

Rapid Detection of Rifampin Resistance in *Mycobacterium tuberculosis* Isolates by Heteroduplex Analysis and Determination of Rifamycin Cross-Resistance in Rifampin-Resistant Isolates

Zeynep Saribaş,* Tanil Kocagöz, Alpaslan Alp, and Ayfer Günalp

Department of Microbiology and Clinical Microbiology, Hacettepe University Faculty of Medicine, Ankara, Turkey

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Direct heteroduplex analysis and a universal heteroduplex generator assay were performed to detect rifampin resistance rapidly in Turkish *Mycobacterium tuberculosis* isolates. Cross-resistance to rifapentine, rifabutin, and rifalazil was investigated. A relationship between specific mutations and resistance patterns, which can guide the choice of an appropriate therapeutic regimen for tuberculosis patients, was identified.

Rifampin is a key component of short-course multidrug antituberculosis therapy. It binds to the β subunit of the DNA-dependent RNA polymerase encoded by the gene *rpoB* and therefore inhibits transcription (7, 8). Ninety-six percent of rifampin-resistant isolates have missense mutations, deletions, or insertions in the 81-bp rifampin resistance-determining region of the *rpoB* gene coding for amino acids 507 through 533. Codons 531, 526, and 516 are reported as the most frequent mutation sites, with codon 531 mutations being the most common (7).

The emergence of rifampin-resistant strains has led to the use of structural analogs of rifampin. Rifapentine, rifabutin, and rifalazil are new rifamycin derivatives and are tested in rifampin-resistant isolates. Recently, several studies showed a correlation between specific mutations in *rpoB* and the level of resistance to rifamycin derivatives (6, 11, 14).

In this study we amplified the resistance-determining region of the *rpoB* gene by PCR and performed direct heteroduplex analysis (1, 15) and a universal heteroduplex generator (UHG) assay (13) for rapid detection of rifampin resistance in clinical isolates of *Mycobacterium tuberculosis*. We performed DNA sequencing for certain isolates and determined the rifamycin cross-resistance in rifampin-resistant isolates and showed the correlation between antimycobacterial activities of rifamycins and mutations in the *rpoB* gene.

Ninety-seven rifampin-resistant and 21 rifampin-susceptible isolates of *M. tuberculosis* were included in this study. All isolates were isolated from different patients in the microbiology laboratory of Atatürk Chest Diseases and Chest Surgery Center, Ankara, Turkey. The rifampin-susceptible strain ATCC 25177 H37Ra was used as a negative control.

Susceptibility testing was performed by the proportion method (2, 3, 4) with Middlebrook 7H10 agar. Each drug (rifampin, rifapentine, rifabutin, and rifalazil) was added at a concentration of 1 $\mu\text{g/ml}$. Isolates with growth on drug-containing media that was >1% greater than growth on control media were considered resistant.

DNA extracts from clinical isolates and from the rifampin-susceptible control strain H37Ra of *M. tuberculosis*, already grown on Löwenstein-Jensen media, were prepared by the boiling method as described previously (5).

A 305-bp region of the *rpoB* gene covering the 81-bp rifampin resistance-determining region was amplified by using TbRif-1 and TbRif-2 primers, and DNA duplexes were obtained as previously described by Williams et al. (9). They were loaded on a 20-cm-long 1 \times mutation detection enhancement (FMC Bioproducts) gel containing 15% urea. The electrophoresis was run at 300 V for 24 h with 1 \times Tris-boric acid-EDTA buffer.

The UHG was a gift from Diana Williams, Hansen's Disease Research Laboratory, Louisiana State University. The heteroduplex generator is a synthetic (Genelab) PCR-amplified (double-stranded) 181-bp DNA fragment. It mimics the genomic DNA and covers the 81-bp rifampin resistance-determining region. It has four 3-bp deletions and three 2-bp substitutions (10). We performed the UHG assay by using primers rpo105 and rpo273 and the protocol described by Williams et al. (10, 12).

