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Rapid Determination of Rifampin Resistance in Clinical Isolates of *Mycobacterium tuberculosis* by Real-Time PCR

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Real-time PCR was used to determine rifampin resistance in clinical isolates of *Mycobacterium tuberculosis*. Ninety-six rifampin-resistant isolates and 23 rifampin-susceptible isolates were included in the study. A 305-bp region covering the 81-bp "rifampin resistance-determining region" of *rpoB* was amplified. Two hybridization probe pairs that covered the most frequent mutation sites in *rpoB*, codon regions 526 to 531 and 513 to 516, were used. The results obtained by real-time PCR were compared to those obtained by the proportion method. For detection of rifampin resistance, the real-time PCR assay yielded a sensitivity of 92.7% and a specificity of 100%. Real-time PCR is a very rapid method, and it can be especially helpful for the reporting of resistant clinical isolates in a very short period of time.

Tuberculosis is still one of the infectious diseases that causes high fatality rates worldwide. In recent years, the emergence of drug-resistant strains has been an important problem and is threatening the control of the disease (4, 5). Therefore, the rapid detection of drug resistance is essential in order to begin effective therapies and protect the community from tuberculosis.

Rifampin was introduced as an antituberculosis drug in 1972, and since then it has been a key component of short-course multidrug antituberculosis therapy. It is a bactericidal agent. It binds to the β subunit of the DNA-dependent RNA polymerase and inhibits the initiation of transcription (2, 15). The widespread use of rifampin and other rifamycin derivatives has led to the emergence of rifampin resistance (3, 8).

Ninety-six percent of the cases of rifampin resistance are associated with mutations in the 81-bp "rifampin resistance-determining region" of the *rpoB* gene coding for amino acids 507 through 533. The frequencies of frequent mutations were reported to be 41% in codon 531, 32 to 36% in codon 526, and 7 to 9% in codon 516 (15, 22).

Real-time PCR is a method that enables both the monitoring of amplification and the detection of mutations with the help of fluorescently labeled DNA probes (1, 11, 24). In previous studies, real-time PCR was used for the detection of rifampin resistance in isolates with known mutations (18, 19, 21). In this study, use of real-time PCR for the detection of rifampin resistance in clinical isolates of *Mycobacterium tuberculosis* was evaluated. The "resistance-determining region" of the *rpoB* gene was amplified, and the melting temperatures (T_ms) of the probes were obtained on a LightCycler instrument (Roche Diagnostics). The change in the T_m was considered an indicator of a mutation, and isolates for which the probe had a T_m other than that for *M. tuberculosis* H37Ra were considered resistant to rifampin (1, 11, 24). The results obtained by real-time PCR were compared to the drug susceptibility results obtained by the conventional proportion method.

We also performed DNA sequencing with certain strains, determined the rifamycin cross-resistance in rifampin-resistant strains, and showed the correlation between the antimycobacterial activities of rifamycins and mutations in the *rpoB* gene.

MATERIALS AND METHODS

Mycobacterial isolates. In this study, 96 rifampin-resistant and 23 rifampin-susceptible isolates of *M. tuberculosis* were included. The isolates were selected on the basis of their rifampin susceptibilities. They were isolated from sputum samples of different patients with tuberculosis in the microbiology laboratory of Atatürk Chest Diseases and Chest Surgery Training and Research Hospital, Ankara, Turkey. The standard strain *M. tuberculosis* H37Ra (ATCC 25177), which has no mutation in the *rpoB* gene and which is susceptible to rifampin, was used as negative control.

Drug susceptibility testing. Rifampin was obtained from Kocak, Turkey; rifapentine was obtained from Hoechst Marion Roussel, France; rifabutin was obtained from Pharmacia and Upjohn, Germany; and rifalazil was obtained from Pathogenesis. Susceptibility testing was performed by the proportion method, as described previously (6, 9). Middlebrook 7H10 agar (Difco) containing oleic acid-albumin-dextrose-catalase was used. Each drug was added at a concentration of 1 μg/ml. Resistance was defined greater than 1% growth on drug-containing medium compared to the number of colonies obtained on the control medium without any drug.

