

Original Article

Genotypic Analysis of Isoniazid and Rifampin Resistance in Drug-Resistant Clinical *Mycobacterium tuberculosis* Complex Isolates in Southern Turkey

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SUMMARY: In this study we aimed to learn about the nature and frequency of *katG*, *inhA*, and *rpoB* gene mutations underlying isoniazid (INH) and rifampin (RMP) resistance in clinical *Mycobacterium tuberculosis* complex isolates. The Silver Sequence DNA sequencing method was used to detect the resistance condition of 22 INH, 6 RMP, and 13 INH and RMP in previously determined drug-resistant clinical *M. tuberculosis* isolates. Thirty of 35 (85.7%) INH-resistant strains and 14 of 19 (73%) RMP-resistant strains were found to have a mutation in the analyzed *katG* gene fragment or *inhA* locus and *rpoB* gene fragment. In the *katG* gene region, the codons of mutation detected were determined to be 315 (23 of 30, 76.6%), 279 (4 of 30, 13.3%), and 293 (1 of 30, 3.3%), a finding that has not been reported previously. Our findings demonstrated that the most frequent mutation pattern was Ser315Thr at codon 315 with a rate of 60% (18 of 30). In 5 (16.6%) isolates, a nucleotide change was detected which is associated with INH resistance from -15th C to T in the *inhA* locus. In the *rpoB* gene region, codons possessing point mutations were 531 (9 of 14, 64.2%), 516 (1 of 14, 7.1%), 524 (1 of 14, 7.1%), and 545 (4 of 14, 28.6%), which has not been reported previously. We believe that our present study supplies important data on the different kinds of mutations occurring at various target loci for associated RMP and INH resistance in clinical isolates of our restricted region.

INTRODUCTION

Mycobacterium tuberculosis has re-emerged as a major public-health threat, and drug-resistant strains have been observed worldwide (1). Rifampin (RMP), isoniazid (INH), streptomycin (SM), and ethambutol (EMB) are first-line anti-tuberculosis drugs (2). In addition, INH and RMP constitute the backbone of a short chemotherapy succession for *M. tuberculosis* infection (3). Multidrug resistant (MDR) tuberculosis has emerged due to *M. tuberculosis* resistance to at least RMP and INH, the two most effective and commonly used anti-tuberculosis drugs (2). Actually, MDR strains develop by sequential acquisition of mutations at different loci, usually because of inappropriate patient treatment (4). Furthermore, the existence of MDR strains of *M. tuberculosis* have encouraged research on the molecular mechanisms responsible for resistance to anti-tuberculosis drugs (5). Drug resistance develops due to random genetic mutations in particular genes responsible for resistance in the *M. tuberculosis* strain (6).

The activity of RMP involves binding to β -subunit of ribonucleic acid (RNA) polymerase, resulting in the inhibition of transcription initiation (7). RMP resistance in *M. tuberculosis* complex strains emerges as the results of point mutations or small deletions or insertions in a limited region of the gene encoding for the β -subunit of the RNA polymerase (*rpoB*) gene (codons 507-533) encoding 27 amino acids (4,7,8). Mutations of the RMP-resistant *M. tuberculosis* isolates are frequently located in an 81-bp core region (the rifampin resistance determining region [RRDR]) of the *rpoB* gene in up to 95-98% of RMP-resistant strains (2,6-11). Up to the

present, more than 35 different mutations have been described in this region (7,8), and a mutation found in a resistant strain is generally accepted to be responsible for the resistant phenotype (5). Nevertheless, most of the strains were determined to be missense mutations in codon 531 (43%) or alterations in codon 526 (36%), and the most common amino acid substitutions were Ser531Leu (42%) or His526Tyr (23%). However, the mechanism of resistance was not identified in 4% of RMP-resistant clinical isolates in which mutations were not detected in the 81-bp core region or elsewhere in the *rpoB* gene (7).

