Characterization of Rifampin Resistance in Pathogenic Mycobacteria

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The emergence of rifampin-resistant strains of pathogenic mycobacteria has threatened the usefulness of this drug in treating mycobacterial diseases. Critical to the treatment of individuals infected with resistant strains is the rapid identification of these strains directly from clinical specimens. It has been shown that resistance to rifampin in Mycobacterium tuberculosis and Mycobacterium leprae apparently involves mutations in the rpoB gene encoding the β-subunit of the RNA polymerases of these species. DNA sequences were obtained from a 305-bp fragment of the rpoB gene from 110 rifampin-resistant and 10 rifampin-susceptible strains of M. tuberculosis from diverse geographical regions throughout the world. In 102 of 110 rifampin-resistant strains 16 mutations affecting 13 amino acids were observed. No mutations were observed in rifampin-susceptible strains. No association was found between particular mutations in the rpoB gene and drug susceptibility patterns of multidrug-resistant M. tuberculosis strains. Drug-resistant M. tuberculosis strains from the same outbreak and exhibiting the same IS6110 DNA fingerprint and drug susceptibility pattern contained the same mutation in the rpoB gene. However, mutations are not correlated with IS6110 profiling outside of epidemics. The evolution of rifampin resistance as a consequence of mutations in the rpoB gene was documented in a patient who developed rifampin resistance during the course of treatment. Rifampin-resistant strains of M. leprae, Mycobacterium avium, and Mycobacterium africanum contained mutations in the rpoB gene similar to that documented for M. tuberculosis. This information served as the basis for developing a rapid DNA diagnostic assay (PCR-heteroduplex formation) for the detection of rifampin susceptibility of M. tuberculosis.

Rifampin is an important component of effective multidrug therapies for tuberculosis and leprosy; however, widespread use has led to the emergence of rifampin-resistant (Rif⁰) strains, threatening its usefulness in treating mycobacterial diseases (4–6, 8, 26, 27). Rapid information about drug susceptibility patterns is critical to the treatment of individuals with mycobacterial disease for which rifampin is indicated. Since conventional drug susceptibility testing can require 2 to 4 weeks after growth detection (identification of Mycobacterium tuberculosis) or up to a year (Mycobacterium leprae) in mouse footpads, improvements are needed to yield accurate analysis in a shorter time. DNA diagnostic assays have the potential to provide rapid analysis of rifampin resistance in mycobacteria because of their high degree of sensitivity and specificity and the fact that they do not rely on in vitro growth for results. Shortening the time between diagnosis and the onset of effective therapy should improve patients’ survival (tuberculosis) or decrease physical deformities and ocular manifestations resulting in disabilities and blindness (leprosy).

Developing such assays requires knowledge of the molecular basis of Rif⁰ in pathogenic mycobacteria. Mutations resulting in the Rif⁰ phenotype in prokaryotes have been mapped to the gene encoding the β-subunit of the DNA-dependent RNA polymerase (rpoB gene) (10, 11). Recently, the entire rpoB genes of M. leprae (7) and M. tuberculosis (17) have been sequenced, and several mutations associated with rifampin resistance have been identified in both species (8, 12, 28, 29). To further characterize mutations associated with the Rif⁰ phenotype in M. tuberculosis, M. leprae, and other pathogenic mycobacteria, we developed a rapid PCR-based, DNA sequencing protocol targeted to a 305-bp region of rpoB. By direct DNA sequencing of PCR products, the nucleic acid sequence within this region was determined in 4 rifampin-susceptible (Rif⁰) and 4 Rif⁰ strains of M. leprae and in 12 Rif⁰ and 110 Rif⁰ strains of M. tuberculosis. In addition, mutations were identified in this region of Rif⁰ strains of Mycobacterium africanum and Mycobacterium avium, the latter causing frequent opportunistic infections in immunocompromised hosts. On the basis of these results we have established conditions for a PCR-heteroduplex formation assay (PCR-HDF) for the rapid detection of the Rif⁰ phenotype in pathogenic mycobacteria.

