**rpoB Gene Mutations and Molecular Characterization of Rifampin-Resistant *Mycobacterium tuberculosis* Isolates from Shandong Province, China**

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Sixty rifampin (RIF)-resistant and 75 RIF-susceptible *Mycobacterium tuberculosis* isolates from Shandong Province, China, were analyzed for *rpoB* gene mutations and genotyped. Mycobacterial interspersed repetitive unit (MIRU) genotype 223325173533 was overrepresented among RIF-resistant isolates. MIRU combined with IS6110 restriction fragment length polymorphism analysis as the second-line genotyping method may reflect epidemiologic links more reliably than each method alone.

Tuberculosis (TB) remains a major public health concern in China, causing 150,000 deaths per year and making China second only to India in TB mortality (22). A significant challenge for TB control in China is the rapid dissemination and high prevalence of drug-resistant *Mycobacterium tuberculosis* (12). Rifampin (RIF), one of the principal first-line antituberculosis drugs, inhibits DNA-directed RNA synthesis of *M. tuberculosis* by binding to the β subunit of RNA polymerase (10). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, have been shown to be strongly associated with RIF-resistant phenotypes in multiple study populations (6, 9, 14, 17). *rpoB* mutations are more likely segregated in an 81-bp region called the RIF resistance-determining region (RRDR). Because up to 90% of RIF-resistant strains carry RRDR mutations within codons 516, 526, and 531, these mutational “hot-spots” are being used to rapidly identify RIF-resistant isolates (8, 15).

The Beijing family, a dominant *M. tuberculosis* genotype in China and the rest of Asia, has been associated with increased frequencies of drug resistance in some populations, but not in others (5). More-reliable genotyping strategies are required to refine the Beijing family and identify possible subgroups, which may explain the drug resistance scenario. With few exceptions (7, 13), there have been few investigations into the molecular characterization of RIF-resistant *M. tuberculosis* isolates from mainland China, reflecting a general lack of understanding of this important topic.

In this study, 60 RIF-resistant and 75 RIF-susceptible *M. tuberculosis* isolates were collected through an *M. tuberculosis* sentinel surveillance network in Shandong Province, China (21). These isolates were obtained from different patients with no familial ties. Drug susceptibility testing was performed by the proportion method on Löwenstein-Jensen medium using the critical drug concentrations for RIF (40 μg/ml), isoniazid (0.2 μg/ml), streptomycin (4 μg/ml), and ethambutol (EMB; 2 μg/ml). The drug resistance profiles of 60 RIF-resistant isolates are shown in Table 1. Seventy-five *M. tuberculosis* isolates were susceptible to all first-line antituberculosis drugs.

Extraction of *M. tuberculosis* genomic DNA was performed by standard methods (20). A 495-bp region of the *rpoB* gene including the RRDR was amplified by PCR with forward primer 5′ GACGACATCGACCAGCTTC and reverse primer 5′ GGTCAGGTACACGATCTCC. PCR fragments were sequenced with an ABI 377 DNA sequencer (Applied Biosystems, Inc., Foster City, CA). The new alleles were confirmed by further PCR and resequencing from the original DNA. Sequence data were independently analyzed by two biologists for quality control purposes.

Three independent genotyping methods were applied to molecularly characterize the 135 *M. tuberculosis* isolates. IS6110 restriction fragment length polymorphism (RFLP) characterization was conducted by following the standard protocol of van Embden et al. (20), with data analyzed by Whole Band Analysis software (version 3.2; BioImage, Ann Arbor, MI). Spoligotyping was carried out by using commercial kits from IsoGen Bioscience BV (Maarssen, The Netherlands). Mycobacterial interspersed repetitive unit (MIRU) typing was performed by PCR amplification of the 12 MIRU loci following the protocol described previously, and samples were given a 12-digit MIRU identification number (3).

Among the 60 RIF-resistant *M. tuberculosis* isolates, 55 (91.7%) were found to have mutations in the *rpoB* RRDR region with 10 different genotypes at six codons (Table 2). Codon 531 had the highest mutational frequency (33/55; 60.0%) followed by codons 526 (16/55; 29.1%), 516 (4/55; 7.3%), 511 (2/55; 3.6%), 515 (1/55; 1.8%), and 518 (1/55; 1.8%).
Codon 526 showed the highest variability of mutations, with five different nucleotide substitutions. One multidrug-resistant isolate (resistant to four first-line drugs) presented rare multiple mutations at three codons, 511, 515, and 518. In the 75 RIF-susceptible isolates, 3 (4.0%) presented with mutations. In the RRDR of rpoB, and they exclusively occurred at codon 533 with a novel nucleotide substitution, CTG (Leu)→ATG (Met). Together, the overall agreement rate between the RDDR mutation of rpoB and the RIF-resistant phenotype was 94.1% (127/135), which was consistent with previous studies of isolates from China (4, 13, 24). The relevance between codon 533 mutation and RIF resistance phenotype has been argued in previous studies, in which M. tuberculosis isolates with codon 533 mutations showed RIF susceptibility (MIC, ≥1.0 mg/liter) (11), low-level resistance (MIC, 12.5 mg/liter) (16), or high-level resistance (MIC, ≥512 mg/liter) (24). Our limited data suggest that codon 533 mutations are not associated with RIF resistance. The variability seen in RIF-resistant phenotypes in the codon 533 mutant isolates may thus be caused by other unknown mutations.