INH is a prodrug and when it enters actively growing tuberculosis bacilli, it is inactive. Bacterial catalase-peroxidase enzyme converts INH to oxidizing organic toxic radicals which effect synthesis of the mycobacterial cell wall component (12). In contrast to RMP, the molecular basis of INH resistance is more complex and is associated with mutations located in a large number of gene regions such as the *katG*, *inhA*, *kasA*, *ahpC*, and *oxyR* genes (7). It was also stated that mutations in *furA*, *iniA*, *iniB*, and *iniC* were associated with INH resistance in a much lower percentage of strains (13). This finding demonstrated that INH resistance is controlled by a complex genetic system including several genes (8,14). Approximately 50-95% of INH-resistant strains have been found to contain mutations in codon 315 of the *katG* gene, while 20-35% contain mutations in the *inhA* regulatory region (6).

Various PCR-based molecular techniques were designed to rapidly detect mutations associated with resistance. These methods include direct sequencing of PCR products, single-stranded conformation polymorphism analysis (SSCP), heteroduplex analysis, dideoxy-fingerprinting, restriction fragment length polymorphism (RFLP), RNase cleavage assay, molecular beacon analysis, and reverse hybridization-based line probe assay (2,7). These methods can also be used to determine a molecular epidemiological marker which

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varies geographically (15).

In this study, we intended to determine how often the common mutation occurred within the *katG*, *inhA*, and *rpoB* target gene regions, which are associated with INH and RMP resistance, in 22 INH-, 6 RMP-, and 13 INH- and RMP-resistant clinical *M. tuberculosis* complex isolates in Mersin province, southern Turkey, from 2003 to 2007. Furthermore, we hoped to obtain geographic information about mutated gene patterns as characterized by the Silver Sequence DNA sequencing assay with respect to RMP and INH resistance in patients in our restricted region.

MATERIALS AND METHODS

Clinical specimens and study groups: All of the *M. tuberculosis* clinical isolates were collected from patients in the microbiology department of Mersin University hospital between 2003 and 2006 in Mersin province in southern Turkey. Primary isolation and identification of the recovered mycobacterial isolates from clinical specimens were performed using the classical Löweinstein-Jensen culture method and radiometric BACTEC 460 TB system (Becton Dickinson, Sparks, Md., USA). A total of 22 INH-resistant, 6 RMP-resistant, and 13 INH- and RMP-resistant clinical mycobacterium isolates were included in this study. For these isolates grown on Löweinstein-Jensen medium, phenotypical drug susceptibility test results for RMP, INH, EMB, and SM were determined with a radiometric BACTEC 460 TB system by following the standard procedures and as recommended by the manufacturer. The RMP and INH susceptibility tests were confirmed according to the agar proportion method and standard procedures with the following critical final drug concentrations: RMP, 1.0 µg/ml; INH, 0.2 µg/ml and 1.0 µg/ml.

Bacterial DNA preparation: A rapid DNA extraction procedure was performed for *M. tuberculosis* colonies on Löweinstein-Jensen slants. A loopful of fresh organism colonies was suspended in 1 ml of sterile distilled water. Then bacteria were lysed by boiling for 20 min at 80°C (9). The cells were centrifuged (12,000 × g for 5 min) and supernatant was discharged. The pellet was mixed with 200 µl of chloroform and 200 µl of sterile water. The mixture was then centrifuged at 12,000 × g for 10 min. The supernatant was used as a template for amplification.

PCR amplification: Particular gene regions of *M. tuberculosis* responsible for the resistance to INH and RMP drugs were amplified by a primer-specific PCR assay. Target regions and concerned primer sequences are listed in Table 1. For INH resistance, the *katG* gen region (9,16) and the regulatory region of the *inhA* locus (9,17) were amplified with primer pairs of TB86-TB87 and TB92-TB93, respectively. Also for RMP resistance, the *rpoB* gene region (9,16) was amplified

using the primer pair of TR9-TR8.