MATERIALS AND METHODS

Mycobacterial strains. Rifampin-susceptible and -resistant strains of M. leprae were isolated initially from homogenates of skin biopsy samples from lepromatous leprosy patients not responding to antileprosy therapy, which included rifampin, and were subsequently defined as resistant to rifampin by the standard mouse footpad drug susceptibility assay (23). These strains were amplified by growth in armadillos or the footpads of BALB/c mice and harvested from tissue homogenates of armadillo spleens or footpads of BALB/c mice as described previously (23, 32). Several M. leprae strains were obtained directly from homogenates of 4- to 8-mm skin biopsy samples from lepromatous leprosy patients (32).
TABLE 1. Sequence analysis of rifampin-resistant isolates of pathogenic mycobacteria

| Isolate and phenotype | Amino acid(s) affected<sup>a</sup> | Amino acid(s) change(s) | No. of isolates (frequency [%]) | Origin (no.) of isolates |
|-----------------------|----------------------------------|-------------------------|--------------------------------|-------------------------|
| *M. tuberculosis*      |                                  |                         |                                |                         |
| Resistant<sup>b</sup>  | 533                               | Leu → Pro               | 3 (2.7)                        | United States (2), Philippines (1) |
| Resistant             | 531                               | Ser → Leu               | 46 (41.7)                      | Belgium (1), Japan (5), Rwanda (9), Canada (2), Philippines (1), Vietnam (1), Yemen (4), United States (21), Peru (2) |
| Resistant             | 526                               | His → Tyr               | 20 (18.2)                      | United States (17), Yemen (1), Peru (1), Philippines (1) |
| Resistant             | 526                               | His → Arg               | 6 (5.5)                        | Rwanda (2), Japan (4) |
| Resistant             | 526                               | His → Asp               | 6 (5.5)                        | Switzerland (1), United States (5) |
| Resistant             | 526                               | His → Leu               | 3 (1.0)                        | Rwanda (1), United States (2) |
| Resistant             | 526                               | His → Pro               | 2 (2.7)                        | Japan (1), Peru (1) |
| Resistant             | 522                               | Ser → Leu               | 2 (1.9)                        | United States (1), Japan (1) |
| Resistant             | 521                               | Leu → Met               | 1 (0.9)                        | United States (1) |
| Resistant             | 518                               | Asn deletion            | 1 (0.9)                        | United States (1) |
| Resistant             | 516                               | Asp → Gly               | 1 (0.9)                        | United States (1) |
| Resistant             | 516                               | Asp → Val               | 7 (6.4)                        | United States (3), Switzerland (1), Peru (1), Yemen (2) |
| Resistant             | 514                               | Phe insertion           | 1 (0.9)                        | United States (1) |
| Resistant<sup>c</sup> | 513                               | Gln → Pro               | 1 (0.9)                        | Philippines (1) |
| Resistant<sup>c</sup> | 509, 526                           | Ser → Thr, His → Asp    | 1 (0.9)                        | Canada (1) |
| Susceptible           | 527, 526                           | Lys → Gln, His → Pro    | 1 (0.9)                        | United States (1) |
| Susceptible           | None                              | None                    | 8 (7.3)                        | Rwanda (2), Burundi (1), United States (5) |
| *M. leprae*           |                                   |                         |                                |                         |
| Resistant             | 531                               | Ser → Leu               | 4 (100)                        | United States (2), Thailand (1), Philippines (1) |
| Susceptible           | None                              | None                    | 4                              | United States (2), Philippines (2) |
| *M. avium*            |                                   |                         |                                |                         |
| Resistant<sup>d</sup> | 531                               | Ser → Trp               | 1 (25)                         | Belgium (1) |
| Resistant             | 531                               | Ser → Leu               | 1 (25)                         | United States (1) |
| Susceptible           | None                              | None                    | 2 (50)                         | Belgium (1), United States (1) |
| Susceptible           | None                              | None                    | 2                              | Belgium (2) |
| *M. africanum*        |                                   |                         |                                |                         |
| Resistant             | 531                               | Ser → Leu               | 1 (50)                         | Sierra Leone (1) |
| Susceptible           | None                              | None                    | 1 (50)                         | Sierra Leone (1) |
| Susceptible           | None                              | None                    | 1                              | Sierra Leone (1) |

<sup>a</sup> Numbers correspond to *E. coli* numbering system for RNA polymerase β-subunit.

<sup>b</sup> Resistant to rifampin at 1 μg/ml by the proportion method.

<sup>c</sup> Two mutations in separate codons of the *rpoB* gene in the same isolate.

<sup>d</sup> Resistant to rifampin at 40 μg/ml by the proportion method.

