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Genotypic Assessment of Isoniazid and Rifampin Resistance in *Mycobacterium tuberculosis*: a Blind Study at Reference Laboratory Level

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Progress in understanding the basis of resistance to isoniazid (INH) and rifampin (RMP) has allowed molecular tests for the detection of drug-resistant tuberculosis to be developed. Consecutive isolates (n = 95) of *Mycobacterium tuberculosis*, from a Spanish reference laboratory investigating outbreaks of multidrug-resistant tuberculosis, were coded and sent to two external laboratories for genotypic analysis of INH and RMP resistance by PCR–single-strand conformation polymorphism (SSCP) analysis of specific regions of four genes: part of the coding sequence of *katG* and the promoter regions of *inhA* and *ahpC* for INH and the RMP resistance region of *rpoB*. After correction for the presence of outbreak strains and multiple isolates from single patients, RMP resistance was detected successfully by PCR-SSCP in >96% of the RMP-resistant strains. PCR-SSCP had a sensitivity of 87% for INH resistance detection, and mutations in *katG*, *inhA*, *katG-inhA*, *ahpC*, and *katG-ahpC* were identified in 36.8, 31.6, 2.6, 13.2, and 2.6%, respectively, of the unique strains. Specificity was 100%. Molecular detection of resistance to the two main antituberculous drugs, INH and RMP, can be accomplished accurately by using a strategy which limits analysis to four genetic regions. This may allow the expedient analysis of drug resistance by reference laboratories.

In the last few years there has been considerable progress in our understanding of the mechanisms of action of the antimycobacterial agents and the basis of resistance to these compounds. To date, there is information about 12 genes involved in resistance in *Mycobacterium tuberculosis* (2, 20). Of greatest interest is the investigation of the basis of resistance to the two key drugs, isoniazid (INH) and rifampin (RMP), as effective genotypic analysis of resistance to these compounds would likely influence patient care and the utilization of hospital resources.

The mechanism of resistance to RMP involves missense mutations in a well-characterized region of the *rpoB* gene (encoding the β subunit of the RNA polymerase) (21); thus, investigation of rifampin resistance is relatively straightforward. In contrast, resistance to INH is associated with a variety of mutations affecting one or more genes such as those encoding catalase-peroxidase (*katG*) (28), the enoyl-acyl carrier protein reductase involved in mycolic acid biosynthesis (*inhA*) (1), and the recently described alkyl-hydroperoxide reductase (*ahpC*), which is involved in the cellular response to oxidative stress (7, 17, 27). Therefore, cost- and time-efficient investigation of INH resistance will require a strategy for targeted mutation analysis.

We performed a blind assessment of the accuracy of targeted genotypic examination of drug resistance. Analysis was limited to selected regions of four genes: *katG*, *inhA*, and *ahpC* for INH and the rifampin resistance region of *rpoB*. We also estimated the strain diversity in the population studied to address the confounding factor introduced when studying isolates from a defined geographical region. Our goal was to define the sensitivity and specificity of this approach at the reference laboratory level.

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MATERIALS AND METHODS

Study setting, patients, and isolates. The mycobacteriology laboratory at the Instituto Carlos III in Madrid, Spain, serves as reference laboratory for drug-resistant *M. tuberculosis* for the Madrid hospitals and for other Spanish medical centers. Consecutive isolates of *M. tuberculosis*, corresponding to susceptible, resistant, and multiresistant strains, were coded, and DNA lysates were sent to the Institut Pasteur, Paris, France (for INH resistance mutation analysis), and to the Institute for Medical Microbiology of Bern, Switzerland (for species confirmation, typing, and RMP resistance mutation analysis).

Upon completion of the study, the code was broken, and discrepancies were analyzed by (i) reculturing and susceptibility testing of the original isolate and, when necessary, of a second isolate, (ii) revision of previously obtained mutation analysis results, and (iii) genetic retesting of the original and second isolates, including sequencing of the corresponding gene(s).

In total, 95 isolates from 67 patients (13.5% new cases of tuberculosis in patients without AIDS, 31% cases of AIDS-related multidrug-resistant tuberculosis [MDR-TB], and 55.5% retreatment cases of tuberculosis) were investigated. Resistance testing was performed by the proportional method with 7H11 medium and the following critical drug concentrations: INH, 0.2 μg/ml; RMP, 1 μg/ml. Resistant organisms were further tested at higher concentrations (INH, 1 and 10 μg/ml; RMP, 64 μg/ml).