IS6110 RFLP identified seven RFLP clusters and 117 unique patterns. The IS6110 copy numbers ranged from 8 to 24. The largest cluster consisted of six epidemiologically linked RIF-susceptible isolates. Among 60 RIF-resistant isolates, there were 8 (13.3%) isolates falling into four IS6110 RFLP clusters; similarly, 10 (13.3%) of 75 RIF-susceptible isolates were identified in three clusters (Table 3). Spoligotyping determined four clusters and 12 unique patterns. Of the 135 isolates, 116 (85.9%) belonged to the Beijing family. Beijing family isolates were found more often among RIF-resistant isolates (90.0%) than among RIF-susceptible isolates (82.7%), but the difference was not statistically significant. Fifty-seven MIRU genotypes were found from all isolates in this study, including 40 unique patterns and 17 clusters (including 95 isolates) (Table 3). The largest cluster of 44 (32.6%) isolates shared MIRU genotype 223325173533 and are referred to as the “Shandong cluster” in this study. Interestingly, all isolates from the “Shandong cluster” were members of the Beijing family. The “Shandong cluster” isolates were found more often among RIF-resistant isolates (41.7%; 25/60) than among RIF-susceptible isolates (25.3%; 19/75) (P = 0.04). A comparative analysis using the Houston Tuberculosis Initiative database showed that print 33 from Houston, Texas, and strain 210 from Los Angeles, California (25), had the same MIRU genotype as the “Shandong cluster.” Previously, strain 210 has been reported to be more virulent (25) with clonal expansion observed in several U.S. populations (1, 23). These findings suggest that the “Shandong cluster” may be an important subcluster of the Beijing family.

Among all molecular typing methods for M. tuberculosis, IS6110 RFLP has been considered the most discriminatory method and standardized for extensive applications in tracking M. tuberculosis transmission. However, the reliability of IS6110 RFLP reflecting epidemiological links between TB patients has been a concern in some studies (2, 18, 19). In this study, the rpoB genotypes and drug resistance phenotypes, together with the sentinel surveillance sites of the clustered and rpoB mutant M. tuberculosis isolates, are presented in Table 4. Four IS6110 RFLP clusters (A to D) carried rpoB mutations in the RRDR. Isolates from clusters A and B showed the intracluster consistency between the sites, MIRU and rpoB genotypes, and drug resistance.
profiles, except for a slight difference of the EMB resistance phenotype in cluster A. In contrast, isolates in clusters C and D presented intracluster inconsistency between sites, MIRU and \( \text{rpoB} \) genotypes, and drug resistance profiles (Table 4). Besides the “Shandong cluster,” five MIRU clusters (clusters a to e) had \( \text{rpoB} \) mutations. Four (clusters a, b, d, and e) of the five MIRU clusters showed intracluster consistency between \( \text{rpoB} \) genotypes, drug resistance profiles, and sites. However, none of them showed intracluster identity in IS6110 RFLP patterns. These observations suggest the complexity of \( M. \) tuberculosis genomic variation and in agreement with a recent study (19) suggest that MIRU typing may more likely reflect epidemiologic links between TB patients than IS6110 RFLP. However, “Shandong cluster” isolates presented with a high prevalence in our study (32.6%), as well as a recent study from Hong Kong, China (21.6%) (7), indicating that MIRU alone does not provide enough discriminatory power for differentiating the “Shandong cluster” unless IS6110 RFLP is used as the second-line genotyping method.

In general, our data provide additional molecular insights into RIF-resistant \( M. \) tuberculosis strains in China. The newly identified “Shandong cluster,” showing an association with the RIF resistance phenotype and strain 210, justifies further investigations into its virulence, transmissibility, and molecular mechanisms of drug resistance. Additionally, our results also provided important supplemental evidence for a recent strategy of subdividing the Beijing family, which suggested that the addition of RFLP to MIRU offered a higher discriminatory value than the addition of MIRU to RFLP (7).

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### Table 4. Epidemiological analyses of clustered and \( \text{rpoB} \) mutant \( M. \) tuberculosis isolates

<table>
<thead>
<tr>
<th>Method and cluster</th>
<th>Isolate designation</th>
<th>Surveillance site designation</th>
<th>Drug resistance profile(^c)</th>
<th>No. of IS6110 copies</th>
<th>MIRU type</th>
<th>( \text{rpoB} ) mutation</th>
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<tr>
<td>IS6110(^a)</td>
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<td>27</td>
<td>RRRR</td>
<td>21</td>
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<tr>
<td></td>
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<tr>
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<td>E</td>
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<td>RRRR</td>
<td>16</td>
<td>22324173534</td>
<td>531 TTG (Leu)</td>
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<tr>
<td>MIRU(^b)</td>
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<td>SRSS</td>
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</table>

\(^a\) Clusters A to D are based on IS6110 RFLP fingerprinting.
\(^b\) Clusters a to e are based on MIRU typing.
\(^c\) Drug resistance profile sequence: isoniazid, RIF, streptomycin, and EMB, respectively. R, resistant; S, susceptible.

### References