*M. tuberculosis*, *M. africanum*, and *M. avium* strains were obtained as either cultures from the American Type Culture Collection, purified DNA preparations, or clinical isolates from various geographically distinct laboratories throughout the world (Table 1). In addition, *M. tuberculosis* strains were obtained directly from sputum specimens of tuberculosis patients. All mycobacterial strains were characterized to the species level by either standard biochemical procedures (13, 30) or DNA probe analysis (Gen-probe). In addition, the IS6110 restriction fragment length polymorphism patterns of several *M. tuberculosis* isolates were determined to establish the individuality of strains (1, 2).

**Drug susceptibility testing.** All *M. leprae* isolates were tested for rifampin susceptibility by the mouse footpad drug susceptibility assay as previously described (23). All Rif isolates were resistant to 0.01% rifampin in food. *M. tuberculosis* and *M. africanum* isolates were tested for rifampin susceptibility by the proportion plate method (1 μg of rifampin per ml) (30). Isolates with greater than 1% growth on rifampin-containing media as compared to control media were considered resistant. In addition, selected *M. tuberculosis* isolates were analyzed for isoniazid (INH), streptomycin (SM), ethambutol (EMB), ethionamide, and kanamycin susceptibility by the same methodology. The susceptibility of *M. avium* strains to rifampin was determined by the proportion method with 40 μg of rifampin per ml on Löwenstein-Jensen slants.

**Sample preparation for PCR.** Crude bacterial cell lysates of mycobacterial strains were prepared from either mouse footpad homogenates, human skin biopsy sample homogenates (*M. leprae*), or bacterial suspensions (other mycobacterial species) containing approximately 10⁷ acid-fast bacilli in 100 μl of sterile deionized H₂O and subjected to three cycles of snap freeze-thawing (−160°C for 5 min and then 95°C for 5 min). In addition, 250 μl of *N*-acetyl-l-cysteine–sodium hydroxide-treated sputum sediments (15, 30) obtained from tuberculosis patients was fixed in 70% ethanol, washed in 1× sputolysin (Calbiochem-Behring Diagnostics, San Diego, Calif.), resuspended in 50 μl of deionized H₂O, and boiled for 10 min.

**PCR amplification of mycobacterial strains.** Aliquots of purified mycobacterial DNA (1 ng) or 2 μl of crude bacterial cell lysates was added to PCR reagents and primers with the following sequences: TbRif-1, 5′CAG ACG TTG ATC AAC ATC CG3′, and TbRif-2, 5′TAC GGC GTT ATG AAT C3′. These amplifiers target a 305-bp fragment of the *rpoB* gene in *M. tuberculosis* (17).

The reactants were incubated at 94°C for 5 min, and then DNA was amplified with a Perkin-Elmer 9600 thermocycler with a three-step program—30 s at 94°C, 30 s at 60°C, and 90
strains. of was cycler DNA PCR program: manufacturer's instruction with the burg, primer ThRif-1 (Promega Corp.) corresponding region at 55°C for 100 cycles. Approximately 10 fmol of each purified PCR product was added to fmol DNA Sequencing System (Promega Corp.) reagents and [γ-32P]dATP end-labeled sequencing primer TbRif-1 and TbRif-2 and sequenced as per the manufacturer's instructions with the following cycle program: 2 min at 95°C and then a two-step sequencing program (step 1, 30 s at 95°C; step 2, 30 s at 70°C) for 30 cycles. The reactions were terminated by adding 3 μl of DNA loading dye and then spinning the mixture for 10 s at 10,000 × g. Samples were heated at 72°C for 2 min and separated for 1.5 h at 55°C in 1× Tris-borate-EDTA buffer on a 6% polyacrylamide sequencing gel. The gel was dried, and autoradiography was performed.

PCR-HDF analysis. For HDF analysis, 5 μl of the 305-bp PCR product of each strain was mixed with 5 μl of the 305-bp PCR product from rifampin-susceptible M. tuberculosis H37Rv. Samples were heated at 95°C for 5 min in a thermal cycler and cooled slowly to room temperature with a 2-min ramp time for each 5°C decrease in temperature. The resulting DNA duplexes were analyzed by electrophoresis on a 40-cm mutation detection enhancement gel (MDE; Hydrolink AT BioChemical, Malvern, Pa.) for 16 h at 700 V in 0.6× Tris-borate-EDTA buffer at room temperature. The gels were stained with 0.1 μg of ethidium bromide per ml for 15 min, and bands were visualized by UV transillumination.