Standard PCR reactions for each gene region were carried out in a 50-µl volume, containing 3 µl of template DNA obtained from *M. tuberculosis* cell lysates, 0.5 µM/L each of primers (sense and anti-sense primers), 0.2 mM of nucleotide mix, 1 × *Taq* polymerase buffer, 1.25 units *Taq* DNA polymerase and 1.5 mM MgCl₂. PCR amplification conditions were carried out after 5 min pre-denaturation at 95°C for 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C (for *rpoB*), 67°C (for *katG*), or 64°C (for *inhA*) for 30 s, and extension at 72°C for 45 s, followed by final extension at 72°C for 10 min in a thermal cycler (Eppendorf Mastercycler, Hamburg, Germany).

The products of the PCR were analyzed by electrophoresis on 1% agarose gel under ultraviolet light after staining with ethidium bromide. After electrophoresis, PCR products were purified with Wizard® PCR Preps DNA Purification System (cat no. A21180; Promega, Madison, Wis., USA) for Silver Sequence DNA sequencing analyses.

Silver Sequence DNA sequencing method: Sequence analyses of drug-resistant strains were performed by electrophoresis-based methods, and each base position in the DNA chain was determined individually. Sequence reaction was performed with the Silver Sequence™ DNA Sequencing System (Q4130; Promega), which combines a thermal cycle sequencing system with a silver staining protocol to detect bands in a DNA sequencing gel, and with several modifications.

The sequencing reaction mixture was prepared for each set of four sequencing reactions using 3 µl of purified PCR product, 3 µl of single template-specific sense primer (2 pmol/µl), 5 µl of 5 × DNA sequencing buffer, 1 µl of Sequencing Grade *Taq* DNA polymerase, and a final volume completed to 16 µl with nuclease-free water. Then the mixture was dispensed to each tube containing 2 µl of an A, C, G, and T mix of deoxy- and dideoxynucleotides. Further, reaction mixture tubes were cycled through 60 cycles of denaturing for 30 s at 95°C, annealing for 30 s at 42°C, and extension for 1 min at 70°C.

After the reaction was completed, the products were run on standard sequencing gel, which was prepared with 4-6% polyacrylamide gel in 7M urea in TBE buffer with 0.4 mm spacers. The products were visualized by silver staining the sequencing gel according to the recommendations of the manufacturer.

RESULTS

DNA sequences of 209 bp of *katG* and 248 bp of *inhA* fragments were determined for 35 INH-resistant *M. tuberculosis* isolates and DNA sequences of 158 bp of *rpoB* fragment were determined for 19 RMP-resistant *M. tuberculosis*

Table 1. Primers used for PCR amplification of INH and RMP-resistant *M. tuberculosis* isolates

Gene region	Primer	Primer sequence (5'-3')	GenBank accession no.	Nucleotides	Product size (bp)	Reference
<i>katG</i>	TB86 (sense)	GAAACAGCGGCGCTGATCGT	X68081	2759-2778	209	9, 16
	TB87 (anti-sense)	GTTGTCCCATTTCTCGGGG		2968-2948		
<i>inhA</i>	TB92 (sense)	CCTCGCTGCCAGAAAGGGA	U66801	56-75	248	9, 17
	TB93 (anti-sense)	ATCCCCGGTTTCTCCGGT		303-248		
<i>rpoB</i>	TR9 (sense)	TCGCCGCGATCAAGGAGT	L27989	2335-2352	158	9, 16
	TR8 (anti-sense)	GTGCACGTCGCGACCTCCA		2492-2473		

isolates. The results of the nucleotide change that is responsible for the resistance of the entire clinical *M. tuberculosis* complex isolates are given in Table 2.

