Mutations in the rpoB Gene of Multidrug-Resistant Mycobacterium tuberculosis Isolates from Brazil

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According to the World Health Organization, 8 million cases of tuberculosis (TB) occur each year, resulting in 3 million deaths (12). Strains of Mycobacterium tuberculosis resistant to at least two drugs, such as rifampin and isoniazid, are considered multidrug resistant (MDR-TB). These multidrug-resistant strains arise by sequential acquisition of resistance-conferring mutations in several genes as a consequence of antibiotic selection. This situation causes great concern worldwide because of the prolonged infectivity, which increases the risk of transmission (1).

Rifampin is one of the most important chemotherapeutic agents used to combat infections by M. tuberculosis and can be assumed to be a surrogate marker for MDR-TB (3, 11). Resistance to this drug has been shown to be due to alteration of rpoB, which codes the β subunit of RNA polymerase (5, 15). This putative rifampin resistance is associated with mutations that occur within a 69-bp region of the rpoB gene, which encodes the β subunit of RNA polymerase. The types of mutations include single-nucleotide changes and in-frame deletions and insertions (8, 15, 18).

In this study, we have used DNA sequencing to characterize mutations in the 69-bp region of the rpoB gene for 82 rifampin-resistant (Rifr) and 16 rifampin-susceptible (Rifs) isolates from three states of Brazil (Rio Grande do Sul [RS], São Paulo [SP], and Rio de Janeiro [RJ]). SP and RJ are located in the southeast region, and RS is located in the southern region.

Isolates used. One hundred M. tuberculosis isolates from different states of Brazil were analyzed by sequencing. From RS, 38 Rifr and 16 rifampin-susceptible (Rifs) clinical isolates were isolated in the Laboratório Central do Rio Grande do Sul. From RJ, 26 samples were obtained from the Centro de Referência Professor Hélio Fraga (25 Rifr and 1 Rifs). Twenty isolates were provided by Instituto Adolfo Lutz and Faculdade de Ciências Farmacêuticas Araraquara, both in SP (19 Rifr and 1 Rifs). The samples were collected from 1996 to 1998.

Culture and susceptibility testing. Cultures were grown on Ogawa medium. Rifampin susceptibility was determined on Löwenstein-Jensen medium by the proportional method of Canetti et al. (2). The isolates were also tested for susceptibility to isoniazid, ethambutol, pyrazinamide, and streptomycin by the same method (Table 1).

Sequencing of rpoB. To detect the mutations associated with Rifr, a 157-bp region of the rpoB gene was sequenced. A loopful of bacteria was suspended in 500 μl of TE (10 mM Tris, 1 mM EDTA, pH 8), and the DNA was extracted using cetlytrimethyl ammonium bromide as described previously (17). Purified M. tuberculosis DNA from the clinical isolates and reference strain H37RV was used to produce a 157-bp fragment of the rpoB gene, from nucleotide 1846 to 2002 (GenBank accession no. U12205) by using the primers TR9 (5′-TC GCCGCGATCAAAGGAGT) and TR8 (5′-TGACGTCGCGGACCTCCA) by the protocol described by Telenti et al. (15). The unincorporated nucleotides and primers were separated from the amplified DNA using MicroSpin columns (Pharmacia Biotech). Manual sequencing was carried out with the Thermus Sequenase Radiolabeled Terminator Cycle Sequencing kit (Amersham) according to the manufacturer’s instructions and using the primers described above. For each sample the sequence was examined twice in one direction, using as a template the products of two independent amplification reactions. The isolates that showed new mutations were sequenced again using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI Prism 310 genetic analyzer (Perkin-Elmer).

Mutations in the rpoB gene of Rifr. M. tuberculosis isolates from RS. In this group, DNA sequence analysis of 38 resistant isolates revealed 9 different kinds of missense mutations within a 157-bp region of the rpoB gene. All isolates had a single-point mutation, and the highest frequency of mutation was observed in the codon Ser-531 (55%). Point mutations in codons 526 (29%), 516 (8%), 511 (3%), and 522 (3%) were also observed (Table 2). We found no mutations in the 157-bp rpoB segment sequenced from one Rifr isolate and 16 Rifs isolates.