DNA extraction. DNA extracts from clinical isolates and rifampin-susceptible control strain *M. tuberculosis* H37Ra, which were grown on Löwenstein-Jensen medium, were prepared by suspending a loopful of colonies in TE (10 mM Tris, 1 mM EDTA, pH 8) buffer, washing three times, and boiling for 20 min in the same buffer. After the cell debris was eliminated by centrifugation (12,000 × *g*), the supernatant that contained the template DNA was used for PCR (10).

Real-time PCR. A 305-bp region covering the 81-bp "rifampin resistance-determining region" of the *rpoB* gene was targeted; and primers TbRif1 (5′-CAG ACG TTG ATC AAC ATC CG-3′) and TbRif2 (5′-TAC GGC GTT TCG ATG AAC-3′) (positions 2307 and 2611R, respectively; GenBank accession no. L27989) were used for the amplification (22). For mutation detection, we designed two pairs of hybridization probes (Synthegen). The first pair, which covers codons 531 and 526, was (anchor probe) RpoAP1 (5′-CAC GCT CAC GTG ACA GAC CGC CGG GC-3′-fluorescein) and (sensor probe) RpoSP1 (LightCycler Red 640-5′CCA GCG CCG ACA GTC **T**GC GCT TGT GGG TC-3′-phosphate) (positions 2441 and 2411, respectively; GenBank accession no. L27989). In order to obtain a lower T_m for the sensor probe (RpoSP1) than for the anchor probe (RpoAP1), a G→T substitution was applied (mutation indicated in boldface in sequence above). The other probe pair was RpoAP2 (5′-TCG CCG CGA TCA AGG AGT TCT TCG GCA CCA-3′-fluorescein) and

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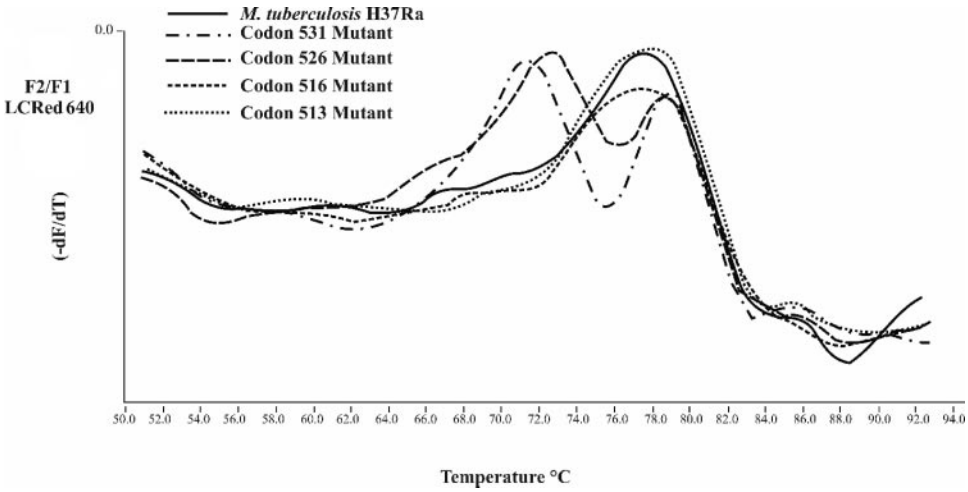

FIG. 1. Melting temperature analysis for different *M. tuberculosis* mutants by use of the RpoSP1 and RpoAP1 probe pair.

RpoSP2 (LightCycler Red 705-5′-CAG CTG AGC CAA TTC ATG GAC CAG AAC-3′-phosphate), which covers codons 516 and 513 (positions 2335 and 2367, respectively; GenBank accession no. L27989).

