Comparison of different drug susceptibility test methods to detect the rifampin heteroresistance in *Mycobacterium tuberculosis*

Zhijian Zhang¹,², Yufeng Wang², Yu Pang¹,²,*, Changting Liu¹,*

Respiratory Diseases Department of Nanlou, Chinese People's Liberation Army General Hospital¹; National Center for Tuberculosis Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China²

*Corresponding author. Mailing address for Yu Pang: National Center for Tuberculosis Control and Prevention, Chinese Center for Disease Control and Prevention, No. 155, Chang Bai Road, Changping District, Beijing 102206, People’s Republic of China. Phone: 86 10 5890 0779. Fax: 86 10 5890 0779. E-mail: pangyu@chinatb.org.

Mailing address for Changting Liu: Respiratory Department of Nanlou, Chinese People's Liberation Army General Hospital, 28# Fuxing Road, Haidian District, Beijing 100853, People’s Republic of China. Phone: 86 10 6687 6272. Fax: 86 10 6687 6272. E-mail: liuchangting301@163.com.
Abstract

We compared the efficiency of different drug susceptibility testing methods to detect the rifampin (RIF) heteroresistance in *Mycobacterium tuberculosis*. Our data revealed that broth dilution method found more resistance than MGIT ($P=0.046$) for low resistance group. Similarly, broth dilution method was more sensitive to detect RIF heteroresistance in subpopulation with slow grow rate than MGIT ($P=0.033$). In conclusion, our data demonstrated that the broth dilution method was more sensitive than MGIT to detect RIF heteroresistance.

Drug-resistant tuberculosis (TB) can be acquired through the transmission of already resistant strains, or it can happen *de novo* during treatment due to improper diagnosis and poor compliance of TB patients (1, 2). In the latter condition, selection of drug-resistant organisms can occur during discontinuous exposure to subtherapeutic drug levels, initially producing heteroresistant and finally fully resistant bacterial populations (2-4). The early diagnosis of heteroresistance is essential to generate effective drug regimen and prevent the emergence of full resistant populations (5, 6). Unfortunately, heteroresistance in clinical isolates is difficult to detect using conventional and genotypic drug susceptibility testing (DST) methods (7). In this study, our aim was to compare the efficiency of different drug susceptibility testing methods to detect the rifampin (RIF) heteroresistance in *Mycobacterium tuberculosis* (MTB), including Bactec MGIT DST, broth dilution method and Sanger sequencing of *rpoB* gene, to detect the RIF heteroresistance in MTB isolates.
Seven RIF-resistant and one RIF-susceptible MTB strains, classified as Beijing family, were obtained from the National Tuberculosis Reference Laboratory of China (8, 9). The drug susceptibility of MTB isolates was determined by conventional proportion method recommended by World Health Organization (10). Genomic DNA of MTB was extracted from freshly cultured bacteria as previously reported (11). A 450-bp region of the *rpoB* gene containing the 81-bp RRDR was amplified by PCR (12). The primers were synthesized by Invitrogen Company (Shanghai, China). The amplicons were sent to Tsingke Company for DNA sequencing service.

The Bactec MGIT 960 system (Becton Dickinson, USA) was used to evaluate the growth rate of RIF-resistant MTB isolates. Tubes were incubated at 37°C in the BACTEC MGIT 960, and time to detection (TTD) was calculated as the time between the date of culture inoculation and the earliest date the instrument recorded positive growth. The RIF-resistant suspensions were serially diluted into RIF-susceptible suspension to the following percentages: 0.5%, 1%, 2%, 5%, 10%, 20% and 50%. Additionally, three separate experiments were performed for each diluted suspension to detect the reproducibility of different DST methods.

To determine minimal inhibitory concentrations (MICs) of MTB strains with different proportion of RIF-resistant subpopulations, a microplate Alamar blue assay (MABA) was performed as described previously (12, 13). Final RIF concentrations were 0.125
to 256 μg/ml. The MIC breakpoints concentration for RIF was defined as 0.5 μg/ml (12, 14). In addition, the RIF susceptibility of heteroresistant mixture was detected by the Bactec MGIT 960 automated system according to the manufacturer’s instructions for DST to RIF (15).

We performed a chi-square test or Fisher’s exact test to evaluate the efficiency of drug susceptibility test methods to detect the RIF heteroresistance. Statistical analysis was performed in SPSS 11.5 (SPSS Inc., USA). Differences with a $P$ value of 0.05 or less were considered statistically significant.

A total of five mutant types in RRDR of $rpoB$ gene were observed among seven RIF-resistant isolates, including 2 isolates with Ser531Leu, 2 with His526Arg, 1 with Ser531Trp, 1 with Asp516Val, and 1 with Leu533Pro. The RIF MICs of strains with mutations at codon 531 and 526 were no lower than 256 μg/mL, while the strains harboring 516Val or 533Pro mutation had MICs ranging from 16 to 32 μg/mL. The TTD was also analyzed with MGIT system. As shown in Table 1, five RIF-resistant isolates showed similar growth rate with RIF-susceptible control isolate, the TTD of which ranged from 169 to 189 hours. In addition, we also found that the TTD of two RIF resistant isolates was 248 and 264 hours, respectively.