Mutations in the rpoB gene of Rifr. M. tuberculosis isolates from SP. Twenty M. tuberculosis isolates (19 Rifr and one Rifs) were analyzed. Single-point mutations were detected in 18 of the isolates with the resistant phenotype. Seven different kinds of nucleotide substitution were revealed in four codons of the rpoB gene. The mutations were located in codon 531 (58%) and in codon 526 (26%). Two samples had changes of two bases in the same codon. Mutations in codons 522 (5%) and 533 (5%) were also detected (Table 2). One M. tuberculosis Rifr isolate contained no mutation within the region of the rpoB gene examined. Two new alleles (526CTG [encoding Leu] and 533CCT [encoding Pro]) were identified in this group of isolates (isolates SP28 and SP22). No mutations were observed in the Rifr isolate.
Mutations in the rpoB gene of Rifr M. tuberculosis isolates from RJ. The analysis of 26 M. tuberculosis isolates (25 Rifr and 1 Rifd) from RJ showed 10 mutations within a 157-bp region of the rpoB gene, affecting 12 amino acids in this region. Sixteen isolates presented one point mutation in codons 531 (52%), 516 (8%), and 513 (4%), while one isolate (4%) had a 4-codon deletion (515 to 518) and another isolate contained a point mutation in one codon (513) and a 3-codon deletion (514 to 516). Four Rifr isolates (16%) of M. tuberculosis contained point mutations in two separate codons, resulting in two amino acid substitutions for each isolate (513 and 514; 511 and 515; 531 and 526; and 511 and 516). One isolate had point mutations in three separate codons (524, 525, and 526) and a one-codon deletion (527) (Table 2). One M. tuberculosis Rifr isolate contained no mutations within the 157-bp region of the rpoB gene. Four other isolates were identified in this group, all involving changes in two or more codons. Isolate RJ37 showed changes in codons 514 and 531. In isolate RJ48, the codons 524, 525, and 526 changed, together with a deletion of codon 527. Isolate RJ49 showed a deletion of codons 514 to 516 associated with a change in codon 513, and isolate RJ55 showed a deletion of four codons (515 to 518).

General analysis. Twenty-one different types of mutations were identified in 82 Rifr M. tuberculosis clinical isolates, and six new alleles were identified (Table 2). Most of them were single-nucleotide mutations (81%) involving seven codons. Eleven isolates (13%) exhibited more complex mutations. No silent substitutions were observed in the rpoB gene region examined for any of the M. tuberculosis isolates analyzed in this study. The codons most frequently affected by point mutations were 531, 526, and 516, with frequencies of 54%, 21%, and 7%, respectively. Although Codon 526 was the second most affected, in Rifr isolates from RJ no mutation restricted to this codon was observed. No mutations were revealed in the rpoB segment sequenced from 18 Rifd isolates. Three Rifd isolates (4%) contained no mutations in this sequenced region, although these isolates were resistant to rifampin as determined by the proportional method.

The presence of mutations in a restricted region of the rpoB gene has been found in more than 96% of M. tuberculosis strains with various levels of rifampin resistance (4, 6, 10, 15, 18). While more than 20 distinct missense mutations within the 69-bp hypervariable region of rpoB accounting for rifampin resistance in M. tuberculosis have been reported (9), two of these mutations (Ser531→Leu and His526→Tyr) account for ≥65% (61% in our study) of rifampin resistance (6, 15, 16). These substitutions were considered important to the acquisition of rifampin resistance, and it is clear that these two amino acids are critical sites for this characteristic.

In our study, we observed that 82% of the M. tuberculosis isolates with the Rifr phenotype contained missense mutations which led to amino acid substitutions at the Ser531 (54%), His526 (21%), and Asp516 (7%) residues. Similar mutations and frequencies of codon substitution in Rifr M. tuberculosis have been reported previously (6, 7, 13, 14, 15). Other missense mutations as well as deletions were found in 21% of the Rifr M. tuberculosis strains. A characteristic finding was the high frequency of double mutations occurring in two separate codons (16%) in the RJ isolates and more complex mutations, such as a combination of point mutations with a deletion of one or more codons in two cases [CAA(Gln33)→CAC(His), with a deletion of codons encoding Lys524, Tyr525, and Leu516; TGG(Leu524)→TGG(Trp), AAC(Tyr526)→CCC(Pro), and CAC(His526)→CAG(Gln), with a deletion of the codon for Phe524 and deletion of four codons (encoding Tyr525, Leu516, Val517, and Leu518) in one isolate.

