results were retested with the MODS and APM, but the repeat susceptibility results were unchanged.

3.1. Ofloxacin and ciprofloxacin resistance

The sensitivity of MODS was 96.2% for OFX and 88.1% for CIP, whereas the specificity of MODS was 98.8% for OFX and 99% for CIP. There were 12 discrepant results, of which 4 (33.3%) were major errors (false-resistant strains): 2 for OFX and 2 for CIP. Eight discrepant results (66.7%) were very major errors (false-susceptible strains): 3 for OFX and 5 for CIP. We next analyzed the glymA/glyR gene to investigate resistance-conferring mutations for OFX and CIP. The most frequent mutation was D94G (50%, 6/12) in glyA gene. DNA sequencing results for the discrepant isolates between MODS assay and APM are shown in Table 2.

3.2. Kanamycin, amikacin and capreomycin resistance

The sensitivity of MODS was 90.7% for KM, 93.9% for AMK and 88.4% for CAP, whereas the specificity of MODS was 97.9%–100% for KM, AMK and CAP. There were 19 discrepant results, of which 6 (31.6%) were major errors: 4 for KM and 2 for CAP. 13 discrepant results (68.4%) were very major errors (false-susceptible strains): 3 for OFX and 5 for CIP. We next analyzed the nt 1400 region of the rrs gene to investigate resistance-conferring mutations for KM, AMK and CAP. The most frequent mutation was A1401G (21.1%, 4/19) (Table 2). In addition, the fliA gene was sequenced for all the CAP discrepant isolates. DNA sequencing identified a nucleotide change at position 33 (A→G) of the fliA gene in 2/5 isolates that were phenotypically resistant to CAP.

3.3. Cycloserine, para-aminosalicylic acid and ethionamide

The sensitivity of MODS was 96.2% for CYC, 100% for PAS and 88.5% ETH, whereas the specificity of MODS was 92.3%–95.9% for CYC, PAS and ETH. There were 45 discrepant results, of which 38 (84.4%) were major errors: 15 for ETH, 14 for CYC and 9 for PAS. Seven discrepant results (15.6%) were very major errors: 6 for ETH and 1 for CYC. No minor errors were observed (Table 1).

3.4. Turnaround time

The median turnaround time for DST results by MODS was 7 days (range 5–18). With the MODS assay, results for 67.5% were available by day 7, 83.7% by day 10 and by day 18 all results were available. With the conventional APM, results for only 52.8% of the isolates were available at day 21.

4. Discussion

Drug-resistant TB, especially MDR- and XDR-TB, is now a major threat to global TB control. China has been described as a global “hot spot” for drug-resistant TB. The National TB control programs in China are however unable to routinely screen or do surveillance for MDR- and XDR-TB due to lack of affordable rapid tests [3].

The MODS assay is an inexpensive, liquid culture-based diagnostic test that has been endorsed by the WHO for rapid screening of patients suspected of having MDR-TB in resource-limited settings [24]. Most of the previous work related to MODS has been done for early detection of MDR-TB cases focusing on rifampicin and isoniazid, the two most important drugs for the treatment of TB, showing a high sensitivity and specificity [9–14]. However, there are currently only a few studies evaluating the use of the MODS assay for the detection of resistance to second-line drugs. In 2009, Devasia et al. [16] reported the first evaluation of the MODS.

Table 1

<table>
<thead>
<tr>
<th>Strains (n = 246)</th>
<th>APM</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
<th>PPV %</th>
<th>NPV %</th>
<th>Total agreement %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td></td>
<td>Susceptible</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OFX</td>
<td>Resistant</td>
<td>75</td>
<td>96.2</td>
<td>98.8</td>
<td>97.4</td>
<td>98.2</td>
</tr>
<tr>
<td></td>
<td>Susceptible</td>
<td>3</td>
<td>166</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KM</td>
<td>Resistant</td>
<td>49</td>
<td>90.7</td>
<td>97.9</td>
<td>92.5</td>
<td>97.4</td>
</tr>
<tr>
<td></td>
<td>Susceptible</td>
<td>5</td>
<td>188</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMK</td>
<td>Resistant</td>
<td>46</td>
<td>93.9</td>
<td>100</td>
<td>100</td>
<td>98.5</td>
</tr>
<tr>
<td></td>
<td>Susceptible</td>
<td>3</td>
<td>197</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAP</td>
<td>Resistant</td>
<td>38</td>
<td>88.4</td>
<td>99</td>
<td>95</td>
<td>97.6</td>
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<tr>
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<td>Susceptible</td>
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<td>201</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>PAS</td>
<td>Resistant</td>
<td>28</td>
<td>100</td>
<td>95.9</td>
<td>75.7</td>
<td>100</td>
</tr>
<tr>
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<td>Susceptible</td>
<td>0</td>
<td>209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETH</td>
<td>Resistant</td>
<td>46</td>
<td>88.5</td>
<td>92.3</td>
<td>75.4</td>
<td>96.8</td>
</tr>
<tr>
<td></td>
<td>Susceptible</td>
<td>6</td>
<td>179</td>
<td></td>
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<tr>
<td>CYC</td>
<td>Resistant</td>
<td>25</td>
<td>96.2</td>
<td>93.6</td>
<td>64.1</td>
<td>99.5</td>
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<tr>
<td></td>
<td>Susceptible</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIP</td>
<td>Resistant</td>
<td>37</td>
<td>88.1</td>
<td>99</td>
<td>94.9</td>
<td>97.6</td>
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<tr>
<td></td>
<td>Susceptible</td>
<td>5</td>
<td>201</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MODS, microscopic observation drug susceptibility; APM, agar proportion method; OFX, ofloxacin; KM, kanamycin; AMK, amikacin; CAP, capreomycin; PAS, para-aminosalicylic acid; ETH, ethionamide; CYC, cycloserine; CIP, ciprofloxacin; NPV, negative predictive value; PPV, positive predictive value.

