New Multiplex PCR for Rapid Detection of Isoniazid-Resistant
*Mycobacterium tuberculosis* Clinical Isolates

Laura Herrera-León,* Tamara Molina, Pilar Saíz, Juan Antonio Sáez-Nieto, and Maria SoledadJiménez

Mycobacterial Reference Laboratory, Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain

Received 4 February 2004/Returned for modification 26 June 2004/Accepted 11 September 2004

In this study, we describe a multiplex PCR to detect a AGC→ACC (serine to threonine) mutation in the *katG* gene and a −15 C-to-T substitution (*inhA*Δc−15T) at the 5′ end of a presumed ribosome binding site in the promoter of the *mabA-inhA* operon. These mutations have been reported in the majority of previous studies as the most frequent mutations involved in the resistance to isoniazid (INH) of *Mycobacterium tuberculosis* clinical strains with high levels of resistance. The method was optimized and validated after an analysis of 30 *M. tuberculosis* clinical isolates with known sequences of the relevant part of the *katG* gene and the regulatory region of the *mabA-inhA* operon. We analyzed 297 INH-resistant *M. tuberculosis* isolates collected in Spain from 1996 to 2003 by PCR-restriction fragment length polymorphism (using the *katG* gene), DNA sequencing, and the newly developed multiplex PCR. The results were concordant for all 297 isolates tested. The analysis revealed that 204 (68.7%) of the isolates carried one or both of the mutations. This finding suggests that with further development this multiplex PCR will be able to detect the majority of the INH-resistant *M. tuberculosis* clinical isolates from Spain and other countries where a high frequency of similar mutations occur.

Currently, throughout the world, isoniazid (INH) and rifampin (RIF) together represent the backbone of short-course chemotherapy for *Mycobacterium tuberculosis* infections. The number of multidrug-resistant strains of *M. tuberculosis*, defined as resistant to INH and RIF, has been increasing over the years, and several outbreaks have been reported (5, 9, 24). The development of resistance to these two drugs reduces the efficacy of standard antituberculosis (anti-TB) treatment to 77%. It is very important, therefore, to identify these strains as soon as possible to allow for adjustments in treatment and to minimize the transmission of drug-resistant strains. Phenotypic drug susceptibility testing by conventional methods on solid media (6, 8) requires 10 to 30 days after the primary culture has been isolated. This time can be reduced by the use of rapid methods such as BACTEC, which requires 5 to 10 days.

Resistance to RIF has been shown to be caused by an alteration of the β subunit of RNA polymerase, which is encoded by the *rpoB* gene. More than 95% of RIF-resistant strains are associated with mutations within an 81-bp region of the *rpoB* gene (encoding the RNA polymerase β subunit). Specific mutations, insertions, and deletions have been described in several countries by several authors, and this 81-bp region has been termed the rifampin resistance determinant region (7, 14, 20, 26, 27, 30). Numerous methods exist to detect resistance to rifampin (10, 12, 18, 23).

In contrast, resistance to INH is more complicated, as mutations in several genes can lead to drug resistance. For most INH-resistant strains, mutations have been found in two genes, i.e., the *katG* gene, encoding catalase-peroxidase (31), and the *mabA-inhA* regulon (4), encoding a target of activated prodrug, enoyl-acyl carrier protein reductase (1–3, 11, 13, 15, 17, 21, 27). For some other INH-resistant strains, however, mutations in the *ahpC* promoter region (located in the 105-bp *oxyR-ahpC* intergenic region) or within the β-ketocyl acyl carrier protein synthase gene *kasA* have also been reported (19, 25). Most studies have examined the mutations present in these genes by DNA sequencing or analyses of a portion of the *katG* gene after PCR amplification and digestion with the restriction enzyme MspI or SatI (2, 3, 17).

Molecular methods have been developed to detect resistance to INH and RIF as an alternative to conventional tests because of their ability to provide results rapidly. Upon the elucidation of the genes involved in resistance to rifampin and INH, several studies describing various PCR-based molecular genetic techniques for the detection of resistance were published (12).

In the present study, we report a simple, rapid, and inexpensive assay based on allele-specific PCR methodology targeting an AGC→ACC mutation in the *katG* gene and an *inhA*Δc−15T mutation in the regulatory region of the *mabA-inhA* operon to detect INH-resistant *M. tuberculosis* strains and to identify the *M. tuberculosis* complex in the same PCR tube for each sample.

**MATERIALS AND METHODS**

**Description of study samples.** (i) **Set one.** Thirty *M. tuberculosis* strains with known sequences for the relevant part of the *katG* gene and the regulatory region of the *mabA-inhA* operon served as samples for optimization of the PCR assay conditions. The strains had the following distribution of *katG* and *mabA-inhA* operon alleles: wild type, 6 strains; AGC→ACC mutation, 10 strains; *inhA*Δc−15T mutation, 10 strains; and both the AGC→ACC and *inhA*Δc−15T mutations, 4 strains.

