rpoB Gene Mutations in Rifampin-Resistant 
*My*cobacterium *tuberculosis* Identified by 
Polymerase Chain Reaction Single-Stranded 
Conformational Polymorphism

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The use of polymerase chain reaction-single-stranded conformational polymorphism (PCR-SSCP) to study rpoB gene mutations in rifampin-resistant (RIFr) Mycobacterium tuberculosis has yielded contradictory results. To determine the sensitivity of this method, we analyzed 35 RIFr strains and 11 rifampin-susceptible (RIFs) strains, using the DNA sequencing of the core region of rpoB for comparison. Of the RIFr, 24 had a PCR-SSCP pattern identical to that of H37Rv; the other 11 had four different patterns. The 11 RIFs had PCR-SSCP patterns identical to that of H37Rv. The sensitivity of the assay was 31.4%; its specificity was 100%. We observed a strong correlation between the degree of resistance and the type of mutation.

In the developed world, tuberculosis (TB), once considered to have been essentially eliminated, has rebounded and is increasingly caused by drug-resistant strains. In developing countries, however, TB has been an unrelenting scourge. Increasing international travel and migration contribute to its widespread dissemination. Consequently, in 1993, the World Health Organization declared TB to be a global emergency (1).

Drug-resistant TB is a widespread phenomenon, with primary isoniazid-resistance rates as high as 32% and primary multidrug resistance close to 15% in the former Soviet Union. In Latin America, primary resistance to isoniazid varies from 1% in Uruguay to 20% in the Dominican Republic, and primary multidrug resistance is as high as 7% in the Dominican Republic and 5% in Argentina (2). In 1995, we reported increasing resistance rates to isoniazid and rifampin, four times higher than previously reported rates for Mexico (3). Since then, several studies have addressed this issue in different settings: urban, semi-urban, and rural areas. The common finding has been a high rate of primary resistance to isoniazid and to the combination of isoniazid and rifampin (4,5). In 2000, a collaborative effort between the Centers for Disease Control and Prevention and the Mexican TB control program reported an 11% rate of primary isoniazid resistance and 2% of primary multidrug resistance (6).

From the public health perspective, the impact of resistance on disease and death has recently been emphasized (7) in settings where HIV is highly prevalent. However, its impact is also high in semi-urban settings without the influence of HIV infection (8). Thus, reliable methods are urgently needed to rapidly detect resistance, particularly to rifampin (a marker for multidrug resistance), without cumbersome traditional methods or use of radioactivity (9).

Several techniques use polymerase chain reaction (PCR)-based strategies to rapidly detect mutations known to confer resistance. One such method is single-stranded conformational polymorphism (SSCP) analysis, which involves amplification by PCR of a segment of the gene encoding for the specific drug target and comparison of PCR products of drug-sensitive and drug-resistant strains by SSCP, in which mutations usually result in an altered pattern (9,10). This technique is relatively simple and was promising initially, but recent studies have questioned its sensitivity and specificity (10). We investigated the usefulness of PCR-SSCP to detect mutations in the rpoB gene of Mycobacterium tuberculosis strains with a wide range of rifampin resistance and whether specific mutations in this gene are associated with degree of rifampin resistance.

Methods

Clinical Isolates

Forty-six clinical isolates of M. tuberculosis were included in this study; all isolates were recovered from sputum samples of patients from Mexico City and were fully characterized by conventional methods (11). All strains were resistant to at least one primary antituberculosis agent (isoniazid 0.1 µg/mL, rifampin 2 µg/mL, streptomycin 6 µg/mL, etc.).
or ethambutol 7.5 µg/mL). Thirty-five strains were rifampin resistant (RIFr), and 11 were rifampin sensitive (RIFs). MICs to the primary antituberculosis drugs were determined by the radiometric method (Becton Dickinson, Cockeysville, MD) (12).

**PCR Amplification**

Chromosomal DNA was extracted by conventional methods (13). A 157-bp fragment of the rpoB gene was amplified by PCR with primers Tb8 (5’TGCACGTGCACGACCCCTCA3’) and Tb9 (5’TGCACGTGCATCAAGAGGT3’). PCR was carried out in 50 µL of a reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM MgCl₂, 100 µM of deoxynucleoside triphosphates (dNTPs), 1U Taq polymerase, 10 pmol of each set of primers, and 10 ng of chromosomal DNA. Samples were then subjected to one cycle at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, and a final cycle at 72°C for 8 min to complete the elongation of the PCR intermediate products. PCR products were then run on 2% agarose gels and examined for the presence of the 157-bp band after ethidium bromide staining.