In general, isolates from RS and SP showed some similarity in the mutation frequencies. Samples from RJ showed a wider range of the mutations described above. Some of the mutations observed in RJ isolates were not found in other isolates analyzed in this work and in isolates from other published studies.
Further studies must be done to find an explanation for such differences.

Sequence analysis identified no mutation in three of the isolates tested, although these isolates were resistant to rifampin. Similar observations have been reported by others (6, 7, 15) and suggest that mutations located outside the region of analysis can result in rifampin resistance. Another possibility is that in these resistant strains, changes have occurred in genes whose products participate in antibiotic permeation or metabolism (6). No silent mutations were observed in the sequenced regions of rifampin-susceptible or -resistant M. tuberculosis isolates in this analysis.

No association was found between particular mutations in the rpoB gene and drug susceptibility patterns of MDR-TB isolates, supporting the view that the mutations leading to rifampin resistance are independent events unrelated to these isolates affecting the development of resistance to the other antibiotics tested (18).

In this study, we identified mutations in rifampin-resistant M. tuberculosis strains from Brazil that were commonly found in strains from other parts of the world. We found six new alleles, four of them in isolates from Rio de Janeiro.

**Table 2. Mutations of the rpoB gene found in Rif' M. tuberculosis isolates from Brazil**

<table>
<thead>
<tr>
<th>Mutated codon</th>
<th>Specific mutation</th>
<th>No. (%) of mutated sites/origin of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>511</td>
<td>CTG(Leu)—CCG(Pro)</td>
<td>1 (1.2)/RS</td>
</tr>
<tr>
<td>513</td>
<td>CAA(Asp)—CCA(Pro)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>516</td>
<td>GAC(Asp)—GTC(Val) TAC(Tyr)</td>
<td>5 (6.2)/RS and RJ</td>
</tr>
<tr>
<td>522</td>
<td>TCG(Scr)—TTG(Leu)</td>
<td>2 (2.4)/RS</td>
</tr>
<tr>
<td>526</td>
<td>CAC(His)—CTC(Leu) TAC(Tyr) CCG(Arg) TGC(Cys) GAC(Asp) CTTG(Leu)</td>
<td>2 (2.4)/RS and SP</td>
</tr>
<tr>
<td>531</td>
<td>TCG(Scr)—TTG(Leu) TGG(Trp)</td>
<td>41 (50)/RS, SP, and RJ</td>
</tr>
<tr>
<td>533</td>
<td>CTG(Leu)—CTT(Pro)</td>
<td>1 (1.2)/SP</td>
</tr>
<tr>
<td>531 and 514</td>
<td>TCG(Scr)—TTG(Leu) and TTC(Phe)—GTC(Val)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>531 and 526</td>
<td>TCG(Scr)—TTG(Leu) and CAC(His)—TGC(Cys)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>511 and 516</td>
<td>CTG(Leu)—CGG(Arg) and GAC(Asp)—GTC(Val)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>511 and 515</td>
<td>CTG(Leu)—CCG(Pro) and ATG(Met)—ATA(Ile)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>524, 525, 526, and del 527</td>
<td>TTG(Leu)—TTG(Trp), ACC(Thr)—CCC(Pro), CAC(His)—CAG(Val), and del AA(Val)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>513 and 514, 515, and 516</td>
<td>CAA(Asp)—CAC(His) and del TTC(Phe), ATG(Met), and GAC(Asp)</td>
<td>1 (1.2)/RJ</td>
</tr>
<tr>
<td>Del 515, 516, 517, and 518</td>
<td>Del ATG(Met), GAC(Asp), CAG(Val), and AAC(Val)</td>
<td>1 (1.2)/RJ</td>
</tr>
</tbody>
</table>

*a* Codon numbers correspond to the Escherichia coli numbering system for the RNA polymerase β subunit. The mutated bases are shown in bold. Del, deletion of.

*b* Double mutations in the same codon.

*c* The Rif’ isolates showing no mutation within the 69-bp hypervariable region (3 of 82) were not included.

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**REFERENCES**