(ii) **Set two.** A total of 297 isolates collected from 1996 to 2003 in Spain and identified as INH-resistant *M. tuberculosis* strains by standard biochemical methods served to validate the developed multiplex PCR assay. Susceptibility testing with INH (0.2 and 1.0 μg/ml) was performed with Lowenstein-Jensen medium by
Fig. 1. Schematic presentation of multiplex allele-specific PCR to detect the AGC→ACC mutation in codon 315 of the katG gene (A) and the −15 C-to-T substitution (inhAΔC−15T) at the 5’ end of a presumed ribosome binding site in the promoter of the mabA-inhA operon (B). Short arrows indicate the primers, and long double-headed arrows depict the resulting PCR fragments. The targeted sequences are shown in shaded boxes. The mutated bases are underlined and in bold, and the mutated bases are underlined.

The proportional method of Canetti et al. (6). A total of 50 M. tuberculosis strains that were susceptible to isoniazid were used as controls. All sequence data were assembled and edited electronically with the EDITSEQ, ALIGN, and MEGALIGN programs (DNASTAR, Madison, Wis.) and were compared with the published sequences for the EDITSEQ, ALIGN, and MEGALIGN programs (DNASTAR, Madison, Wis.) and were sequenced by use of a Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems). This fragment was the individual positive control and needed to be present in every sample. The absence of this amplification product would show that the PCR was inhibited and that the result should be ignored.

An analysis of 297 INH-resistant and 50 INH-susceptible M. tuberculosis clinical isolates from TB cases by katG PCR-RFLP and automated DNA sequencing showed that none of the 50 INH-susceptible strains had the AGC→ACC mutation (S315T) at codon 315 of katG or the less frequent mutations in that codon. The AGC→ACC missense mutation was observed in 130 (43.8%) INH-resistant strains by PCR-RFLP with an MspI endonuclease treatment (Fig. 2, lanes 2 and 3), and 17 INH-resistant strains showed a different mutation in codon 315 by PCR-RFLP with a SatI endonuclease treatment (Fig. 2, lanes 10 and 11). The use of the MspI endonuclease only detected the AGC→ACC mutation, whereas SatI was able to detect any mutation in codon 315 that affected the GC nucleotides at that position. Of the 17 strains with different mutations, 12 (4.0%) showed an AGC→AAC mutation at codon 315 of katG and 5 (1.7%) showed an AGC→ACA mutation by automated DNA sequencing. The results are summarized in Table 2.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Sequencing primers</th>
<th>Primer designation</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>katG</td>
<td>katGF</td>
<td>GTGCCCGAGACACACCCACCC</td>
</tr>
<tr>
<td></td>
<td>katGR</td>
<td>CGACGCTGACCTGTCGGAGG</td>
</tr>
<tr>
<td>mabA-inhA</td>
<td>mabAF</td>
<td>CGAAGTGCTGACTGACCCCG</td>
</tr>
<tr>
<td></td>
<td>inhAR</td>
<td>CCCACCGGAATGGCGGTG</td>
</tr>
<tr>
<td>gwB</td>
<td>MTUBI</td>
<td>TCGACGCGTATGGGATATC</td>
</tr>
<tr>
<td></td>
<td>MTUBr</td>
<td>ATCAGTCTTGGACCTTGGC</td>
</tr>
<tr>
<td>katG</td>
<td>katGF</td>
<td>CGACGCTGACCTGTCGGAGG</td>
</tr>
<tr>
<td></td>
<td>katGR</td>
<td>CGAAGTGCTGACTGACCCCG</td>
</tr>
<tr>
<td>mabA-inhA</td>
<td>mabAF</td>
<td>CGAAGTGCTGACTGACCCCG</td>
</tr>
<tr>
<td></td>
<td>inhAR</td>
<td>CCCACCGGAATGGCGGTG</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

M. TUBERCULOSIS
The $inhA^{C-15T}$ mutation was found in 70 (23.6%) INH-resistant isolates, with 4 of these strains showing the AGC→ACC mutation as detected by PCR-RFLP, and in none of the 50 INH-susceptible strains (Table 2).