Thirty of 35 (85.7%) INH-resistant strains were found to have mutations in the analyzed *katG* gene fragment or the *inhA* locus by the Silver Sequence DNA sequencing method. We detected 8 different mutation patterns in the *katG* gene fragment (Table 3). These mutations were determined in codon 315 in 23 (76.6%) and codon 279 in 4 (13.3%) INH-resistant isolates. The determined mutation patterns of the Ser315 codon were substituted as follows: AGC→ACC (Ser→Thr) in 18 (60%) isolates, AGC→ATC (Ser→Ile) in 3 (10%) isolates, AGC→AAC (Ser→Asn) in 1 (3.3%) isolate and AGC→ACA (Ser→Thr) in 1 (3.3%) isolates. Mutation patterns of codon 279 were also determined as follows: GGC→ACC (Gly→Thr) in 2 (6.6%) isolates, GGC→CGC (Gly→Arg) in 1 (3.3%) isolate and GGC→ATC (Gly→Ile) in 1 (3.3%)

isolate (Table 3). Most of the mutations in the *katG* gene fragment were characterized as single nucleotide changes except for the patterns AGC→ACA at codon 315 and GGC→ATC at codon 279. Furthermore, in contrast with previous findings, the mutation at codon 293 (GCT→ACT, Ala→Thr) in the *katG* gene was detected in only one isolate. A single point mutation, -15th C→T, at the 5' end of the ribosome binding site in the promoter of the *inhA* regulator gene was detected in 5 isolates (16.6%). The prevalence and patterns of mutations in the *katG* and *inhA* gene regions which could be responsible for INH resistance among INH-resistant isolates are summarized in Table 3.

In this study, we detected a dual mutation in 3 (10%) of the INH-resistant detected strains. Two of these isolates had a mutation in both of the *katG* gene region and the *inhA* gene locus, which were located at the codon 279 form of GGC→ATC and -15th C→T, at the codon 279 form of GGC→ACC

Table 2. Results of resistance for the entire clinical *M. tuberculosis* complex isolates

Isolate code	Phenotypic resistance pattern		Determined mutations of target region		
	BACTEC 460 TB		<i>rpoB</i>	<i>katG</i>	<i>inhA</i>
	RMP	INH			
EY-I	S	R	NP	AGC 315 ACC	-
AU-I	S	R	NP	GGC-279-ATC	-15 th C→T
FO-I	S	R	NP	AGC-315-ACC	-
HB-I	S	R	NP	GGC-279-CGC & AGC-315-ATC	-
MU-I	S	R	NP	AGC-315-ACC	-
HC-I	S	R	NP	GCT-293-ACT	-
AB-I	S	R	NP	AGC-315-ATC	-
HO-I	S	R	NP	AGC-315-ACC	-
EC-I	S	R	NP	AGC-315-ACC	-
AY-I	S	R	NP	AGC-315-ACC	-
FP-I	S	R	NP	AGC-315-ACC	-
MOS-I	S	R	NP	-	-15 th C→T
MOK-I	S	R	NP	AGC-315-ACA	-
HA-I	S	R	NP	-	-
HO-I	S	R	NP	-	-
MOR-I	S	R	NP	-	-
SZ-I	S	R	NP	AGC-315-ACC	-
FH-I	S	R	NP	AGC-315-ACC	-
FM-I	S	R	NP	-	-
EA-I	S	R	NP	GGC-279-ACC	-15 th C→T
ND-I	S	R	NP	AGC-315-ACC	-
HB-I	S	R	NP	AGC-315-ACC	-
VS-R	R	S	CTG-545-ATG	NP	NP
HS-R	R	S	-	NP	NP
HE-R	R	S	CTG-545-ATG	NP	NP
RE-R	R	S	CTG-545-ATG	NP	NP
FD-R	R	S	TCG-531-TTG	NP	-
NA-R	R	S	-	NP	-
RB-I	R	R	TCG-531-TTG	AGC-315-ACC	-
AO-RI	R	R	TCG-531-TTG	-	-
IB-RI	R	R	TCG-531-TTG	AGC-315-ACC	-
TD-RI	R	R	TCG-531-TTG	GGC-279-ACC	-
NA-RI	R	R	-	AGC-315-ACC	-
MA-RI	R	R	TCG-531-TTG	AGC-315-ACC	-
MSY-RI	R	R	-	AGC-315-ATC	-
MD-RI	R	R	-	AGC-315-ACC	-
IUU-RI	R	R	CTG-545-ATG & TCG-531-GCG	AGC-315-ACC	-
AU-RI	R	R	TCG-531-TTG	AGC-315-AAC	-
BK-RI	R	R	CAC-516-GTC	AGC-315-ACC	-
SK-RI	R	R	TCG-531-TGG	-	-15 th C→T
FG-RI	R	R	TTG-524-TTA	-	-15 th C→T