A total of 20 μl of PCR mixture was prepared. It included 2 μl LightCycler DNA master hybridization probes (Roche Diagnostics) ready reaction mixture containing reaction buffer, *Taq* polymerase, deoxynucleoside triphosphates, and $MgCl_2$. The final concentration of $MgCl_2$ was 2.63 mM. Primers were added at a final concentration of 0.5 μM. The final concentrations of the probes were 0.1 μM for RpoAP1, 0.4 μM for RpoSP1, 0.2 μM for RpoAP2, and 0.2 μM for RpoSP2. Two microliters of DNA was used for each isolate. The real-time PCR was performed in capillary tubes in the LightCycler instrument (Roche Diagnostics). The cycling conditions were denaturation at 95°C for 1 min, followed by 45 cycles of amplification at 95°C for 5 s, 58°C for 10 s (with a single acquisition of fluorescence), and 72°C for 20 s. The melting program was 30 s at 95°C, 2 min at 55°C, and 95°C for 0 s, with a rate of increase of 0.3°C/s (with continuous acquisition of fluorescence) and cooling at 40°C for 30 s. The fluorescence of LightCycler Red 640-labeled probe pair RpoAP1 and RpoSP1 was monitored through channel F2 (λ = 640 nm) of the LightCycler instrument, while the fluorescence of LightCycler Red 705-labeled probe pair RpoAP2 and RpoSP2 was monitored through channel F3 (λ = 705 nm). The fluorescence of the fluorescein was visualized through channel F1 (λ = 570 nm). In order to neglect the background effect of this fluorescence, the F2/F1 and F3/F1 ratios were used during monitoring.

DNA sequencing. We performed DNA sequencing for 14 isolates showing different melting temperatures, with the expectation that different mutations would be detected. Seven rifampin-resistant isolates that could not be detected by real-time PCR were analyzed by DNA sequencing as well. The PCR products were separated from unincorporated nucleic acids and primers with a PCR Preps DNA purification system (Promega), according to the manufacturer's instructions. DNA sequencing reactions were performed with a DNA sequencing kit, the Silver Sequence DNA sequencing system (Promega). Approximately 120 fmol of each purified PCR product was added to each reaction mixture. We designed a new primer, TbRif0 (5′-AAC CGA CGA CAT CGA CCA CT-3′), in order to observe frequent mutation sites more clearly. We used primer TbRif2 to sequence the PCR products amplified with primers TbRif1 and TbRif2. Primer TbRif0 was used in the sequencing reaction of the PCR products amplified with primers TbRif0 and TbRif2. The sequencing program used was denaturation at 95°C for 2 min and then 30 s at 95°C, 30 s at 58°C, and 1 min at 70°C for 55 cycles. The reactions were terminated by the addition of 3 μl of stop solution. Samples were heated to 95°C for 2 min and immediately loaded onto a 5% polyacrylamide gel containing 7 M urea. Electrophoresis was performed with 1× TBE (Tris-borate-EDTA) buffer at 55°C and 1,600 V for 3 h. The bands were visualized by silver staining, according to the manufacturer's instructions.

RESULTS

Drug susceptibility testing. Ninety-six rifampin-resistant isolates and 23 rifampin-susceptible isolates were included in the study. Fifty of 96 (52.1%) rifampin-resistant isolates were resistant to the three other rifamycin derivatives. Thirty-two (33.3%) isolates were resistant to rifampin and rifapentine. Nine (9.3%) isolates were resistant to rifampin only. Three isolates were resistant to rifampin, rifapentine, and rifabutin. Two isolates were resistant to rifampin, rifapentine, and rifalazil. All rifampin-susceptible isolates were susceptible to other rifamycin derivatives.

Real-time PCR and identification of mutations. Eighty-nine of 96 rifampin-resistant isolates were detected by real-time PCR. All 23 rifampin-susceptible isolates were found to be susceptible by this method. The overall sensitivity and specificity of the real-time PCR were 92.7% and 100%, respectively.