We performed DNA sequencing for 14 strains showing different drug susceptibility and heteroduplex patterns. The PCR products were separated from unincorporated nucleic acids and primers with PCR Preps DNA purification system (Promega) according to the manufacturer's instructions. DNA-sequencing reactions were performed with a DNA sequencing kit (Silver Sequence DNA sequencing system; Promega). Primers TbRif-1 and TbRif-2 and a new primer, TbRif-0 (5' AAC CGA CGA CAT CGA CCA CT 3'), designed to show frequent mutation sites more clearly, were used for PCR and DNA-sequencing reactions.

Fifty-one of 97 (52.6%) rifampin-resistant isolates were resistant to all four rifamycin derivatives tested. Thirty-five (36%) isolates were resistant to rifampin and rifapentine, and nine (9.3%) isolates were resistant to rifampin only (Table 1). All rifampin-susceptible strains were susceptible to other rifamycin derivatives.

Among 97 rifampin-resistant isolates, 90 were classified as resistant to rifampin by direct heteroduplex analysis. All 21 rifampin-susceptible isolates were classified as susceptible to rifampin by direct heteroduplex analysis. The sensitivity and

* Corresponding author. Mailing address: Department of Microbiology and Clinical Microbiology, Hacettepe University Faculty of Medicine, 06100 Sıhhiye, Ankara, Turkey. Phone: 90-312-305-1560. Fax: 90-312-311-5250. E-mail: zsaribas@superonline.com.

TABLE 1. Comparison of three methods for determining the drug resistance of *M. tuberculosis* isolates

Drug(s)	No. of rifampin-resistant isolates found to be resistant to indicated drug(s) by:		
	Proportion method	Direct heteroduplex analysis	UHG analysis
Rifampin + rifapentine + rifabutin + rifalazil	51	50	48
Rifampin + rifapentine + rifabutin	1	1	1
Rifampin + rifapentine + rifalazil	1	0	1
Rifampin + rifapentine	35	30	31
Rifampin	9	9	7
Total	97	90	88

specificity of direct heteroduplex analysis, compared to the sensitivity and specificity of conventional drug susceptibility testing, were 92.7 and 100%, respectively. The proportion of agreement was 94%.

M. tuberculosis H37Ra and rifampin-susceptible isolates were expected to form a single band belonging to homoduplex DNA, and resistant isolates were expected to form extra bands due to heteroduplex DNA with mismatches moving at different speeds than the homoduplex DNA in electrophoresis (Fig. 1).

The UHG assay detected 88 of 97 rifampin-resistant strains. All 21 rifampin-susceptible isolates produced the electrophoretic patterns expected from susceptible strains with the UHG assay. The sensitivity and specificity of this method, compared to those of the proportion plate method, were 90.7 and 100%, respectively. The proportion of agreement was 92.4%.

Figure 2 shows rifampin-resistant and -susceptible isolates. *M. tuberculosis* H37Ra forms a four-band pattern containing homoduplexes at 181 and 193 bp and heteroduplexes running in approximately the range of electrophoresis corresponding to homoduplex double-stranded DNA with sizes of 400 and 500 bp. The isolates showing the same band pattern as that of *M. tuberculosis* H37Ra were considered susceptible, and isolates showing a band pattern different from that of *M. tuberculosis* H37Ra were considered rifampin resistant. A single band at 181 bp indicates that there is no *M. tuberculosis* in the sample.

A comparison of the two heteroduplex analysis methods is

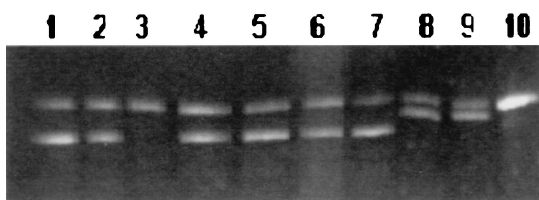


FIG. 1. Electrophoretic patterns obtained by direct heteroduplex analysis of *rpoB* from *M. tuberculosis* isolates. Lanes 1, 2, and 4 to 9, rifampin-resistant isolates; lane 3, rifampin-susceptible isolate; lane 10, rifampin-susceptible control strain H37Ra.