S, sensitive; R, resistant; NP, not performed.

Table 3. Frequency of mutations concerned with INH resistance in *katG* and *inhA* gene region detected by silver sequencing

Analyzed gene region	Location of mutation	Nucleotide change(s)	Amino acid change(s)	No. (%) of strains <i>n</i> = 30
<i>katG</i> -related mutations				
<i>katG</i>	codon 315	AGC→ACC	Ser→Thr	18 (60)
		AGC→ATC	Ser→Ile	2 (6.6)
		AGC→AAC	Ser→Asn	1 (3.3)
		AGC→ACA	Ser→Thr	1 (3.3)
	codon 279	GGC→ACC	Gly→Thr	1 (3.3)
	codon 293	GCT→ACT	Ala→Thr	1 (3.3)
<i>katG</i> dual	codon 279 & 315	GGC→CGC & AGC→ATC	Gly→Arg & Gly→Ile	1 (3.3)
<i>inhA</i> -related mutations				
<i>inhA</i>	-15 th locus	C→T	C→T	3 (10)
<i>katG</i> & <i>inhA</i> -related mutations				
<i>katG</i> & <i>inhA</i>	codon 279 & -15 th locus	GGC→ATC & C→T	Gly→Ile & C→T	1 (3.3)
		GGC→ACC & C→T	Gly→Thr & C→T	1 (3.3)

Table 4. Frequency of mutations concerned with RMP resistance in *rpoB* gene region detected by silver sequencing

Location of mutations	Nucleotide changes	Amino acid changes	No. (%) of strains <i>n</i> = 14
Codon 531	TCG→TTG	Ser→Leu	7 (50)
	TCG→TGG	Ser→Tryp	1 (7.1)
Codon 516	CAC→GTC	Asp→Val	1 (7.1)
Codon 524	TTG→TTA	Leu→Leu	1 (7.1)
Codon 545	CTG→ATG	Leu→Met	3 (21.4)
Codon 531 & 545	TCG→GCG & CTG→ATG	Ser→Ala & Leu→Met	1 (7.1)

and -15th C→T, respectively. The other isolate had a dual mutation only at the *katG* gene region, which was located at the 279 form of GGC→CGC and the codon 315 form of AGC→ATC (Table 2). In 5 (14.3%) INH-resistant isolates, no nucleotide changes were detected at the *katG* gene fragment or the *inhA* regulator region.

Fourteen of 19 (73.6%) RMP-resistant strains were found to have a mutation in the analyzed *rpoB* gene fragment. We detected 6 different patterns of mutations (Table 4). All of them included single nucleotide changes. The most affected codon (64.2%) was found to be codon 531. The following mutation patterns were determined: TCG→TTG (Ser→Leu) in 7 (46.1%) strains, TCG→GCG (Ser→Ala) in 1 (7.1%) strain and TCG→TGG (Ser→Tryp) in 1 (7.1%) strain at codon 531 of *rpoB* gene; CAC→GTC (Asp→Val) in 1 (7.1%) strain at codon 516 of *rpoB* gene; TTG→TTA (Leu→Leu) in 1 (7.1%) strain at codon 524 of *rpoB* gene; and CTG→ATG (Leu→Met) in 4 (28.6%) strains at codon 545 of *rpoB* gene (Table 4). One of the RMP-resistant strains also had a dual mutation in both codon 545 (CTG→ATG, Leu→Met) and codon 531 (TCG→GCG, Ser→Ala) in the *rpoB* gene region (Table 2). In 5 (26.4%) phenotypically RMP-resistant isolates no nucleotide changes were detected at the *rpoB* gene fragment.