The multiplex PCR that we developed was evaluated for its detection of the AGC→ACC mutation in codon 315 of the $katG$ gene and the $inhA^{C-15T}$ mutation in the promoter of the $mabA$-$inhA$ operon in the same PCR tube for each sample. The 130 INH-resistant strains with the AGC→ACC mutation produced a 296-bp band (Fig. 3, lanes 1 to 4), while the 70 strains with the $inhA^{C-15T}$ mutation produced a 146-bp band (Fig. 3, lanes 5 to 12). The four strains with both mutations produced two bands, one at 296 bp and the other at 146 bp (Fig. 3, lanes 13 and 14). All of the strains, including the susceptible strains (Fig. 2, lanes 16 to 18), produced a 1,020-bp band by amplification of a partial sequence of the $gyrB$ gene. The multiplex PCR results were concordant with those generated by PCR-RFLP analysis and DNA sequencing for all of the strains tested (Table 2). The specificity of the multiplex PCR for the detection of INH resistance in resistant strains carrying the AGC→ACC mutation at codon 315 of $katG$ and/or the $inhA^{C-15T}$ mutation in our setting was 100%. The sensitivity of the multiplex PCR for the detection of INH resistance depended on the prevalence of these two mutations among the INH-resistant strains in each geographic area. In our setting, the sensitivity was 68.7%. The negative predictive value was 35%, and the positive predictive value was 100%. In Spain, the prevalence rates of the AGC→ACC allele at codon 315 of $katG$ and of the $inhA^{C-15T}$ mutation are 47.2 and 25%, respectively (unpublished data), whilst other mutations in codon 315 are found in 6.6% of INH-resistant strains. Consequently, 72.2% of the INH-resistant strains in our country could be detected with the multiplex PCR.

Mutations at the Ser315 codon of $katG$ and at the regulatory region of the $mabA$-$inhA$ operon occur most frequently in INH-resistant isolates (1–3, 11, 13, 15, 17, 21, 27). These mutations, AGC→ACC and $inhA^{C-15T}$, respectively, may be responsible for 70% of INH-resistant $M. tuberculosis$ cases.

The prevalence of mutations in codon 315 of $katG$ varies depending on the geographical region studied, with percentages ranging from 35% in Beirut (2) to 91% in Latvia and Russia (21, 27), while the prevalence of the $inhA^{C-15T}$ mutation varied from 32.7% in a study by Bakonyte et al. (3) to 3.3% in a study by Van Rie et al. (29), with several studied strains being similar to those in Bakonyte et al.’s study. Torres et al. (26) reported that this mutation was found with a frequency of 4.3% in strains isolated from Seville, Spain, but the present study shows a much higher frequency of 23.6%. The frequency of other mutations in codon 315 (5.7%) was found to be lower than those in other studies (1, 11).

The high prevalence of the AGC→ACC mutation in the $katG$ gene and of the $inhA^{C-15T}$ mutation in the promoter of the $mabA$-$inhA$ operon in the strains isolated from Spain suggests that the multiplex PCR technique can be used as a single, rapid tool for detecting INH resistance in $M. tuberculosis$ clinical strains with a high probability. The assay is easy to perform and interpret and could be implemented as part of the routine

---

**TABLE 2. Comparison of INH susceptibility test results obtained by the different methods used in this study**

<table>
<thead>
<tr>
<th>No. of strains</th>
<th>Proportional method&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR-RFLP</th>
<th>DNA sequencing&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td>R</td>
<td>AGC &gt; ACC</td>
<td>AGC &gt; ACC</td>
<td>AGC &gt; ACC</td>
</tr>
<tr>
<td>12</td>
<td>R</td>
<td>S</td>
<td>AGC &gt; ACC</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>R</td>
<td>S</td>
<td>AGC &gt; ACC</td>
<td>S</td>
</tr>
<tr>
<td>70</td>
<td>R</td>
<td>S</td>
<td>$inhA^{C-15T}$</td>
<td>$inhA^{C-15T}$</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>AGC &gt; ACC</td>
<td>AGC &gt; ACC + $inhA^{C-15T}$</td>
<td>AGC &gt; ACC + $inhA^{C-15T}$</td>
</tr>
<tr>
<td>76</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>50</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

<sup>a</sup> R, resistant; S, susceptible. When indicated, mutations were detected by the various methods.

<sup>b</sup> Performed on Lowenstein-Jensen medium.

<sup>c</sup> DNA sequencing of the $katG$ gene and the promoter of the $mabA$-$inhA$ operon.
practices of clinical laboratories in areas with a high prevalence of multidrug-resistant TB strains. For easier and unambiguous interpretations of multiplex PCR profiles, each run should include a wild-type strain (as a positive control for no amplification due to mutation) and one strain with a known mutation (as a positive control of amplification due to mutation). Furthermore, the procedure is inexpensive and requires only standard PCR and electrophoresis equipment. However, all negative results should be confirmed by conventional methods based on cell culture.

ACKNOWLEDGMENTS

We thank A. Valverde and M. A. García-Aranda for excellent technical assistance and A. Echeita and S. Herrera for their time and advice. We are indebted to Malcolm Yates for revisions of the English language in the manuscript.

This work was supported by Instituto de Salud Carlos III (0017/99).

REFERENCES