Rapid Direct Detection of Multiple Rifampin and Isoniazid Resistance Mutations in *Mycobacterium tuberculosis* in Respiratory Samples by Real-Time PCR

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Rapid detection of resistance in *Mycobacterium tuberculosis* can optimize the efficacy of antituberculous therapy and control the transmission of resistant *M. tuberculosis* strains. Real-time PCR has minimized the time required to obtain the susceptibility pattern of *M. tuberculosis* strains, but little effort has been made to adapt this rapid technique to the direct detection of resistance from clinical samples. In this study, we adapted and evaluated a real-time PCR design for direct detection of resistance mutations in clinical respiratory samples. The real-time PCR was evaluated with (i) 11 clinical respiratory samples harboring bacilli resistant to isoniazid (INH) and/or rifampin (RIF), (ii) 10 culture-negative sputa spiked with a set of strains encoding 14 different resistance mutations in 10 independent codons, and (iii) 16 sputa harboring susceptible *M. tuberculosis* strains, the mutation encoding resistance was successfully detected. No mutation was detected in any of the susceptible sputa. The test was applied only to smear-positive specimens and succeeded in detecting a bacterial load equivalent to 10^5 CFU/ml in sputum samples (10 acid-fast bacilli/line). The analytical specificity of this method was proved with a set of 14 different non- *M. tuberculosis* bacteria. This real-time PCR design is an adequate method for the specific and rapid detection of RIF and INH resistance in smear-positive clinical respiratory samples.

Tuberculosis has been treated effectively for many years. Nevertheless, it is still the second-leading cause of death from an infectious disease worldwide, and it has been reported that resistance to antituberculous drugs is increasing dramatically (28). One of the most important challenges in the control of tuberculosis is a rapid diagnosis of cases and the optimization of antituberculous treatment, mainly to prevent the development of resistance and the dissemination of resistant strains.

Isoniazid (INH) and rifampin (RIF) are considered the mainstays of antituberculous treatment (1, 29). The knowledge of the genetic basis of resistance to both drugs has provided molecular tools to rapidly detect the principal mutations conferring resistance to INH and RIF (2, 14, 16, 19). Ninety-five percent of RIF resistance mutations in *Mycobacterium tuberculosis* are located in an 81-bp region of the *rpoB* gene (20). The molecular basis of INH resistance is less well characterized, and mutations in several genes have been associated with it, although mutations in codon 315 of the *katG* gene are considered to be the most prevalent mutations encoding higher levels of resistance to INH (26).

Despite the use of new liquid medium cultures, the isolation of *M. tuberculosis* is still time consuming and leads to delays in obtaining susceptibility patterns. Rapid methods to detect resistance are necessary to optimize antituberculous treatment and avoid the transmission of resistant strains. In the last few years, several molecular methods to detect resistance mutations in *M. tuberculosis* have been described (2, 6, 14); however, they are labor intensive and frequently require well-grown cultures. Recently, real-time PCR has made the rapid detection of resistance mutations from *M. tuberculosis* isolates possible (4, 5, 7, 22). Nevertheless, little effort has been made to adapt these rapid methods to the detection of resistance directly from clinical samples (8, 10, 11, 13, 24, 27); therefore, genotypic susceptibility patterns still depend on culture.

Our aim is to adapt a real-time PCR design, recently developed in our laboratory (7), to assess resistance against RIF and INH in *M. tuberculosis* directly from clinical respiratory samples.

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MATERIALS AND METHODS

Clinical samples. Fifteen auramine-positive clinical respiratory samples whose cultures were positive for drug-resistant *M. tuberculosis* were recovered from our laboratory collection. Sixteen stain-positive respiratory samples harboring susceptible *M. tuberculosis* isolates were used as controls. The specimens were decontaminated by the N-acetyl-L-cysteine NaOH method (9) and conserved frozen at −70°C until use.

Spiked control specimens. An *M. tuberculosis*-negative sputum sample spiked with 10^4 CFU of *M. tuberculosis* H37Rv/ml was used as a susceptible control. To increase the variety of resistance mutations assayed, we obtained a set of 10 resistant controls after spiking a pool of *M. tuberculosis*-negative sputa with 10^4 CFU of 10 different *M. tuberculosis* strains/ml encoding 11 different resistance mutations in *rpoB* (codons 511, 513, 514, 515, 516, 517, 526, 531, and 533) and three different substitutions in codon 315 of *katG* (see Table 2). The resistance mutations assayed for the *rpoB* gene represent the different mutations detected in Spain up to the year 2002 because they are a selection of resistant strains compiled nationwide (provided by the Mycobacterium Reference Laboratory, Madrid, Spain).