Sample preparation, PCR-RFLP, and rep-PCR. Preparation of isolates for shipping and PCR was accomplished by simply boiling a loopful of colonies for 30 min in 100 μl of distilled water, followed by pelleting of debris and storage at −20°C or shipping at room temperature. Supernatant (2.5 μl) was used as the template in all PCR mixtures, which contained 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 200 μM (each) deoxynucleoside triphosphate, 1.25 U of *Taq* polymerase, and 0.5 mM (each) primer. Species confirmation was performed by PCR-restriction fragment length polymorphism (RFLP) of *hsp65* as previously reported (23). Strain relatedness was investigated by rep-PCR (modified from reference 26). For this purpose, PCRs containing a single degenerate IRIS primer [5′-GAGTCTCCGGAC(A/T)(T/C)(G/A)CCGGGGCGG TTCA], representing the inverted repeats of the IS*6110* element in an outward direction, were performed using the following conditions: 5′ denaturation at 95°C, then 35 amplification cycles (each cycle was 94°C for 15 s, 45°C for 30 s, and 72°C for 30 s), and a final extension cycle of 7 min at 72°C in a Perkin-Elmer 2400

TABLE 1. Primers used in this study

Target	Primer	Product size (bp)
rpoB	TR8, 5′-TGCACGTCGCGGACCTCCA TR9, 5′-TCGCCGCGATCAAGGAGT	157
katG	TB86, 5′-GAAACAGCGGCGCTGGATCGT TB87, 5′-GTTGTCCCATTTCGTCGGGG	209
inhA	TB92, 5′-CCTCGCTGCCCAGAAAGGGA TB93, 5′-ATCCCCCGGTTTCCTCCGGT	248
ahpC	TB90, 5′-CCGATGAGAGCGGTGAGCTG TB91, 5′-ACCACTGCTTTGCCGCCACC	236

Cycler. Establishing strain relatedness allowed better estimates of the relative frequency of different mutations by avoiding the bias introduced by investigating multiple samples from single patients or from outbreak isolates.

PCR-SSCP. Fluorescently labelled primers for amplification of *rpoB*, *katG*, *ahpC*, and *inhA* are listed in Table 1. Postamplification single-strand conformation polymorphism (SSCP) mutation analysis of *rpoB* was performed by overnight electrophoresis in a Pharmacia ALF automated sequencer using mutation detection enhancement gel solution (FMC Bioproducts, Rockland, Maine) as previously described (22). SSCP mutation analyses of *katG*, *inhA*, and *ahpC* were performed by overnight electrophoresis on an Applied Biosystems 373A automated sequencer by using a 6% polyacrylamide gel containing 1× Tris-borate-EDTA buffer and 10% glycerol, and the run conditions were as recommended by Applied Biosystems. The electrophoretic mobility of each strand was determined with respect to Rox-labelled internal Genescan size markers (Applied Biosystems no. 401100), using the GENESCAN software of Applied Biosystems which enables efficient comparisons between different samples to be made. For negative and positive control purposes, DNA from the fully susceptible reference strains H37Rv or *Mycobacterium bovis* BCG Pasteur was used.

Additional mutation analyses. The presence of *rpoB* mutations was also investigated in a subset of 44 isolates by using the Inno-Lipa RTB kit (Innogenetics N.V., Zwijndrecht, Belgium) with the manufacturer's primers, PCR reagents, and recommendations. In addition, all isolates were tested for the *katG2-katG3* allele (9) by amplifying a 269-bp region with primers TB84 (5′-CCGGCACCT ACCGCATCCAC) and TB85 (5′-GCCCCAATAGACCTCATCGG). For INH-resistant isolates with no identified mutation, an additional region of *katG* was investigated for the presence of the *katG1* allele (R463L) (9). Direct sequencing of selected isolates was performed with Pharmacia or Applied Biosystems automated sequencers and corresponding sequencing kits from the same manufacturers.

RESULTS

All isolates were confirmed to be *M. tuberculosis* complex by PCR-RFLP analysis of *hsp65*. To determine the extent of strain relatedness the rep-PCR method was employed. This was successful in 91 of the 95 samples, and the isolates were assigned to 36 rep-PCR patterns. Of these, 26 patterns (36 isolates) corresponded to single isolates or multiple samples from the same patient, while one pattern corresponded to 17 isolates from an outbreak of MDR-TB among 10 human immunodeficiency virus-infected individuals. The remaining 38 isolates could be assigned to 18 individual genotypes by combining the data from rep-PCR and mutation analysis. Thus, a minimal estimate of 45 individual strains with singular genotype was established for the study collection.

