**Lab Note**

**Detection of mutations associated with resistance to rifampicin and isoniazid in Mycobacterium tuberculosis by polymerase chain reaction-ligase detection reaction**

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*Mycobacterium tuberculosis* (MTB) infection remains a serious infectious disease worldwide, causing 8.8 million new infections and 1.45 million deaths in 2010 [1]. The emergence of drug-resistant strains of MTB poses a significant threat to the control of the disease globally. Multidrug-resistant MTB (MDR-TB), defined as being resistant to at least rifampicin (RMP) and isoniazid (INH), and extensively drug-resistant TB (XDR-TB), defined as being additionally resistant to any fluoroquinolone and one injectable second-line drug, are associated with higher treatment failure rates. China is the second largest nation in terms of the number of the MTB infection, and 9.3% of the cases are MDR-TB [2]. MDR-TB arises from spontaneous chromosomal mutations at low frequency, but clinical MDR-TB largely occurs as a result of man-made selection during disease treatment of these genetic alterations through erratic drug supply, suboptimal physician prescription, and poor patient adherence [3]. S315T/M mutation in *katG* and mutations in the promoter region of *inhA* operon are the main mechanisms of INH resistance [4,5]. Mutations at positions 516, 526, and 531 in *rpoB* are the most frequent mutations in RMP-resistant MTB strains [6]. Since the transmission of the selected MDR-TB strains causes a large number of MDR-TB cases [7,8], the need for rapid, reliable, and cost-effective drug susceptibility testing (DST) is urgent.

Polymerase chain reaction (PCR) followed by ligase detection reaction (LDR) method has been widely used to detect various single nucleotide polymorphisms and drug-resistant mutations with high sensitivity and low cost [9–12]. In the present study, we established a PCR-LDR assay for the simultaneous detection of the mutations related to MDR-TB, and evaluated its performance using isolates and clinical samples. Here, a total of 120 MTB isolates obtained from pulmonary TB patients at The Third Hospital of Changzhou, during July, 2011 and March, 2012 were collected and subjected to DST for RMP and INH in the BACTEC MGIT 960 instrument with medium, and DST supplement (Becton Dickinson and Company, Sparks, USA) as recommended by the manufacturer. All the 120 MTB isolates were detected by phenotypic DST, PCR-LDR, and DNA sequencing.

The specific primers and probes (Supplementary Tables S1 and S2) for both wild type and mutant type targeting codon 315 of the *katG* gene, codon 15 of the *inhA* promoter region, codons 516, 526, and 531 of *rpoB*. All the primers and probes were synthesized by Invitrogen (Shanghai, China). Genomic DNA of MTB was extracted by a commercial kit (Fosun Diagnostics, Shanghai, China) according to manufacturer’s instruction. All isolates were detected by both the PCR-LDR assay and sequencing. The multiplex PCR-LDR method was the same as described in previous studies [11,12]. The amplifications and LDR were performed in a PE 9600 thermal cycler (Life Technology, Foster City, USA). The LDR products were electrophoresed on an ABI 377 DNA Sequencer (Life Technology) for 30 min. Results were analyzed using the GeneMapper software (Life Technology). Meanwhile, the PCR products of *rpoB* and *inhA* promoter region were purified and sequenced by Life Technology.

The results of phenotypic DST, PCR-LDR, and DNA sequencing were summarized in Table 1. Phenotypic DST of MTB isolates indicated that 26 (21.7%) at low level (1 μg/ml) and 11 (9.2%) at high level (10 μg/ml) resistance to INH, while 17 (14.2%) at low-level and 9 (7.5%) at high-level resistance to RMP. Among them, 13 (10.8%) were resistant to both INH and RMP at low level, or MDR. PCR-LDR assays for the detection of MDR-TB were carried out on 120 isolates. The S315M in *katG* was detected in 16 (13.3%) of 120 isolates. No S315T mutation in *katG* and G15T mutation in the promoter region of *inhA* was detected. The D516G/15, H526Y/D, S531I/L/W mutations in *rpoB* were detected in 1 (0.8%), 2 (1.7%), and 8 (6.7%) of 120 isolates, respectively. The typical results of PCR-LDR were shown in Fig. 1 and Supplementary Fig. S1.
Drug-resistant mutations were detected in 16 (61.5%) of 26 low-level INH-resistant isolates and 6 (54.5%) of 11 high-level INH-resistant isolates, respectively. Among the 17 low-level and 10 high-level RMP-resistant isolates, 10 (58.8%) and 9 (90.0%) drug-resistant mutations were detected by PCR-LDR, respectively. Among the 13 MDR isolates, 8 (61.5%) with mutations in both \( \text{katG} \) and \( \text{rpoB} \) were detected by PCR-LDR. Thirteen isolates were detected as S315M by PCR-LDR, but 2 of them were sensitive to INH at low level, and 11 of them were sensitive to INH at high level, as tested by phenotypic DST. One isolate with S531L/W mutation was sensitive to RMP at both low and high levels. One isolate with H526Y/D mutation was resistant to RMP at low level but sensitive at high level.