We were able to detect 78 of 96 (81.2%) rifampin-resistant isolates with the probe pair RpoSP1 and RpoAP1, which covered codons 531 and 526 of *rpoB*. The T_m of the probe for *M. tuberculosis* H37Ra and the susceptible isolates was 77.50 ± 0.4°C. Fifty-eight rifampin-resistant isolates created a drop of 6.03°C in the T_m of the probe (T_m = 71.47 ± 0.4°C). For 20 rifampin-resistant isolates, a drop of 4.75°C in the T_m of the probe was observed (T_m = 72.75 ± 0.5°C). The codon 531 mutation (Ser→Leu) was detected in isolates for which the probe showed a drop of 6.03°C in the T_m. Isolates for which the probe showed a 4.75°C drop in the T_m were codon 526 mutants (His→Glu) (Fig. 1).

The other probe pair, RpoSP2 and RpoAP2, which covered codons 513 and 516, allowed detection of 11 rifampin-resistant isolates. The T_m of the probe for *M. tuberculosis* H37Ra and susceptible isolates was 66.29°C ± 0.5°C. The resistant isolates created a drop in the probe T_m from 3.23°C to 5.76°C. Isolates creating a 3.23°C drop in the probe T_m were identified as codon 513 mutants (Gly→Leu). Isolates showing a 3.75°C drop in the probe T_m were 516 mutants (Asp→Val), while isolates with 5.76°C drop in the probe T_m were 516 mutants (Asp→Tyr) (Fig. 2).

A heterogeneous population was not detected for any of the isolates by drug susceptibility testing; therefore, the probes for all of the isolates created single melting curves. The T_ms of the probes for strains with identified *rpoB* mutations and *M. tuberculosis* H37Ra are summarized in Table 1.

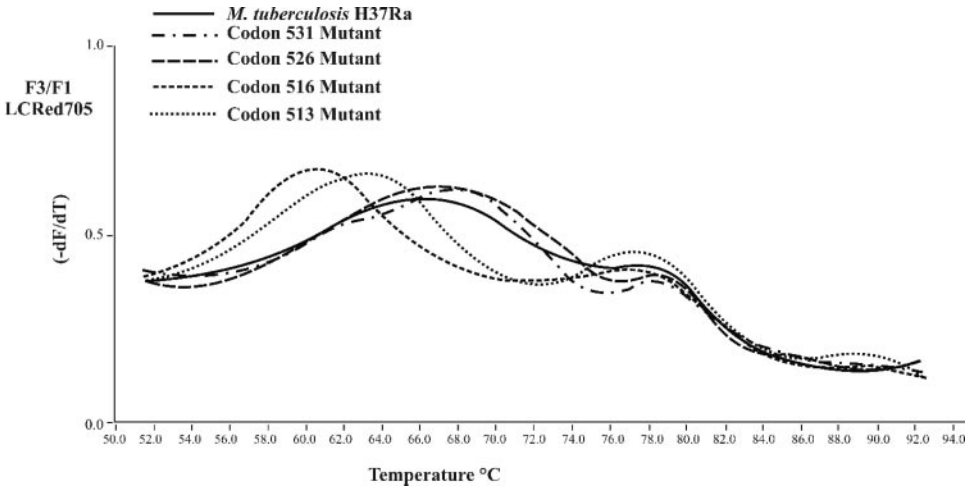

FIG. 2. Melting temperature analysis for different *M. tuberculosis* mutants by use of the RpoSP2 and RpoAP2 probe pair.

Seven rifampin-resistant isolates that could not be detected by either of the probe pairs were analyzed by DNA sequencing. Four of them did not have any mutations in the region sequenced. Two of them contained a mutation at a single codon, which were codons 511 and 522, respectively. One contained mutations both at codon 510 and at codon 516.