DISCUSSION

The purpose of our study was to determine the frequency of mutations constituting the basis of drug resistance (RMP and INH) in *M. tuberculosis* isolates in Mersin in southern Turkey. A total of 41 INH- or RMP-resistant clinical isolates, of which 22 were INH-resistant, 6 RMP-resistant, and 13 INH- and RIF-resistant, were included in this study. Nucleotide

changes that may be the cause of the drug resistance were analyzed by the Silver Sequence DNA sequencing method.

The present study is the first investigation involving genotypic analysis of INH and RMP resistance in southern Turkey. In our study, 8 different patterns of mutations in the *katG* gene fragment and 6 different patterns of mutations in the *rpoB* gene fragment including the same or separate codons were detected. The majority of INH-resistant isolates had mutations at Ser315Thr of the *katG* gene region. The high percentage of mutations at position 315 of the *katG* gene (76.6%, 23 of 30 isolates) demonstrates the importance of this codon for the development of INH resistance in *M. tuberculosis* strains in our region. Codon Ser531 mutation (64.2%) in the *rpoB* gene region was also the most common mutation in our RMP-resistant isolates (Table 4). For this reason, we suggest that the codons that were found to most commonly undergo mutation, namely position 315 (76.6%) of *katG* and position 531 (64.2%) of *rpoB* were exposed were found in various geographic regions all around the world (18). These results suggest that the determination of the frequency of mutations associated with drug resistance in isolates from various geographical regions is improving rapid and specific molecular genetic techniques such as commercially available DNA strip assays.

In the *katG* gene region, the most effected codons were determined to be at positions 315 (76.6%) and 279 (13.3%) in our geographical region. We also detected what is to the best of our knowledge a new mutation which was not previously noted in codon 293 (GCT→ACT) of the *katG* gene in one isolate, but its contribution to INH resistance, i.e., whether it alters the catalase-peroxidase enzyme activity, is unknown to us. In previous studies in Turkey, the mutation rate of codon 315 was found to be 73% in the western region (19) and 63.0%

in the eastern region (20) of our country. A comparison of our results with the above regional data showed regional differences with the strains found in eastern populations and similarity with the strains found in western populations.

Most of the studies suggest that mutations in various gene loci and those that contribute to drug resistance display geographic variation (13,21-23). Caws et al. mentioned that it is not clear for feature of strains that are secondarily acquired, *M. tuberculosis* strain subtypes, or other strains (21).

It was reported that the most prevalent observed Ser→Thr mutation at codon 315, which is a significant loss of catalase-peroxidase activity (17), was determined to have a prevalence of 83.9% in Lithuania (3), 71% in Vietnam (21), 65.4% in Australia (18), 66.2% in Poland (9), 60% in South Africa (23), 55.2% in China (13), 59.2% in the Philippines (22) and 41.3% in Spain (17). We found the Ser315Thr mutation in 63.3% of INH-resistant isolates in the *katG* gene region in southern Turkey (Table 3). This percentage is significantly different from the data (41.3%) from Spain (17).

Several investigations demonstrated that *inhA* regulator region mutations appeared with low frequency, namely 10 to 25.8% of INH-resistant isolates (3,9,12,13,17,22). Five (16.6%) isolates had a nucleotide change on -15th C to T in the *inhA* regulator region in the current study. We did not observe another nucleotide substitution on this 5' side of the presumed ribosome binding site. However, one study demonstrated a nucleotide substitution which had not been reported previously, on -15th G to T in the *inhA* locus (22).