Determination of drug resistance by phenotypic and genotypic methods. Susceptibility of *M. tuberculosis* isolates to INH and RMP was determined by the internationally accepted reference technique, the proportional method (8). On final analysis of the 95 isolates, 6 strains were characterized as fully susceptible, 85 were characterized as INH resistant, and 75 were characterized as RMP resistant. In total, 71 isolates were INH-RMP resistant (MDR-TB).

Preliminary mutation data analysis and resolution of discrepancies. Preliminary analysis of data from the 95 isolates revealed 9 discrepancies in the case of RMP between the drug susceptibility patterns determined by the proportion method and those obtained by PCR-SSCP. Seven discrepancies represented potential false negatives of SSCP (resistant by culture but susceptible by PCR-SSCP). Four of these isolates were found to be susceptible upon reculturing, and three were confirmed as resistant. The latter discrepancies were due to the abnormal SSCP profile being missed in two strains from the same patient, with the H526R mutation, while in the third isolate the SSCP pattern of a mixed population of resistant and susceptible strains was wrongly interpreted as being of the susceptible type. There were two potential false positives of SSCP (susceptible by culture but predicted to be resistant by PCR-SSCP). On reculturing two isolates were demonstrated to be resistant.

In the case of INH, there were 11 discrepancies between the drug susceptibility patterns determined by the proportion method and those obtained by PCR-SSCP. All discrepancies represented potential false negatives, and there were no false positives. Reculturing revealed four isolates to be susceptible. Six isolates were resistant, but no mutation was identified in the regions examined. An additional resistant strain, present in a mixed population, was falsely identified as susceptible.

After correction for errors in phenotype assessment or sample handling, PCR-SSCP results indicated a sensitivity of 96% for RMP resistance detection and of 91.7% for INH resistance detection among the collection of 95 isolates (Table 2). These values would represent real-life experience, as they reflect both the limitations of the tests (failure to detect all mutations with the proposed strategy) and operator errors (failure to recognize particular SSCP mutation patterns).

Correction for operator-related failures in the assessment of PCR-SSCP patterns allowed us to establish the best possible outcome for the defined four-gene detection strategy. Final corrected values indicated a sensitivity of 100% and a specificity of 100% for the detection of RMP resistance mutations; the sensitivity and specificity values remained at 91.7 and 100%, respectively, for INH detection.

The bias introduced by the presence of outbreak strains and multiple isolates from single patients was corrected by estimating the number of singular strains, defined by a unique genotype, in the collection (Table 2). PCR-SSCP successfully detected >96% of the RMP-resistant singular strains and 87% of the INH-resistant strains.

Solid-phase hybridization assay for RMP resistance. The performance of a commercially available solid-phase hybridization assay was evaluated in a subset of 44 isolates and

TABLE 2. Performance of PCR-SSCP for the detection of INH and RMP resistance mutations[a]

Genotype	No. of:			
	Isolates (n = 95)		Singular strains (n = 45)	
	R	S	R	S
INH				
R	78	0	33	0
S	7	10	5	7
RMP				
R	72	0	30	0
S	3	20	1	14

[a] To correct for the presence of multiple samples from single patients and for outbreak strains, results are also presented for singular strains, defined by a unique genotype. R, resistant; S, susceptible.

TABLE 3. Relative frequency of mutations associated with INH resistance in the three regions examined

Isolates	Localization of mutations [no. (%)]					
	katG	*katG*/*inhA*	*inhA*	*ahpC*	*ahpC*/*katG*	None
INH resistant (n = 85)	27 (31.8)	1 (1.2)	39 (45.9)	9 (10.6)	2 (2.4)	7 (8.2)
INH-resistant singular strains (n = 38)	14 (36.8)	1 (2.6)	12 (31.6)	5 (13.2)	1 (2.6)	5 (13.2)

compared with that of PCR-SSCP. The kit correctly identified 41 of the 42 RMP-resistant isolates and the two susceptible controls. Like SSCP, it missed the sample which contained a mixed isogenic population of susceptible and resistant strains.