The frequency of mutations in promoter region of \( \text{inhA} \) was low in this study. This result was consistent with previous studies in China [13]. Globally, S315T mutation is the most common mutation in INH-resistant strains, accounting for 50%–90% of INH-resistant clinical isolates [4]. Mutations at codons 516, 526, and 531 are the most frequent mutations in RMP-resistant strains and generally result in high-level resistance [3]. In this study, these three mutations were detected in 88.9% of the high-level RMP-resistant isolates but only 58.8% of the low-level resistant isolates. Low-level resistance to RMP is associated with specific mutations at codons 511, 516, 518, and 522 [3]. For the PCR-LDR assay that is targeted mutations, its results were consistent with DNA sequencing in 119 of 120 isolates, indicating that the PCR-LDR is a reliable rapid molecular method for detection of INH and RMP associated mutations in MTB isolates. DNA sequencing, however, detected all of the potential drug-resistant mutations, while PCR-LDR only detected limited mutations that were covered by the assay.

In this study, two isolates with S315M mutation were sensitive to INH at low level by phenotypic DST. More S315M mutations were found in isolates sensitive to high-level INH.

### Table 1. Comparison of phenotypic DST, PCR-LDR, and sequencing

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose</th>
<th>Phenotypic DST (n, %), ( n = 120 )</th>
<th>PCR-LDR (n, %), ( n = 120 )</th>
<th>Sequencing (n, %), ( n = 120 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH</td>
<td>1 µg/ml</td>
<td>Sensitive (94, 78.3%)</td>
<td>S315M (2, 1.7%)</td>
<td>S315M (2, 1.7%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant (26, 21.7%)</td>
<td>S315M (15, 12.5%)</td>
<td>S315M (15, 12.5%)</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>Sensitive (109, 90.8%)</td>
<td>S315M (11, 9.2%)</td>
<td>S315M (11, 9.2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant (11, 9.2%)</td>
<td>S315M (6, 5%)</td>
<td>S315M (6, 5%)</td>
</tr>
<tr>
<td>RMP</td>
<td>50 µg/ml</td>
<td>Sensitive (103, 85.8%)</td>
<td>S315M (6, 5%)</td>
<td>S315M (6, 5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant (17, 14.2%)</td>
<td>S516V/G (1, 0.8%)</td>
<td>S516V/G (1, 0.8%)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>H526Y/D (2, 1.7%)</td>
<td>H526Y/D (2, 1.7%)</td>
</tr>
<tr>
<td></td>
<td>250 µg/ml</td>
<td>Sensitive (110, 91.7%)</td>
<td>S531L/W (7, 5.8%)</td>
<td>S531L/W (7, 5.8%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Resistant (10, 8.3%)</td>
<td>S531L/W (7, 5.8%)</td>
<td>S531L/W (7, 5.8%)</td>
</tr>
</tbody>
</table>

INH, isoniazid; RMP, rifampicin.
is defined as the coexistence of susceptible and resistant organisms to anti-tuberculosis drugs in the same patients [14]. Heteroresistance of MTB is considered as a preliminary stage of full resistance [15]. Two different mechanisms of heteroresistance in MTB have been defined: (i) superinfection with two different strains; and (ii) splitting of a single strain into susceptible and resistant organisms [16].

In conclusion, compared with DNA sequencing, PCR-LDR is a rapid and accurate molecular method for detection of common mutations associated with RMP and INH in MTB. It would be a useful tool for monitoring MDR-TB in hospitals equipped with DNA sequence analyzers.

Supplementary Data
Supplementary data are available at ABBS online.
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References