RESULTS

All mycobacterial DNA produced a single 305-bp amplicon that served as a suitable template for direct sequencing. DNA sequences obtained from 4 M. leprae and 12 M. tuberculosis Rif^R strains were identical to that of previously published sequences for this region of rpoB in other M. leprae and M. tuberculosis rifampin-susceptible strains, respectively (Fig. 1) (7, 17, 28). The DNA sequence of this region from a Rif^R clinical isolate of M. avium showed 90% homology with the sequences of both M. leprae and M. tuberculosis, and that of M. africanaum showed 100% nucleic acid sequence homology to M. tuberculosis (Fig. 1). The deduced amino acid sequences of M. tuberculosi, M. leprae, M. avium, and M. africanaum showed 100% identity, indicating the highly conserved nature of this region of the rpoB gene sequence in pathogenic mycobacteria (Fig. 1).

Evaluation of 110 M. tuberculosis Rif^R strains from 11 countries and 10 states within the United States identified 16 mutations within a 75-bp region of the rpoB gene affecting 13 amino acids in this region (Table 1; Fig. 2). Fourteen were

509 Ser Gln Leu Ser Gln Phe Met Asp Gln Asn Pro Leu Ser Gly Leu Thr His Lys Arg Arg Leu Ser Ala Leu

533 Thr Pro Gly Val Met Leu Arg Asp Leu Pro Tyr

FIG. 2. Substituted amino acids in codons 509 through 533 of the β-subunit of the RNA polymerase in 110 rifampin-resistant M. tuberculosis strains. The positions of substituted amino acids due to point mutations are shown in boxes below the line. The positions of substituted or missing amino acids due to insertion and deletion mutations are shown in boxes above the line. The numbering system is based on the E. coli β-subunit of RNA polymerase (10).

s at 72°C—for 40 cycles followed by a 10-min final extension step at 72°C.

DNA sequencing. The PCR products for direct DNA sequencing were concentrated and separated from PCR primers with Microcon 100 microconcentrators (Amicon, Gaithersburg, Md.). Approximately 10 fmol of each purified PCR product was added to fmol DNA Sequencing System (Promega Corp.) reagents and [γ-32P]dATP end-labeled sequencing primer TbRif-1 and TbRif-2 and sequenced as per the manufacturer's instructions with the following cycle program: 2 min at 95°C and then a two-step sequencing program (step 1, 30 s at 95°C; step 2, 30 s at 70°C) for 30 cycles. The reactions were terminated by adding 3 μl of DNA loading dye and then spinning the mixture for 10 s at 10,000 × g. Samples were heated at 72°C for 2 min and separated for 1.5 h at 55°C in 1× Tris-borate-EDTA buffer on a 6% polyacrylamide sequencing gel. The gel was dried, and autoradiography was performed. For HDF analysis, 5 μl of the 305-bp PCR product of each strain was mixed with 5 μl of the 305-bp PCR product from rifampin-susceptible M. tuberculosis H37Rv. Samples were heated at 95°C for 5 min in a thermal cycler and cooled slowly to room temperature with a 2-min ramp time for each 5°C decrease in temperature. The resulting DNA duplexes were analyzed by electrophoresis on a 40-cm mutation detection enhancement gel (MDE; Hydrolink AT BioChemical, Malvern, Pa.) for 16 h at 700 V in 0.6× Tris-borate-EDTA buffer at room temperature. The gels were stained with 0.1 μg of ethidium bromide per ml for 15 min, and bands were visualized by UV transillumination.
TABLE 2. Rifampin resistance mutations in multidrug-resistant strains of M. tuberculosis