* Major errors.

Very major errors.
Table 2
DNA sequencing results for the discrepant isolates between MODS assay and agar proportion method.

<table>
<thead>
<tr>
<th>Drug, locus</th>
<th>Resistant by MODS, susceptible by APM (Amino acid change[s])</th>
<th>Resistant by APM, susceptible by MODS (Amino acid change[s])</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFX (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gyrA</td>
<td>Mutation present (n = 3) 1 (D94A) 2 (D94G; D94N and S95T)</td>
<td>Mutation absent (n = 2) 1 1</td>
</tr>
<tr>
<td></td>
<td>Mutation absent (n = 0) 0</td>
<td>Mutation absent (n = 0) 0</td>
</tr>
<tr>
<td></td>
<td>Mutation absent (n = 5) 2</td>
<td>CIP (n = 7) 2 3</td>
</tr>
<tr>
<td>gyrB</td>
<td>Mutation present (n = 5) 2 (D95N; A90V) 3 (D94G; A90V and S95T; D94G and S95T)</td>
<td>Mutation absent (n = 2) 0 2</td>
</tr>
<tr>
<td>KM (n = 9)</td>
<td>Mutation present (n = 3) 1 (C1402T) 2 (A1401G; A1401G)</td>
<td>Mutation absent (n = 6) 2 4</td>
</tr>
<tr>
<td></td>
<td>Mutation absent (n = 0) 0</td>
<td>KM (n = 9) 4 5</td>
</tr>
<tr>
<td></td>
<td>Mutation absent (n = 3) 0</td>
<td>Mutation present (n = 3) 1 (C1401G) 2 (A1401G; G1484T)</td>
</tr>
<tr>
<td></td>
<td>Mutation absent (n = 6) 3</td>
<td>Mutation absent (n = 6) 3 3</td>
</tr>
<tr>
<td>AMK (n = 3)</td>
<td>Mutation present (n = 0) 0</td>
<td>Mutation present (n = 3) 1 (C1401G) 2 (A1401G; G1484T)</td>
</tr>
<tr>
<td></td>
<td>Mutation absent (n = 0) 0</td>
<td>Mutation absent (n = 0) 0</td>
</tr>
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<td></td>
<td>Mutation absent (n = 3) 0</td>
<td>Mutation present (n = 3) 1 (C1401G) 2 (A1401G; G1484T)</td>
</tr>
<tr>
<td>CAP (n = 7)</td>
<td>Mutation present (n = 2) 0</td>
<td>Mutation absent (n = 2) 0</td>
</tr>
<tr>
<td></td>
<td>Mutation present (n = 5) 2</td>
<td>Mutation absent (n = 5) 2</td>
</tr>
</tbody>
</table>

MODS, microscopic observation drug susceptibility; APM, agar proportion method; OFX, ofloxacin; KM, kanamycin; AMK, amikacin; CAP, capreomycin; CIP, ciprofloxacin.

1 Major errors.
2 Very major errors.

for detection of OFX resistance and found complete concordance with the proportion method. Fitzwater et al. [17] recently explored the candidate critical concentrations for second-line DST for M. tuberculosis using direct MODS assay. The concordance of indirect MODS for the different drugs ranged between 59% and 89%. But the correctness and suitability of these candidate critical concentrations should be tested in a range of epidemiological settings.

In this study, the sensitivity of MODS was 96.2%, 90.7%, 93.9%, 88.4%, 100%, 88.5%, 96.2%, and 88.1% for OFX, KM, AMK, CAP, PAS, ETH, CYC, and CIP, respectively. The test specificity was over 92% for all drugs. Our results confirm the fact that the MODS can also be used to detection of M. tuberculosis resistance to second-line drugs. For discrepancies, not all mechanisms of drug resistance are fully known and much less is known about such mechanisms for second-line drugs [25]. In our study, for samples with discordant results, MODS and APM were repeated to confirm the results. If discrepancies retained after the repetition, sequencing of known drug-resistance relevant genes was done to provide circumstantial evidence to the judgment of correctness or reliability of the phenotypic tests. For aminoglycosides (KM and AMK) and CAP resistance, according to our results, the most common mutation, rrs A1401G, was detected in 4 of 19 discordant isolates. For OFX and CIP resistance, the most frequent mutation in gyrA detected in our study was D94G (GAC-GGC), which is consistent with other report [25,26]. Quick turnaround time with DST is important for ensuring the patient receives an appropriate treatment regimen. As expected, MODS assay provided far more rapid results than the APM. Although more rapid molecular methods exist for selected second-line agents (eg., OFX), they are not available for all agents or in all areas of the world, nor do they detect all instances of resistance. Therefore, for correct management of XDR-TB patient phenotypic methods such as the MODS assay remain important.

The only equipment needed to perform the MODS assay are an inverted microscope, biological safety cabinet and incubator. As it is not necessary to use sophisticated equipment or reagents to perform this assay, it easier to use this susceptibility test in laboratories with limited financial resources, especially those in developing countries, where the rates of MDR- and XDR-TB are increasing [9,10,16]. However, in our view, an international standard operating procedure and a quality assurance system accredited by WHO should be developed to standardize and maintain accuracy.

In summary, the present work promotes a wider use of the MODS as a new and rapid ethod for the detection of M. tuberculosis resistance to second-line drugs, especially in developing countries where rapid and inexpensive methods are urgently needed. Additional studies on direct MODS testing for second-line drugs are needed.

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Ethical approval: Not required.

References