**Screening of SSCP-PCR Products**

The SSCP of PCR products was analyzed by electrophoresis with 12% acrylamide gels. In brief, 25 µL of the amplified product was diluted with 100 µL of buffer (0.1% sodium dodecyl sulfate, EDTA 10 mM); 3 µL of this dilution was mixed with 3 µL of loading buffer (95% formamide, 20 mM EDTA, and 0.05% each of bromophenol blue and xylene cyanol). The mixtures were boiled for 2 min, cooled in ice for 5 min, and then loaded on the gel at 40V for 10 h at room temperature. The gels were silver stained and allowed to dry. The drug-susceptible strain H37Rv was run side by side with each strain in each lane for comparison. For SSCP analysis, PCR products were sequenced directly on an Applied Biosystems 373A automated DNA sequencer (Perkin Elmer, Foster City, CA). Samples that gave a single band on agarose gels were purified (Wizard PCR Preps, Promega, Madison, WI) to remove excess primers and nucleotides. Sequencing was done with a PRISM dye terminator cycle sequencing kit (Perkin Elmer), following the manufacturer’s instructions.

**DNA Sequencing**

A 411-bp fragment of the rpoB gene, containing the sequence of the 157-bp rpoB fragment, was amplified by PCR using primers TR1 (5’ TACGGTCGGCGAGCT GATCC3’) and TR2 (5’ TACGGCGCTTGTGATGAACC3’). PCR was carried out in 25 µL containing 50 mM KCl, 10 mM Tris (pH 8.0), 0.7 mM MgCl₂, 100 µM dNTPs, 1U Taq polymerase, and 10 ng of DNA template. Samples were then subjected to one cycle at 94°C for 5 min, followed by 10 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 8 min to complete the elongation of the PCR intermediate products. These products were characterized by electrophoresis on 2% agarose gels and stained in 0.5 µg/mL of ethidium bromide.

PCR products were sequenced directly on an Applied Biosystems 373A automated DNA sequencer (Perkin Elmer, Foster City, CA). Samples that gave a single band on agarose gels were purified (Wizard PCR Preps, Promega, Madison, WI) to remove excess primers and nucleotides. Sequencing was done with a PRISM dye terminator cycle sequencing kit (Perkin Elmer), following the manufacturer’s instructions.

**Statistical Analysis**

Sensitivity and specificity of the PCR-SSCP method were determined by using the test for 2X2 contingency tables. Differences in the mean MIC logs among strains with specific mutations were calculated by the two-sample Wilcoxon rank-sum test (Mann-Whitney U test).

**Results**

**Rifr Pattern among M. tuberculosis Isolates**

The 35 RIFr isolates had MIC values as follows: two isolates had an MIC of 2 µg/mL; six of 8 µg/mL; one of 16 µg/mL; one 32 µg/mL; two of 64 µg/mL; two of 128 µg/mL; eight of 256 µg/mL; one of 512 µg/mL; two of 1,024 µg/mL, and ten of 2,048 µg/mL. All 11 rifampin-susceptible isolates had MIC values ≤0.5 µg/mL, but all of them were resistant to at least one other primary antituberculosis agent.

**SSCP Analysis**

SSCP assays were repeated at least five times with three different amplicons for all isolates with 100% reproducibility. On the basis of the SSCP results, the 35 RIFr isolates were grouped in two main categories: group one, 24 isolates (68.6%) with an SSCP identical to that of the control strain H37Rv, and group two, 11 isolates (31.4%) with an SSCP different from that of H37Rv. The MICs were variable in group one. In group two, one polymorphism was observed, with different MICs (Figure). The 11 RIFs isolates showed an SSCP identical to that of H37Rv. Therefore, the overall sensitivity of the assay was 31.4%, with a specificity of 100%. It was not possible to correlate the MIC values with the polymorphisms because each strain had a different MIC.