| Drug resistance patterna | Amino acid substitutedb | No. of isolates |
|--------------------------|-------------------------|-----------------|
| INH, RMP, EMB, SM        | Leu-531                 | 21              |
|                          | Arg-526                 | 3               |
|                          | Tyr-526                 | 6               |
|                          | Leu-526                 | 2               |
|                          | Asp-526                 | 1               |
|                          | Val-516                 | 1               |
|                          | Pro-513                 | 1               |
|                          | Thr-509                 | 1               |
| INH, RMP, EMB            | Pro-533                 | 1               |
|                          | Leu-531                 | 2               |
|                          | Leu-526                 | 1               |
|                          | Tyr-526                 | 5               |
|                          | Asp-526                 | 2               |
|                          | Val-516                 | 2               |
|                          | Gly-516                 | 1               |
|                          | None                    | 1               |
| INH, RMP, SM             | Pro-533                 | 2               |
|                          | Leu-531                 | 4               |
|                          | Arg-526                 | 2               |
|                          | Tyr-526                 | 4               |
|                          | Leu-522                 | 1               |
|                          | Val-516                 | 2               |
| INH, RMP                 | Leu-531                 | 12              |
|                          | Asp-526                 | 3               |
|                          | Pro-526                 | 1               |
|                          | Arg-526                 | 1               |
|                          | Tyr-526                 | 2               |
|                          | Met-521                 | 1               |
|                          | Asn-518                 | 1               |
|                          | Val-516                 | 2               |
| RMP                      | Leu-531                 | 6               |
|                          | Asp-526                 | 1               |
|                          | Tyr-526                 | 4               |
|                          | Phe-514                 | 1               |
|                          | None                    | 3               |

* RMP, rifampin.

b E. coli numbering system for β-subunit of the RNA polymerase.

point mutations (missense), while one isolate had a 3-base deletion (last 2 nucleotides from the Asn-518 codon and the first nucleotide of the Asn-519 codon) resulting in the loss of an asparagine residue, and another isolate contained a 3-base insertion encoding a phenylalanine residue inserted between Phe-514 and Met-515. Two Rif strains of M. tuberculosis contained point mutations in two separate codons resulting in two amino acid substitutions for each strain (Thr-509 and Asp-526 in one strain and Gln-527 and Pro-526 in the other). Eight M. tuberculosis isolates reported to have the rifampin resistant phenotype contained no mutations within the 305-bp region of the rpoB gene examined in this study (Table 2). No silent mutations were observed in the rifampin-resistant isolates analyzed in this study.

In 104 rifampin-resistant isolates of M. tuberculosis for which INH, EMB, and SM drug susceptibility data were available, no single Rif mutation was observed for a particular drug resistance pattern (Table 2).

In Rif isolates of M. tuberculosis which displayed different IS6110 fingerprint (FP) types, there was no apparent association between Rif mutations and FP type (Table 3). However, isolates from the same outbreak exhibited identical FP types and drug resistance patterns (e.g., FP types A, C2, and D2).

M. tuberculosis isolates obtained from a tuberculosis patient before and after the development of rifampin resistance were analyzed for rifampin susceptibility and the presence of mutations in the rpoB 305-bp fragment. Isolates which were susceptible to rifampin (prior to treatment) contained the susceptible genotype, while isolates obtained following the development of rifampin resistance showed a missense mutation, Leu-531. IS6110 FP analysis of these M. tuberculosis isolates showed the identical 10-band FP type both prior to and following acquisition of rifampin resistance.

All four M. leprae strains exhibiting the rifampin resistance phenotype contained a missense mutation in the codon for Ser-531 which resulted in the substitution of a leucine residue (Table 1). In two Rif M. avium strains two missense mutations were observed in the codon for Ser-531. These mutations resulted in the substitution of a tryptophan in one strain and a leucine residue in the other strain. Two other Rif strains of M. avium contained no detectable mutations in the region of the rpoB gene sequenced. In the two Rif strains of M. africanum analyzed, one contained the Leu-531 mutation and the other contained no mutation in this region of the rpoB gene (Table 1).

All rifampin-resistant M. tuberculosis isolates with defined Rif mutations revealed multiple bands when the 305-bp PCR products from these isolates were analyzed by PCR-HDF (Fig. 3). These bands represented the 305-bp homoduplex plus various species of heteroduplexes migrating in the gels distinct from the band representing the homoduplex. In contrast, a single band representing the homoduplex at 305 bp was detected when rifampin-susceptible isolates were analyzed.