As specificity controls, we spiked a pool of *M. tuberculosis*-negative sputa with six mycobacteria (final concentration, 10^6 CFU/ml) of *M. bovis, M. avium, M.
**FIG. 1.** Schematic representation of (a) the rpoB core region and RPO1 and RPO2 FRET probes and (b) the katG region and KAT probes.

In both figures, the most frequent mutant codons are shown in boldface type.
FIG. 2. Melting curves of wild-type (wt) strains and some representatives of mutant strains. (a) Melting curves obtained with RPO1 probes. A, deletion at codons 514 and 515; B, deletion at codons 515 and 516; C, double mutant in codons 511 and 516; D, deletion at codons 516 and 517; E, mutant in codon 513 (CAA→CGA); F, mutant in codon 516 (GAC→GTC). (b) Melting curves obtained with RPO2 probes. G, mutant in codon 531 (TCG→TGG); H, mutant in codon 531 (TCG→TTG); I, mutant in codon 526 (CAC→GAC); J, mutant in codon 533; K, mutant in codon 526 (CAC→TAC). (c) Melting curves obtained with KAT probes and mutations at codon 315 (AGC→ACC, AGC→ACA, and AGC→AAC).
microscopic quantification of the number of bacilli by auramine staining performed with 50 µl of these controls. The spiked sputa were analyzed with a Light-Cycler instrument as described above for clinical samples.

RESULTS

From 2000 to 2002, 30 INH- and/or RIF-resistant *M. tuberculosis* isolates were cultured from respiratory samples in our laboratory. Of these samples, 18 were stain positive and 15 (83%) were available for study from our collection.

**DNA sequencing.** The isolates which had been cultured from the specimens in analysis were sequenced. All samples containing RIF-resistant bacilli encoded mutations in *rpoB* (codons 514 and 515, 515 and 516, 526, and 531), and five samples harboring INH-resistant bacilli encoded mutations in codon 315 of *katG* (Table 1). Four samples harboring INH-resistant bacilli were excluded from the study due to the lack of mutations in *katG* and thus, resistance in these strains could not be detected genotypically.

**Real-time PCR detection of resistance in clinical samples.** The reference *T*\textsubscript{m} values for wild-type sequences were obtained by 10 repetitions of the assay using sputa spiked with the reference susceptibility control (*M. tuberculosis* H37Rv). The average reference values for wild-type sequences were as follows: 67°C for RPO1, 64.88°C for RPO2, and 68.88°C for KAT (standard deviations, 0.1, 0.17, and 0.22°C, respectively).

All clinical samples containing *M. tuberculosis* strains with a mutation in *rpoB* or *katG* were efficiently detected by this real-time PCR design. In all cases, *T*\textsubscript{m} deviations (more than two times standard deviation) were detected with respect to the susceptible reference *T*\textsubscript{m} (Table 1).

All clinical samples containing RIF- or INH-susceptible strains showed a *T*\textsubscript{m} within two times the standard deviation of the reference susceptible *T*\textsubscript{m}.

**Real-time PCR detection of resistance in spiked control specimens.** In order to evaluate this real-time PCR design with a broader spectrum of resistance mutations, we tested 10 control specimens obtained by spiking a pool of negative sputa with different *M. tuberculosis* strains with known resistant mutations: for *rpoB*, nine different substitutions in eight independent codons, one deletion, and one double mutant in two codons were found; and for *katG*, three different substitutions in codon 315 (Table 2).

Again, all samples harboring resistant strains were correctly detected by significant deviations from the reference wild-type *T*\textsubscript{m} values (Table 2).

**Analytical sensitivity and specificity of real-time PCR.** In order to assess the analytical sensitivity of the method, culture-negative sputa were spiked with serial dilutions of *M. tuberculosis* H37Rv and three different resistant strains. The amount of bacilli in each dilution was defined by determining the number of CFU in culture and by microscopic quantification of the number of bacilli. The test succeeded in detecting a bacterial load equivalent to 10⁸ CFU/ml (10 acid-fast bacilli line).

To determine whether the assay was specific for *M. tuberculosis*, we tested a set of negative sputa spiked with 14 nontuberculous mycobacteria and other bacterial genera. Only *M. bovis* (belonging to the *M. tuberculosis* complex) and *M. avium* (only with RPO1) led to a post-PCR melting fluorescent signal. Nevertheless, the *T*\textsubscript{m} value obtained with *M. avium* was completely different from that obtained with *M. tuberculosis* (*T*\textsubscript{m} = 53.7°C).