Localization of mutations conferring resistance to INH. The relative frequency of mutations in the three regions examined, which are associated with INH resistance, is presented in Table 3. Alterations in the *inhA* regulatory region known to cause overexpression (nucleotide substitution C209T) (11) were present in 46% of the 85 INH-resistant isolates and were the most common mutation in the collection investigated. However, when data were reevaluated in terms of unique strains, *inhA* mutations were detected in 34% of the cases, whereas *katG* mutations proved more common and were identified in 42% of the unique strains (isolated or associated with mutations in other targets). Only one of these had a deletion in *katG*, confirming the rareness of this type of event (10, 12, 14, 18), while most had mutations affecting the codon for Ser315 (S315R or S315T), which was examined with primers TB86-TB87. Screening with *katG* primers TB84-TB85 did not add to the targeted strategy, as it identified only a G186A substitution in association with an *inhA* mutation. Mutations in the recently described *ahpC* promoter region were identified in 16% of singular circulating INH-resistant strains, including the isolate with a deletion in *katG*. Details of the base changes detected are shown in Fig. 1. Overall, our strategy, which targeted three sites, failed to account for INH resistance in 13.2% of singular strains (Tables 2 and 3). This could be due to mutations occurring in other parts of *katG* that were not screened in this study or in as yet unidentified genes. All of these isolates were examined for the presence of the *katG1* allele (R463L) that is associated with the lowest level of INH resistance, but none was found to harbor it (data not shown).

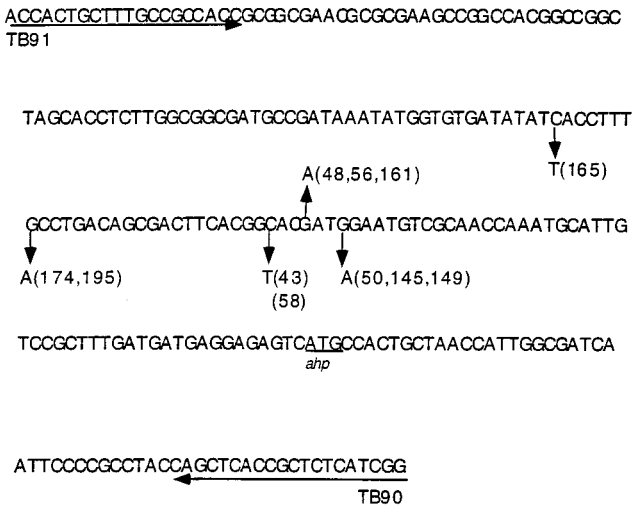

FIG. 1. Nucleotide sequence of the *ahpC-oxyR* intergenic region (GenBank accession number U16243). Indicated are the nucleotide substitutions associated with INH resistance. Numbers indicate the strain identifier.

DISCUSSION

In this study, the feasibility of genotypic testing for resistance to the two main antituberculosis agents, INH and RMP, was evaluated. We used a strategy that limits the number of reactions by targeting the screening to four specific regions in genes involved in resistance to these drugs.

Mutations in *rpoB* were identified in all RMP-resistant isolates and none of the susceptible strains, though operator error contributed to the misidentification of PCR-SSCP patterns for two strains from one patient and one strain present in mixed culture. Thus, in this blind study the real-life sensitivity of the test decreased to 96%. The commercially available Inno-Lipa RTB kit (5) performed equally well in the identification of *rpoB* mutations in a subset of 44 isolates, also missing the one strain present in a mixed culture. The results confirm the validity of *rpoB* testing for the detection of RMP resistance (3) and its potential importance as a surrogate marker for the presence of MDR-TB (21).

In contrast to RMP, genotypic testing for resistance to INH presents obvious difficulties. First, alterations in at least three genes, *katG*, *inhA*, and *ahpC*, are associated with INH resistance. Second, mutations may occur at multiple sites in *katG*, in the regulatory regions of *inhA* (11) or *ahpC* (27), or much more rarely in the *inhA* structural gene (1, 13). Third, some mutations in *katG* are associated with high-level resistance (e.g., deletions or S315T), whereas others may not confer resistance to clinically significant concentrations of INH (e.g., R463L) (9). Fourth, the relative frequency of particular (i.e., rare) mutations may reflect local strain populations and/or epidemic strains, which may be missed by targeting the codons most frequently mutated worldwide. Fifth, the relationship between overexpression of the *ahpC* gene and INH resistance is not yet fully understood (7, 17, 27) except in cases where *katG* has been inactivated (our unpublished data). Although it seems likely that such mutations are indicative of lesions in *katG*, caution is nevertheless required. Despite these potential limitations, the strategy of confining the analysis to the three regions most frequently involved in resistance to INH (10–12) provided satisfactory results in our blind study of Spanish strains: 91.7% of the INH-resistant isolates and an estimated 86.8% of singular, genetically defined, circulating INH-resistant strains were correctly identified.