TABLE 3. IS6110 FP type and rifampin resistance mutations in multidrug-resistant M. tuberculosis strains

| FP type | Amino acid(s) affected | Amino acid(s) substituted | Drug resistance pattern |
|---------|------------------------|---------------------------|-------------------------|
| A       | Leu-533                | Pro                       | INH, RMP, SM            |
| A       | Leu-533                | Pro                       | INH, RMP, SM            |
| A2      | Asp-516                | Val                       | INH, RMP, SM            |
| A4      | His-526                | Asp                       | INH, RMP, SM            |
| B       | Ser-531                | Leu                       | INH, RMP, SM, EMB       |
| C       | His-526, Pro, Gln      | INH, RMP, EMB, ETA        |
|         |                        | Lys-527                   |
| C       | His-526                | Tyr                       | INH, RMP, SM, EMB       |
| C       | His-526                | Tyr                       | INH, RMP, SM            |
| C       | Ser-522                | Leu                       | INH, RMP, SM            |
| C       | His-526                | Tyr                       | INH, RMP, SM, EMB       |
| C       | His-526                | Tyr                       | INH, RMP, SM, EMB       |
| C       | His-526                | Tyr                       | INH, RMP, SM            |
| D2      | His-526                | Asp                       | INH, RMP, SM            |
| D2      | His-526                | Asp                       | INH, RMP, SM            |
| E1      | Asp-516                | Val                       | INH, RMP, SM            |
| F       | His-526                | Tyr                       | RMP                     |
| G1      | His-526                | Asp                       | INH, RMP, SM            |
| W       | His-526                | Tyr                       | INH, RMP, SM, EMB, ETA, KM |
| W1      | Ser-531                | Leu                       | INH, RMP, SM, EMB       |
|         | Ser-531                | Leu                       | INH, RMP, SM, EMB       |
|         | Ser-531                | Leu                       | INH, RMP, SM, EMB       |
|         | Ser-531                | Leu                       | INH, RMP, SM, EMB       |

* IS6110 FP (1) — unique IS6110 FP type.

b E. coli numbering system for RNA polymerase β-subunit.

RMP, rifampin; ETA, ethionamide; KM, kanamycin.

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DISCUSSION

In order to develop a rapid DNA-based assay for direct detection of the rifampin resistance phenotype of mycobacterial pathogens from clinical specimens, it was necessary to define the molecular events which led to development of this resistant phenotype. In this study several mutations within the rpoB gene of mycobacterial pathogens which appear to be associated with the rifampin resistance phenotype are described. It was observed that 82% of the M. tuberculosis isolates with the Rif phenotype contained missense mutations which led to substituted amino acids at Ser-531 (41.9%), His-526 (32.9%), and Asp-516 (7.3%) residues. Other missense mutations, as well as insertion and deletion mutations, were found in 9% of the Rif M. tuberculosis strains. Similar mutations and frequencies of codon substitution in Rif M. tuberculosis isolates have been reported previously (12, 28, 29). In addition, six mutations in this region of the rpoB gene in Rif M. tuberculosis strains which result in amino acid substitutions (Met-521, Thr-509, Leu-526, Gln-527, Gly-516, and Pro-513) and which have not been reported previously in M. tuberculosis are reported here. The Pro-513 substituted amino acid has been previously associated with high-level resistance in Escherichia coli (10). Mutations within the His-526 and Asp-516 codons have been shown to lead to high-level rifampin resistance in both E. coli and M. tuberculosis; therefore, it is anticipated that substitution of Leu-526 and Gly-516 for these 2 amino acids also results in the rifampin resistance phenotype in these isolates. In two Rif strains of M. tuberculosis, each containing two mutations in separate codons resulting in two amino acid substitutions for each strain, one mutation in each strain (Pro-526 and Asp-526) affects a known site associated with high-level rifampin resistance in E. coli and M. tuberculosis (10, 28). The other has not been reported to be associated with rifampin resistance. Therefore, it is not known if the Gln-527 or Thr-509 substituted amino acid could confer rifampin resistance. In addition, the substitution of Met for Leu in codon 521 has not previously been associated with rifampin resistance in either bacterial species. Therefore, it is not clear whether this mutation results in the Rif phenotype. Further characterization using molecular methodologies could potentially substantiate this finding (16, 17).

To demonstrate the association between mutations in the rpoB gene and the development of the rifampin resistance phenotype of M. tuberculosis, serial isolates from a tuberculosis patient were obtained before and after the development of rifampin resistance and analyzed for the presence of mutations in the 305-bp region of rpoB and rifampin resistance by drug susceptibility testing. Susceptibility testing and DNA sequence analysis confirmed the emergence of the rifampin resistance phenotype during the course of therapy. A mutation in the Ser-531 codon resulting in the substitution of Leu-531 was observed in isolates from this patient exhibiting the Rif phenotype. This mutation has been documented to be associated with rifampin resistance in M. tuberculosis and E. coli (10, 28). In addition, IS6110 FP type analysis of these isolates confirmed that the development of rifampin resistance was not due to reinfection with a Rif strain of M. tuberculosis, as others have observed (19, 24), but rather evolved from the original strain, possibly because of patient noncompliance. Therefore, these data document the evolution of a specific mutation in the rpoB gene and the development of the rifampin resistance phenotype in M. tuberculosis.