**DISCUSSION**

A rapid assessment of antituberculous drug resistance is essential for the clinical management of patients with tuberculosis and to avoid the transmission of resistant strains. At present, the quickest way of analyzing the susceptibility of *M. tuberculosis* isolates is by real-time PCR. In recent years, we and other authors have developed real-time PCR designs specialized for detecting mutations encoding resistance to RIF and INH (3–5, 7, 22, 25). Nevertheless, most of these assays have been applied only to cultured isolates and therefore lead to delays in assessing the susceptibility patterns, as the methods still require culture. Furthermore, the few studies which have applied real-time PCR for detecting resistance directly from clinical samples were able to detect only the most prevalent resistance mutations (4, 5, 25).

In the present study, we tried to resolve this dual requirement of rapidity and polyclonal detection of a wide variety of resistance mutations in *M. tuberculosis*. A real-time PCR assay based on rapid cycle technology, recently developed by García de Viedma et al. (7) to detect multiple resistance mutations in a single reaction tube, was adapted to analyze *M. tuberculosis* resistance directly from clinical samples. This adaptation required (i) the real-time PCR design to be converted to an asymmetric format in order to preferentially amplify the DNA strands complementary to the probes and (ii) the three PCRs (with RPO1, RPO2, and KAT probes) to be performed in three independent reaction tubes.

In this sense, this design is methodologically similar to the design recently published by Ruiz et al. (17), which used FRET probes and rapid-cycle PCR to detect resistance to RIF and INH directly from clinical samples. Ruiz et al. (17) succeeded in detecting mutations from four patients with RIF-resistant isolates and seven patients with INH-resistant isolates. The present study is different in several ways. First, we evaluated a much wider variety of mutations encoding resistance (14 different mutations in 10 independent codons). Secondly, in the study by Ruiz et al. (17), sequencing data were not available; therefore, neither the correlation of real-time PCR data with the genotypic “gold standard” nor the efficiency in detecting certain nucleotide substitutions in different codons can be assessed. In this study, sequencing data are available for all 46 specimens, and thus, we can assure (i) 100% correlation with DNA sequencing and (ii) the efficiency of this system for detecting not only some specific prevalent mutations but also a huge variety of different mutations, some of them very rare. This result makes our system suitable for different geographic settings, as has been proved in a study done in Poland (18) in which part of this design has been applied, allowing the detection of mutations different from those tested here. In addition, in our method, the search of all *rpoB* and *katG* regions shares PCR conditions and allows all mutations to be detected in a single run, which makes for earlier availability of results. As a potential methodological limitation, it could be argued that this design does not detect INH-resistant mutations out of *katG*315. Resistance to INH is less frequently found to be associated with mutations out of *katG*, and it is important to
TABLE 1. Genotypic and phenotypic characteristics of M. tuberculosis isolates cultured from the clinical samples studied and Tm s obtained with the real-time PCR assay

<table>
<thead>
<tr>
<th>Clinical sample no. (type)</th>
<th>Acid-fast auramine stain result</th>
<th>Resistance phenotype</th>
<th>Mutant codon</th>
<th>Nucleotidic substitution</th>
<th>Amino acid substitution</th>
<th>Tm (°C) (deviation from wild type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (sputum)</td>
<td>/H11001 (10/line)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>57.20 (9.8)</td>
</tr>
<tr>
<td>2 (bronchial aspirate)</td>
<td>/H11001 (50/field)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TCA-TGG55.0</td>
<td>Ser</td>
<td>71.15 (2.27)</td>
</tr>
<tr>
<td>3 (sputum)</td>
<td>/H11001 (5/field)</td>
<td>R</td>
<td>rpo31</td>
<td>CTC-GAC</td>
<td>Ser</td>
<td>58.72 (6.16)</td>
</tr>
<tr>
<td>4 (sputum)</td>
<td>/H11001 (50/field)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>59.05 (5.83)</td>
</tr>
<tr>
<td>5 (sputum)</td>
<td>/H11001 (10/line)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>58.77 (6.11)</td>
</tr>
<tr>
<td>6 (sputum)</td>
<td>/H11001 (20/line)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>59.85 (6.34)</td>
</tr>
<tr>
<td>7 (sputum)</td>
<td>/H11001 (10/line)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>59.83 (6.34)</td>
</tr>
<tr>
<td>8 (sputum)</td>
<td>/H11001 (20/line)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>59.85 (6.34)</td>
</tr>
<tr>
<td>9 (sputum)</td>
<td>/H11001 (10/line)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>58.77 (6.11)</td>
</tr>
<tr>
<td>10 (sputum)</td>
<td>/H11001 (20/field)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>58.77 (6.11)</td>
</tr>
<tr>
<td>11 (sputum)</td>
<td>/H11001 (50/field)</td>
<td>R</td>
<td>rpo31</td>
<td>Deletion TGG</td>
<td>Ser</td>
<td>58.77 (6.11)</td>
</tr>
</tbody>
</table>

Wild-type Tm s for RPO1, RPO2, and KAT are 67.0, 64.88, and 68.88°C, respectively.