The techniques used in this study for identifying mutations, SSCP, solid-phase hybridization with mutation-specific oligonucleotides, and direct sequencing, represent some of the potential tools for genotypic resistance testing. Each technique has its drawbacks and advantages (24). Automated SSCP is simple and allows very high sample processing (e.g., simultaneous multiplexed evaluation of four targets in 24 isolates in under 24 h) but is relatively costly, as it requires sophisticated equipment (Fig. 2). However, cheaper manual SSCP strategies are available (4, 6), and the technique could be applied to other targets such as detection of fluoroquinolone, pyrazinamide, streptomycin, or ethambutol resistance (4, 16, 19, 25). The commercial kit used in this study for detection of drug

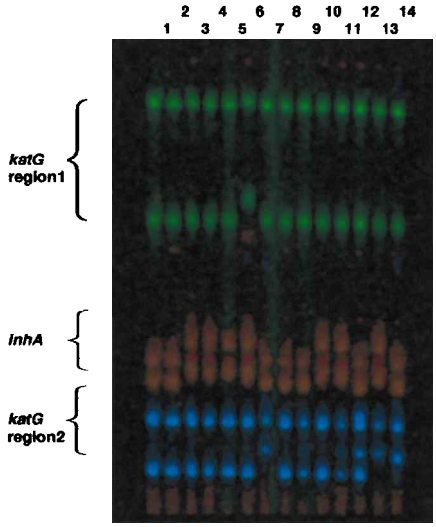

FIG. 2. Multiplex PCR-SSCP analysis of INH resistance. Three targets are analyzed simultaneously in an automated ABI sequencer. Shown are samples with susceptible patterns in all three regions (lanes 1, 2, 8, and 9), samples with a single mutation in *inhA* (lanes 3, 4, 5, 10, and 11) or in *katG* codon 315 (lanes 7 and 14), and examples of multiple mutational events (lanes 6 and 13) and of a mixed population of susceptible and resistant strains (lane 12).

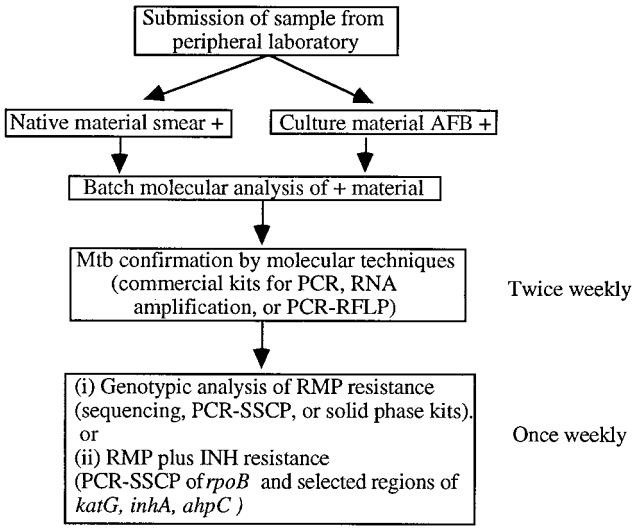

FIG. 3. A model for the implementation of genotypic detection of resistance at the reference laboratory level. AFB, acid-fast bacillus; Mtb, *M. tuberculosis.*

resistance mutations signals the arrival of test systems using technology familiar to diagnostic laboratories, although, as yet, the price of such systems is high and they are limited to the determination of RMP resistance (5). Direct sequencing is the "gold standard," and though significantly more expensive and time-consuming than other alternatives (24), it may represent the best strategy for laboratories with access to a sequencing facility offering daily service.

Where and how could these tests be incorporated into routine use? Certainly, there are settings where the rapid identification of drug resistance may be highly desirable and prove to be cost-effective. A primary example would be urban populations with a significant prevalence of MDR-TB and HIV coinfection, where data on the resistance genotype may result in better patient management and in a more effective utilization of isolation rooms. A model for a cost-effective implementation of these strategies is presented in Fig. 3. Optimally such implementation would be integrated within the fast track concept discussed previously (15).

In summary, in the population investigated, molecular detection of resistance to the two main antituberculous drugs, INH and RMP, can be accomplished with a sensitivity approaching or exceeding 90% and with excellent specificity by using a diagnostic strategy which limits the number of genetic regions to be investigated. Implementation of these techniques, or the future development of more user-friendly tests based on these data, may allow the expedient analysis of drug resistance in reference laboratories.

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