**DNA-Sequencing Analysis**

No mutations were found in the core region of the rpoB gene in the 11 RIFs isolates. All 35 RIFr isolates showed a mutation by sequence analysis. Seven different missense mutations were observed, with all but one detected within the core region. These mutations produced 13 changes in amino acid content (Table). Mutations at specific codons were associated with the level of resistance; significantly higher MICs were observed when point mutations occurred in codon 513 (median MIC 2,048 µg/mL; p=0.001), in codon 526 (median MIC 2,048 µg/mL; p=0.002), and in codon 531 (median MIC 256 µg/mL, p=0.002), compared with mutations at codon 516 (median MIC 8 µg/mL). Single strains

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**Figure.** Representative polymerase chain reaction-single-stranded conformational polymorphism (SSCP) patterns of rifampin resistance in Mycobacterium tuberculosis strains. Lanes: 1, 18, molecular weight ΦX174/HaeIII; lanes: 2, 17, M. tuberculosis rifampin susceptible control strain H37Rv; lanes: 3, 4, 5 (MIC's 2,8,64 µg/mL), pattern 1 rifampin-resistant strains with a pattern indistinguishable from that of M. tuberculosis H37Rv; lanes 6, 7, 8 (MIC's 2, 8, 2048 µg/mL), pattern 2a; lanes 9, 10, 11 (MIC's 2048, 256, 256 µg/mL), pattern 2b; lanes 12, 13, 14, 15 (MIC's 2048, 2048, 256, 1,024 µg/mL), pattern 2c; lane 16 (MIC 128 µg/mL), pattern 2d.
with low-level resistance had mutations at codons 522 (MIC 8 µg/mL), 533 (MIC 2 µg/mL), and 572 (MIC 2 µg/mL) (Table). Three mutations in codon 531 had not been described previously. Neither insertions nor deletions were detected in this group of strains.

**Discussion**

PCR-SSCP has been used extensively to search for genetic diseases (14,15) and recently to detect missense mutations associated with antibiotic resistance in *M. tuberculosis* (9,10,16,17). In spite of extensive and comprehensive standardization of the PCR-SSCP method, our data show that this procedure was highly specific but had poor sensitivity for detecting mutations in the rpoB gene in rifampin-resistant clinical isolates of *M. tuberculosis*, since two thirds of the resistant isolates had a PCR-SSCP pattern similar to that of the *M. tuberculosis* susceptible control strain H37Rv.

Our results differ from those of the investigators who first tested this technique to detect rifampin resistance in *M. tuberculosis* (11,19). They compared the PCR-SSCP and DNA sequencing methods in detecting rifampin resistance and found that the SSCP method performed poorly. This is consistent with our results, where 33 of 41 strains (80.5%) had mutations detected by DNA sequencing but not by PCR-SSCP. However, our findings confirm a recent report of a rifampin-resistant strain identified by PCR-SSCP in Korea (10,11). Moreover, we found that PCR-SSCP may be a useful tool for detecting resistance in some strains, especially in strains with resistance to other anti-tuberculosis drugs (12).

**Table. Mutations of the rpoB gene found in 35 rifampin-resistant Mycobacterium tuberculosis isolates**

<table>
<thead>
<tr>
<th>Mutated rpoB codon</th>
<th>Specific Mutation</th>
<th>Strain n</th>
<th>MIC (µg/mL)</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td>513</td>
<td>CAA/AAA(Gln/Lys)</td>
<td>1</td>
<td>2,048</td>
<td>0.01</td>
</tr>
<tr>
<td>526</td>
<td>CAC/TAC(His/Tyr)</td>
<td>1</td>
<td>1,024</td>
<td>0.01, 0.002</td>
</tr>
<tr>
<td>531</td>
<td>TCG/TTG(Ser/Leu)</td>
<td>1</td>
<td>32</td>
<td>0.002</td>
</tr>
<tr>
<td>572</td>
<td>ATC/TTC(Ile/Phe)</td>
<td>1</td>
<td>2,048</td>
<td>0.002</td>
</tr>
</tbody>
</table>

*This mutation is located outside the core region.*

*Not previously described.*

*Mann-Whitney test.*

In conclusion, the practical implications of our study are that the PCR-SSCP method may not be a reliable tool for the detection of resistance to rifampin in *M. tuberculosis*. However,
if our observation of a strong correlation between specific mutations and the level of resistance is confirmed in other settings, the level of rifampin resistance may be predictable by DNA sequence-based resistance detection methods.

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References