Obtained at a magnification of x250, positive stain.

R, resistant; S, susceptible; I, intermediate.
specify that isolates with mutations in katG315 show high-level resistance to INH, whereas isolates with mutations in loci other than katG are associated with low or intermediate levels of resistance to INH. In this context (7), in 92% of the resistant strains with mutations other than those at katG, high MICs of INH (>3 μg/ml) were obtained, whereas most of the resistant strains included in the population (26). Taking all these data together, we believe that this real-time PCR approach could be criticized for having used only stain-positive samples obtained over 3 years, only three (17%) had a lower bacterial load. This finding means that this method would succeed in analyzing the susceptibility patterns of the majority of the stain-positive respiratory pathogens used as PCR templates. No amplification signal was found with 14 different non-M. tuberculosis pathogens. The only pathogens which led to an amplification signal were M. bovis and M. avium. It is not unexpected that M. bovis would lead to an amplification signal because it is included in the M. tuberculosis complex. With regard to M. avium, only the RPO1 probe produced a hybridization signal, and the Tm of the probe was easily differentiated from that obtained from M. tuberculosis (53.7 versus 67°C). This finding rules out the possibility of a misinterpretation of the results.

Second, the analytical sensitivity of the assay was calculated to be 10^3 CFU/ml (10 acid-fast bacilli/line) for the three regions assayed (RPO1, RPO2, and KAT), for both wild-type and mutant strains. In our laboratory, of the stain-positive samples obtained over 3 years, only three (17%) had a lower bacterial load. This finding means that this method would succeed in analyzing the susceptibility patterns of the majority of the stain-positive respiratory samples in this context. This method could be criticized for having used only stain-positive sputum samples. How useful this design is going to be in smear-negative specimens remains to be established. Few studies have data from stain-negative samples (10, 15, 27), and those studies are all based on traditional PCR and detect either rifampin or isoniazid resistance only, but never both. In our opinion, this limitation of analytical sensitivity should not be cause for concern.

When searching for resistant mutations directly from clinical samples, the two main requirements an assay should be its ability to (i) guarantee specificity in the detection of mutations, considering that respiratory samples frequently include additional bacteria, and (ii) obtain high analytical sensitivity values.

We first tested whether amplification occurred when different respiratory pathogenes were used as PCR templates. No amplification signal was found with 14 different non-M. tuberculosis pathogens. The only pathogens which led to an amplification signal were M. bovis and M. avium. It is not unexpected that M. bovis would lead to an amplification signal because it is included in the M. tuberculosis complex. With regard to M. avium, only the RPO1 probe produced a hybridization signal, and the Tm of the probe was easily differentiated from that obtained from M. tuberculosis (53.7 versus 67°C). This finding rules out the possibility of a misinterpretation of the results.

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bility when they receive the first laboratory evidence of *M. tuberculosis* infection, that is, microscopic observation of bacilli. Additionally, patients with stain-positive samples have a higher risk of causing transmission; therefore, the need for susceptibility patterns is particularly important in these cases. In all these situations, real-time PCR works properly, and requests for further assessment can be met satisfactorily.

For stain-negative samples, it generally makes no sense to apply the real-time PCR, considering the low rate of positivity for *M. tuberculosis* in most mycobacterial laboratories in this context. In this case, *M. tuberculosis* was isolated in only 5.7% of all samples received from requests for mycobacteria culture.

The method described here combines an easy and short DNA extraction from clinical samples with a rapid, sensitive, and specific real-time PCR design. The complete assay, including analysis, enables us to obtain resistance results in 1 1/2 hours. To our knowledge, this assay is the first one based on real-time PCR that is able to detect, simultaneously and directly from clinical samples, numerous different mutations conferring resistance to rifampin and those most frequently associated with high resistance to INH by using several FRET probes simultaneously in a single PCR run. The flexibility of real-time PCR assays to detect resistance mutations in different geographic settings cannot yet be answered, and international panels should be compiled and tested to answer this question.

The results obtained in this study lead us to propose this real-time PCR design as an adequate tool for the direct analysis of *M. tuberculosis* resistance in clinical samples. We are conscious that the introduction of real-time tools in the diagnostic laboratory requires specific installations and personnel to guarantee that the assay can be run upon receipt of the sample while traditional approaches to susceptibility tests are maintained, although this may not be the reality with regard to many laboratories. Efforts should be made, at least in specific reference laboratories, to adapt these new molecular procedures which allow the susceptibility pattern of *M. tuberculosis* to be obtained quickly, thus considerably reducing the time between diagnosis and initiation of individualized therapy.

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