The distribution of different mutations associated with RMP resistance in the *rpoB* region has been previously reported from around the world. The frequency of occurrence of particular mutations was determined at codons 531 (41 to 60%), 526 (9 to 31%) and 516 (7.3 to 16%) in various geographic regions (9,17,21,24-26).

In the *rpoB* gene region, codons where point mutations were detected were 531 (9 of 14, 64.2%), 516 (1 of 14, 7.1%), 524 (1 of 14, 7.1%), and 545 (4 of 14, 28.6%) in the present study. In Turkey, the frequency of point mutations at codon 531 in the *rpoB* gene region was first reported as 56.1% in the Aegean Region in western Turkey (15). Another study suggested that the rate of point mutation was found to be 52.1% at codon 531 in the same geographic region in western Turkey (27). In addition, the mutation rate at the most effected codon 531 in *rpoB* gene region was determined to be 51.4% in isolates from northwestern and central Turkey (28). In eastern Turkey, the detected mutation rate at codon 531 was found to be 47.6% (20). The next most frequent mutations affecting codons in this gene region were reported at 526, 516, and 513 from various geographic regions of Turkey (15,20,27,28). We did not detect any mutations at codons 526 and 513 but mutations that had not been previously reported were found at codons 524 (7.7%) and 545 (30.7%). This result indicates the mutation patterns and prevalence and demonstrates the varying geographic distribution of the *rpoB* gene in distinct areas of the same country.

In the current study, mutations in 4 isolates at *rpoB* codon 545 (Leu→Met) might represent a novel mutation which was not reported previously. This codon is outside of 81-bp RRDR (7), and in this respect, this amino acid substitution may cause resistance to RMP by causing conformational changes in the β -subunit of RNA polymerase encoding *rpoB* rather than direct inhibition of the RMP binding site. Furthermore, we detected a synonym mutation (Leu→Leu) at codon 524 in one isolate. This codon site is inside the active site (codons 507-

533) of RMP binding in the *rpoB* gene region (7); therefore, this nucleotide substitution (TTG→TTA) may cause a mutation that disturbs the RMP binding.

In 5 (14.3%) phenotypically INH-resistant and 5 (26.4%) RMP-resistant isolates no nucleotide changes were detected in the *katG* gene fragment or the *inhA* regulator region and *rpoB* gene fragment. This situation could be due to mutations occurring in other parts of the screened gene region in this study, such as the *kasA*, *ahpC*, *oxyR*, or *furA* genes (4,7), or could be the result of an insufficient technique for the detection of mutations or an application mistake. Furthermore, the results of one investigation indicated that a mutation whose role is unclear was detected in 5 isolates in the *oxyR-ahpC* intergenic region without *katG* or *inhA* mutations (22). Also, similar to our findings, no carry mutations of 9 isolates (24.3%) in the *katG* and *inhA* gene regions were determined in western Turkey (19). In any event, the mechanism of resistance was not identified in 4% of RMP-resistant clinical isolates, as no mutations were detected in the 81-bp core region or elsewhere in the *rpoB* gene region (7). It has also been found that some RMP-resistant strains will not be detected using only rapid molecular methods, but these isolates with RMP resistance can be confirmed by conventional phenotypic drug susceptibility testing (8). It was also suggested that these isolates may contain heteroresistance strains, i.e., a mixed population of resistant and susceptible organisms (9,21).

In conclusion, the present study supplies important data on the frequency and different kinds of mutations occurring at various target loci related to RMP and INH resistance in clinical isolates in our restricted region. We also detected a new mutation which was not previously observed in codon 293 (GCT→ACT) of the *katG* gene. In the *rpoB* gene region, a synonym mutation (Leu→Leu) at codon 524 and a novel mutation at codon 545 (Leu→Met) were detected. The sequence analysis technique that was used in this study is non-radioactive and safer to perform than conventional radioactive methods. Results were obtained in one working day and the technique was fairly economical given our laboratory conditions. In this respect we think that this method may be very useful for revealing prevalent mutations among *M. tuberculosis* complex isolates in our region.

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