We firstly evaluated whether the mutant type or MIC level of RIF-resistant isolates affected the efficiency of DST methods to detect the RIF heteroresistance. The five
RIF resistant strains with similar growth rate were enrolled in this analysis. As shown in Table 2, MGIT DST system showed various abilities to detect heteroresistance containing different mutations. For substitutions 531Leu, 531Trp and 526Arg conferring high level RIF MICs, MGIT DST was able to find 2.0%, 1.0% and 0.5% RIF resistance, respectively. For substitutions 516Val and 533Pro conferring low level RIF resistance, we found that MGIT DST was able to detect RIF resistance when 10% and 5% of the bacteria were resistant, respectively. In comparison with MGIT DST, broth dilution method had better efficiency to detect heteroresistance. For positions 531 and 526, broth dilution method was able to detect RIF resistance if 0.5% resistant bacteria were present. For substitutions 516Val and 533Pro, the limit of detecting RIF heteroresistance had been both increased to 2% (Fig. 1A). We also analyzed the rpoB sequencing chromatograms of suspensions containing various proportions of RIF-resistant and RIF-susceptible bacteria. As shown in Fig. S1, the detection limit of dideoxynucleotide sequencing was 10% in the mutated populations in differential graphs, indicating that this method was less sensitive than MGIT DST and broth dilution method. In contrast to phenotypic drug susceptibility testing methods, DNA sequencing showed similar ability to detect heteroresistance harboring different mutation types (data not shown). When we compared the efficiency of two phenotypic methods in the different MIC groups (1% of heteroresistance was set as cut-off value), statistical analysis revealed that broth dilution method (12/14) found more resistance than MGIT DST (6/14) (P=0.046) for low MIC group (MIC≤32 μg/mL), while there was no significant difference observed among high MIC group (MGIT: 20/21 vs. MGIT DST: 10/14) (P=0.046).
We also analyzed the effect of growth rate on the ability of phenotypic DST methods to detect heteroresistance. Our data revealed that the heteroresistant suspension containing RIF-resistant populations with high growth rate showed higher MIC level than that with slow growth rate. For substitutions 531Leu, MGIT DST was able to only detect 20% for slow growth rate group, and broth dilution method was able to find resistance if 2.0% resistant bacteria were present. Similarly, comparing with the fast growth rate group harboring His526Arg substitution, the detection limit of MGIT DST had been decreased from 1.0% to 20% for the slow growth rate group, while the broth dilution method was able to detect the recommended 1.0% RIF resistance (Table 2 and Fig. 1B). We also found that broth dilution method (13/14) was more sensitive to detect RIF heteroresistance with slow grow rate RIF-resistant subpopulations than MGIT DST (7/14) (P=0.033).

Heteroresistance with presence of susceptible and resistant subpopulations in MTB has been considered as a major reason for discordant phenotypic DST results (16). Firstly, RIF heteroresistance with specific ropB mutation conferring low-level RIF resistance is difficultly detected by MGIT DST. When comparing with MTB populations harboring high MIC level, the low-level resistant isolates may reveal lower fitness under the presence of RIF. Consequently, the growth of RIF-resistant populations will be severely inhibited due to the loss of fitness, failing to reach a GU.
value of 100 by the end of detection. Secondly, our data reveal that the slow growth is another important factor responsible for the ability of phenotypic DST methods to detect RIF heteroresistance. The slow growth rate is associated with loss of fitness, which might explain why MGIT failed to detect RIF heteroresistance in our study.

Surprisingly, another growth-based method, broth dilution method, presents higher efficiency to detect RIF heteroresistance in MTB. One possible explanation is the larger inoculum size for broth dilution method. Similar to our findings, a recent study by Van Deun found that low-level RIF resistance related to specific mutations was easily missed by the rapid MGIT DST system (17). The different detection limits of RIF heteroresistance with various rpoB mutations and slow growth rate highlight the requirement for revision of the MGIT DST methods (16). In addition, the RIF critical concentration used in Bactec methods may be too high, and lowering the RIF breakpoint suggested by Suo et al. may be another more accurate variant of MGIT DST system (16, 18). Considering the complicacy of modifying the MGIT procedures, our findings indicate that the microdilution MIC method may be a satisfactory alternative for identifying RIF heteroresistance. Recently, several literatures revealed that the broth dilution method might be more specific for identifying the INH and EMB resistance than conventional proportion method (19, 20). Taken together, we recommend that the broth dilution method can be regarded as an excellent method able to provide rapid and reliable results of RIF susceptibility testing.