We observed that 7% of the M. tuberculosis isolates with 100% resistance to rifampin showed no mutation in the 305-bp region. Similarly, Tellehi et al. (28) showed that 2 of 66 (3.3%) Rif M. tuberculosis isolates lacked mutations in this region. These observations indicate that other mutations, possibly outside the 305-bp region of rpoB, may be responsible for rifampin resistance in these isolates. Two mutations outside the 305-bp region have been shown to confer rifampin resistance in E. coli (10). These mutations result in the substitution of histidine for arginine at position 687 and phenylalanine for valine at position 146. In order to analyze potential mutations at or near the His-687 codon in Rif M. tuberculosis strains lacking mutations in the 305-bp region, a 200-bp fragment which includes the His-687 codon was sequenced in five of these strains. No mutations were observed in this region in any strain when sequences were compared with that of M. tuberculosis isolate with a rifampin-susceptible phenotype (data not shown). Further studies are in progress to sequence this region in other Rif strains and to analyze potential mutations in the Val-146 codon. It is likely that mutations conferring rifampin resistance in these M. tuberculosis strains will be found in the rpoB gene because evidence supporting alternative mechanisms for rifampin resistance in M. tuberculosis has not been reported. Other mycobacterial species analyzed for Rif mutations in the 305-bp region, such as M. africanum and M. avium, also contained Rif isolates lacking mutations in this region of the rpoB gene. In contrast to M. tuberculosis, other mechanisms have been suggested for rifampin resistance in M. avium, such as those associated with cell wall permeability (9, 14, 22).

There appeared to be no geographical clustering of Rif mutations after analyzing M. tuberculosis strains from 11 countries and 10 states within the United States which contained unique FP types. Only a few isolates were studied from most areas; therefore, analysis of larger numbers of isolates from each geographical region may show that clustering of particular Rif mutants does exist. Distinct FP types have been associated with certain outbreaks of tuberculosis, providing evidence for tracking the spread of a defined M. tuberculosis strain.
strain (1, 2). We attempted to identify an association between Rif\(^\text{R}\) mutations and the IS6110 FP types of the \(M.\) \(tuberculosis\) isolates tested in this study. Results showed that mutations affecting the Ser-531, His-526, and Arg-516 codons were found in several different FP types. However, isolates with a specific FP type obtained from different patients in a defined outbreak of tuberculosis did contain the same Rif\(^\text{R}\) mutation (e.g., FP types A, C2, and D2, Table 3). DNA sequencing of the 305-bp region corroborated the FP typing of these isolates and indicated that FP typing could be useful for determining the rifampin susceptibility of isolates in an outbreak situation. In contrast, some FP types, such as C, form a very diverse group of isolates with respect to the geographical area in which samples were obtained, drug susceptibility patterns, and Rif\(^\text{R}\) mutations, limiting the usefulness of FP typing as a general approach for drug susceptibility testing.

No association between specific \(rpoB\) mutations and multidrug resistance patterns were observed, supporting the view that the mutations leading to rifampin resistance are independent events unrelated to those mutations affecting the development of resistance to the other antibiotics tested.

Methods for detecting a single mutation in PCR-generated fragments have been developed. These methods include PCR–single-strand conformational polymorphism and PCR-HDF analyses (20, 21, 31). PCR–single-strand conformational polymorphism analysis has been used as the basis of a rapid assay for detection of the Rif\(^\text{R}\) phenotype of \(M.\) \(tuberculosis\) (28, 29). In the present study, PCR-HDF analysis was employed to detect all Rif\(^\text{R}\) isolates, including strains with either an insertion, deletion, or point mutation located within the 305-bp region of the \(rpoB\) gene of \(M.\) \(tuberculosis\). PCR-HDF analysis gave easily interpretable results and did not require radioactively labeled compounds to complete the test and therefore provides an assay which may show promise for application in the clinical laboratory.

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