***rpoB* mutations and rifamycin cross-resistance.** Fourteen rifampin-resistant *M. tuberculosis* isolates which were detected by real-time PCR were analyzed by DNA sequencing, and five different mutations were identified. While the isolates with codon 531 and 513 mutations were resistant to all four rifamycin derivatives; the isolates with codon 516 and 526 mutations were resistant to rifampin and rifapentine (Table 2).

DISCUSSION

Since detection of drug resistance in *M. tuberculosis* by classical culture methods requires 6 to 8 weeks, molecular methods are important for the determination of drug resistance in *M. tuberculosis* isolates. Characterization of the mutations that cause drug resistance in *M. tuberculosis* has led to the development of new molecular methods. Based on genetic analysis, molecular methods like heteroduplex analysis and single-stranded conformational polymorphism analysis were performed to determine rifampin resistance in *M. tuberculosis* (14, 16, 17, 22). However, these methods are cumbersome. Expensive gel solutions for mutation detection and long electrophoresis periods may be required, especially for detection of single nucleotide mutations. Real-time PCR is a new, rapid, easy-to-perform method that enables monitoring of the amplification and the detection of mutations.

Theoretically, it is possible to detect 96% of rifampin-resistant isolates by identification of the mutations occurring in a 81-bp region of *rpoB*, called the "rifampin resistance-determining region." In this study, it was possible to detect 92.7% of the rifampin-resistant isolates by real-time PCR with two probes covering the regions from codons 513 to 516 and codons 526 to 531 in a single reaction tube. Other mutations that are not covered by the probes used in this study may be responsible for rifampin resistance in the isolates that are not detected by real-time PCR. In previous studies, it was shown that 3.3 to 7% of rifampin-resistant isolates lacked mutations in the 305-bp region of *rpoB* (17, 22).

The rifampin-resistant isolate with a mutation at codon 522 was not detected by real-time PCR since it was not covered by either of the probes.

Although the codon 511 site was covered by detection probe RpoSP2, it was not possible to detect the isolate with a mutation at this site. The T_m value of the probe for this isolate could not be clearly differentiated from that of the probe for *M. tuberculosis* H37Ra. This may be because the codon 511 site is covered by one end of this probe and the nucleotide changes that come across the ends of the probes create only small changes in T_m values.

It was interesting that the isolate with mutations at codons 510 and 516 was not detected. Only codon 516 is covered by

TABLE 1. T_ms and deviations in T_ms of probes RpoSP1 and RpoSP2

Codon (mutation)	RpoSP1 T_m (°C)	ΔT_m[a] (°C) of RpoSP1	RpoSP2 T_m (°C)	ΔT_m (°C) of RpoSP2
M. tuberculosis H37Ra	77.50		66.29	
513 (Gly→Leu) (n = 2)[b]	77.70	0	63.06	3.23
516 (Asp→Val) (n = 5)	77.50	0	62.54	3.75
516 (Asp→Tyr) (n = 4)	77.50	0	60.53	5.76
526 (His→Glu) (n = 20)	72.75	4.75	66.29	0
531 (Ser→Leu) (n = 58)	71.47	6.03	66.29	0

[a] ΔT_m, change in T_m.
[b] n, number of isolates predicted by probe T_ms.

TABLE 2. *rpoB* mutations and resistance to rifamycin derivatives

Codon (mutation)	Cross-resistance to rifamycin derivatives[a]
513 (Gly→Leu) (n = 1)[b]	Rf, Rp, Rb, Rz
516 (Asp→Val) (n = 3)	Rf, Rp
516 (Asp→Tyr) (n = 3)	Rf, Rp
526 (His→Glu) (n = 5)	Rf, Rp
531 (Ser→Leu) (n = 2)	Rf, Rp, Rb, Rz

[a] Rf, rifampin; Rp, rifapentine; Rb, rifabutin; Rz, rifalazil.
[b] n, number of isolates sequenced.

detection probe RpoSP2. The codon 510 mutation comes across the end of anchor probe RpoAP2 where the fluorescein label is attached. This mutation probably disturbs the T_m of the anchor probe and creates a complex effect on the final T_m observed in the real-time PCR.