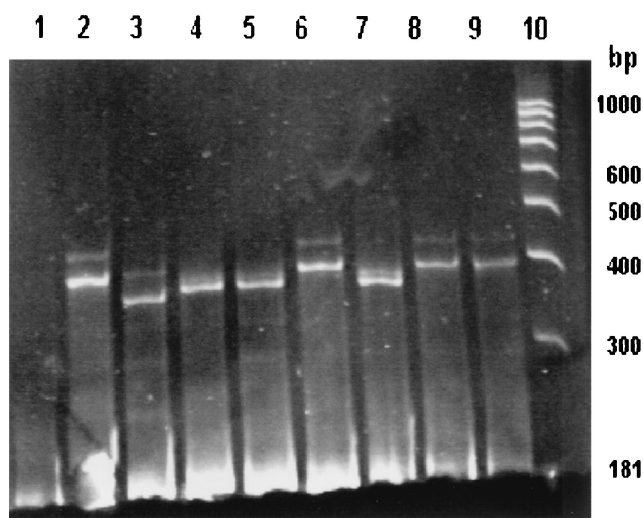


FIG. 2. Electrophoretic patterns obtained by UHG assay from *rpoB* genes. Lane 1, UHG; lane 2, *M. tuberculosis* H37Ra; lanes 3 to 5 and 7 to 9, rifampin-resistant isolates; lane 6, rifampin-susceptible isolate; lane 10, molecular weight standard (100-bp ladder).

shown in Table 2. The difference in detection of rifampin-resistant isolates by the two methods wasn't statistically significant ($P = 0.791$).

Among 14 resistant isolates, seven different mutations were identified (Table 3). All were single nucleotide mutations involving codons 531, 526, 516, and 513, which constitute the sites where mutations are most frequently encountered. While the isolates with mutations in codons 531 and 513 were resistant to all four rifamycin derivatives; the isolates with mutations in codon 516 were resistant to rifampin and rifapentine but susceptible to rifabutin and rifalazil. The isolates with mutations in codon 526 were resistant to either two or four rifamycin derivatives depending on the amino acid change. The isolates with glutamic acid and leucine substitutions were resistant to rifampin and rifapentine but susceptible to rifabutin and rifalazil. Tyrosine substitutions created resistance to all four rifamycin derivatives.

In this study, 46% of rifampin-resistant isolates were susceptible to rifabutin, which is available on the market and may be an important alternative to rifampin.

In conclusion, both heteroduplex methods can be used for rapid diagnosis of rifampin resistance, because their sensitivity and specificity are high. Detection of specific mutations in the *rpoB* gene by DNA sequencing may be very useful for deter-

TABLE 2. Comparison of two methods of heteroduplex analysis

Direct heteroduplex analysis	No. of isolates with UHG analysis result that was:		
	Resistant	Susceptible	Total
Resistant	82	8	90
Susceptible	6	22	28
Total	88	30	118

TABLE 3. Relationship between mutations in *rpoB* and resistance to rifamycins

Mutation	Rifamycin derivatives to which resistance is gained
513 Glycine→leucine	Rifampin plus rifapentine plus rifabutin plus rifalazil
516 Aspartic acid→valine	Rifampin plus rifapentine
516 Aspartic acid→tyrosine	Rifampin plus rifapentine
526 Histidine→glutamic acid	Rifampin plus rifapentine
526 Histidine→leucine	Rifampin plus rifapentine
526 Histidine→tyrosine	Rifampin plus rifapentine plus rifabutin plus rifalazil
531 Serine→leucine	Rifampin plus rifapentine plus rifabutin plus rifalazil

mining the rifamycin resistance phenotypes, offering patients infected with rifampin-resistant isolates the option to be treated with another rifamycin derivative.

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