Several literatures have demonstrated that the existing molecular methods are difficult
to detect RIF heteroresistant MTB, especially direct DNA sequencing (3, 11). In consistent to former findings, we identified that DNA sequencing could only detect 10% RIF heteroresistance, even by reviewing the original sequencing chromatograms. Although phenotypic DST methods show better efficiency to detect RIF heteroresistance, an obvious advantage of DNA sequencing should not be ignored, that various \textit{rpoB} mutations and slow growth rate have no influence on detecting heteroresistant populations. In order to overcome the shortcoming of Sanger sequencing for amplifying low abundance mutations, numerous novel techniques have been developed to enable preferential amplification of minority alleles from a mixture of wild-type and mutant sequences (11). By combining COLD-PCR with high-resolution melt technology, the mutation detection limit of RIF heteroresistant in MTB has been increased from 20% to 2.5%, which is higher than 5% of MTBDR\textit{plus} by Folkvardsen et al. (3, 11).

In conclusion, our data demonstrated that the broth dilution method was more sensitive than MGIT DST to detect RIF heteroresistance in MTB. The ability for identifying RIF heteroresistance may be determined by both various \textit{rpoB} mutations and growth rate of RIF resistant populations in mixtures. Our findings indicate that the micro broth dilution MIC method may serve as an alternative technique in detecting RIF heteroresistance in MTB isolates.

ACKNOWLEDGEMENTS
This work was supported by the National Natural Science Foundation of China (81301509) and National Key Research Program of China (2013ZX10003-003). We are grateful to members of the National Tuberculosis Reference Laboratory at the Chinese Center for Disease Control and Prevention for their cooperation and technical help.

REFERENCES


Figure Legends

Figure 1 MICs of mixtures with different proportion of RIF resistant bacteria detected by broth dilution method. (A) MICs of RIF-resistant MTB isolates with different mutant types in rpoB gene. (B) MICs of RIF-resistant MTB isolates with different growth rate.
Table 1 Mutation, minimal inhibitory concentration (MIC) and growth rate detected in RIF resistant *M. tuberculosis* isolates

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Mutation</th>
<th>MIC (μg/mL)</th>
<th>TTD(^a) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB1</td>
<td>Ser531Leu</td>
<td>256</td>
<td>169</td>
</tr>
<tr>
<td>TB2</td>
<td>Ser531Trp</td>
<td>&gt;256</td>
<td>186</td>
</tr>
<tr>
<td>TB3</td>
<td>His526Arg</td>
<td>&gt;256</td>
<td>248</td>
</tr>
<tr>
<td>TB4</td>
<td>His526Arg</td>
<td>&gt;256</td>
<td>189</td>
</tr>
<tr>
<td>TB5</td>
<td>Asp516Val</td>
<td>16</td>
<td>182</td>
</tr>
<tr>
<td>TB6</td>
<td>Ser531Leu</td>
<td>256</td>
<td>264</td>
</tr>
<tr>
<td>TB7</td>
<td>Leu533Pro</td>
<td>32</td>
<td>179</td>
</tr>
<tr>
<td>TB8</td>
<td>NA(^b)</td>
<td>0.25</td>
<td>173</td>
</tr>
</tbody>
</table>

\(^a\)TTD: time to detection.

\(^b\)NA: no mutation.
<table>
<thead>
<tr>
<th>Proportion of RIF-resistant bacteria</th>
<th>TB1 (Ser531Leu)</th>
<th>TB2 (Ser531Trp)</th>
<th>TB4 (His526Arg)</th>
<th>TB5 (Asp516Val)</th>
<th>TB7 (Leu533Pro)</th>
<th>Slow Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R</td>
</tr>
<tr>
<td>50%</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R</td>
</tr>
<tr>
<td>20%</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R</td>
</tr>
<tr>
<td>10%</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R</td>
</tr>
<tr>
<td>5.0%</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>S S</td>
</tr>
<tr>
<td>2.0%</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>R R R R R R R</td>
<td>S S</td>
</tr>
<tr>
<td>1.0%</td>
<td>S R R R R R R</td>
<td>S R R R R R R</td>
<td>S R R R R R R</td>
<td>S R R R R R R</td>
<td>S R R R R R R</td>
<td>S S</td>
</tr>
<tr>
<td>0.5%</td>
<td>S S R R R R R</td>
<td>S S R R R R R</td>
<td>S S R R R R R</td>
<td>S S R R R R R</td>
<td>S S R R R R R</td>
<td>S S</td>
</tr>
</tbody>
</table>

* R: resistant; S: susceptible.
* Fast and slow groups were divided according to growth rate determined by TTD of MGIT.
**Figure A**

Minimal inhibitory concentration (µg/mL) of various M. tuberculosis isolates with different mutations in the presence of different proportions of RIF-resistant isolates.

**Figure B**

Minimal inhibitory concentration (µg/mL) of various M. tuberculosis isolates with different mutations in the presence of different proportions of RIF-resistant isolates.