In our study, there was a 3 to 6°C drop in the T_ms of two probes for resistant isolates which could be easily differentiated from that of the probe for rifampin-susceptible strain *M. tuberculosis* H37Ra. The probes for all susceptible clinical isolates had T_ms similar to the T_m of the probe for *M. tuberculosis* H37Ra. It was possible to detect mutations more than 15 nucleotides apart by the use of a single probe. We used two fluorescent dyes, Red 640 and Red 705, for the detection of mutations in different parts of *rpoB*. Since the staining caused by two dyes can be visualized through two different channels, reactions were performed in a single tube. It is practical and economical to screen the frequent mutation sites of *rpoB* and report the rifampin resistance of an isolate in a single reaction.

In the study of Torres et al. (19), 30 rifampin-resistant isolates of *M. tuberculosis* were studied by real-time PCR with two pairs of probes. Two of them were labeled with the fluorescent dye Red 640. By using the first probe pair, which covers *rpoB* codons 531 to 526, 24 isolates were found to be resistant to rifampin. Codon 531 mutations ($n = 19$) created an increase of about 2°C in the T_m, while 526 codon mutations ($n = 5$) resulted in a 6°C drop in the T_m of the probe. The other probe pair covered codons 513 to 516 of *rpoB*. Codon 513, 516, and 518 mutations resulted in 1.7, 2.5, and 6°C drops in the T_ms of the probes, respectively. Codon 526 mutations ($n = 5$) created a 4 to 6°C drop in the T_m of the probe, depending on the amino acid change (19).

In the study of Viedma (21), 11 rifampin-resistant isolates with seven different mutations were analyzed with two pairs of hybridization probes. Two different fluorescent dyes, Red 640 and Red 705, were used; and the reactions were performed in a single tube. By use of the first probe pair, isolates with codon 515 and 516 mutations in *rpoB* were detected as resistant with a 2.48 to 8.39°C drop in the T_ms of the probes. Codon 526 and 531 mutations created drops of 0.66 to 6.92°C in the T_ms of the other probes (21).

In this study, cross-resistance to rifapentine was detected in 90.6% of the rifampin-resistant isolates. A total of 44.7% of the rifampin-resistant isolates were susceptible to rifabutin, and 45.8% were susceptible to rifalazil. While the isolates with codon 531 and 513 mutations were resistant to all four rifamycin derivatives, isolates with codon 516 and 526 mutations were resistant to rifampin and rifapentine (Table 2). The relationship between the site of the mutations in *rpoB* and the cross-resistance between rifamycins was shown in previous studies (13, 23, 25). In one of our previous studies, we have shown that 46% of rifampin-resistant isolates were susceptible to rifabutin (16). For that reason, it is important to detect specific mutations in the *rpoB* gene for determination of the rifamycin resistance phenotypes so that patients infected with rifampin-resistant isolates have a chance to be treated with another rifamycin derivative, like rifabutin. It may be very useful for determination of rifabutin-susceptible isolates in particular, since rifabutin may be an alternative to rifampin. In previous studies it was reported that rifabutin yielded lower MICs in rifampin-susceptible isolates and had some activity against rifampin-resistant isolates (7, 12, 20, 25).

Real-time PCR is a very rapid, easy, and specific method and can be used as a screening test for the detection of resistance to rifampin and related rifamycins. This is very important so that the appropriate treatment regimen for tuberculosis patients can be selected early in the course of infection. Although more than 90% of rifampin-resistant isolates can be detected by this method, determination of rifampin resistance by classical culture methods should also be used to identify rifampin-resistant mycobacterial isolates missed by real-time PCR.

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