Journal of Sciences, Islamic Republic of Iran 16(2): 131-138 (2005) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Mutations in the *rpo*B Gene of Rifampin Resistant *Mycobacterium tuberculosis* Isolated in Isfahan by PCR-SSCP

A. Tavakoli,[1,*] H.G. Safaee,[1] F. Navvabakbar,[1] M. Salehi,[2]
A. Bahremand,[3] and B. Nasr Isfahani[4]

[1] *Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran*
[2] *Department of Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran*
[3] *Pasteur Institute of Iran, Tehran, Islamic Republic of Iran*
[4] *Department of Health Education, School of Health Sciences, Isfahan University of Medical Sciences, Isfahan, Islamic Republic of Iran*

Abstract

Rifampin interacts with the beta-subunit of RNA Polymerase (*rpo*B), thereby hindering transcription. Mutations in the *rpo*B locus confer conformational changes leading to defective binding of the drug to *rpo*B and consequently resistance. Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and direct sequencing have been established as a rapid screening test for the detection of mutation in *rpo*B gene, which is responsible for rifampin resistance of *Mycobacterium tuberculosis* species. In this study, a total of 37 strains of *M. tuberculosis*, 16 sensitive and 15 resistant of Isfahan isolates, and 6 resistant strains obtained from Pasteur institute of Iran, were used. They were confirmed as *M. tuberculosis* by conventional methods and amplification of DR gene a region that belongs to *M. tuberculosis* complex group. Their sensitivity or resistance to Rifampin was initially determined by proportion method. The 193-bp region of the *rpo*B gene was then amplified and PCR-SSCP patterns of rifampin resistance (RIFr) and rifampin susceptible (RIFs) strains were determined by electrophoresis on 10% acrylamide gels and silver staining. Also 8 samples of 193-bp *rpoB* amplicons with different PCR-SSCP patterns from Rifr and one from Rifs were sequenced. By PCR-SSCP analysis, 7 PCR-SSCP distinguishable patterns were recognized in the Isfahan RIFr strains. Although 6 of these patterns were different from sensitive strain, one pattern was identical to sensitive standard strain H37Rv. Six resistant strains from Pasteur institute of Iran demonstrated two patterns that one of them was alike to pattern 4 of Isfahan RIFr but other was different. 15 out of 16 RIFs isolates demonstrated PCR-SSCP banding patterns similar to that of sensitive standard strain H37Rv. However, one of the sensitive isolates demonstrated different pattern. After determinations the rpoB sequences of the resistance strains, different mutations were seen in codon 523(GGG/GGT), 526(CAC/TAC), 531(TCG/TTG) and 511(CTG/TTG). This study demonstrated the high specificity (93.8%) and sensitivity (95.2%) of PCR-SSCP method. Presence of different PCR-SSCP banding patterns that were observed in this study is in accordance with the results of similar studies in other parts of the world. The two most prevalent mutations were missense mutations at the positions Ser-531 (TCG/TTG: Ser/Leu) and His-526 (CAC/TAC: His/Tyr). This finding is comparable to the results of early studies demonstrating the *rpoB* mutation frequencies in isolates from other parts of the world.

Keywords: *Mycobacterium tuberculosis*; *rpo*B gene mutation; Rifampin resistant; PCR-SSCP

Introduction

In the last decade, tuberculosis (TB) has reemerged as one of the leading causes of death with nearly 3 million deaths annually [1]. These death rates, however, only partially depict the global TB threat; more than

* *E-mail: ak_ta_ir@yahoo.com*

80% of TB patients are in the economically productive age of 15 to 49 years. The emergence of AIDS and decline of socioeconomic standards contribute to the disease's resurgence in industrialized countries [2].

Advanced antituberculosis chemotherapy and improved conditions have recently led to a striking decline in the living incidence of tuberculosis in developed countries, however, in developing countries the disease is still one of the major threats to public health. Even in developed countries TB is reemerging in association with human immunodeficiency virus infection [3]. A more alarming aspect of human immunodeficiency virus infection-related tuberculosis is the emergence of multidrug-resistant strains of *M. tuberculosis* (MDRTB) [3-6].

Drug-resistant TB is a widespread phenomenon. The common finding has been a high rate of primary resistance to isoniazid and to the combination of isoniazid and rifampin [7]. In 2000, the Centers for Disease Control reported an 11% rate of primary isoniazid resistance and 2% of primary multidrug resistance in Mexican states [8]. An alarming rate of resistant (17.2%) to all the four commonly used antimycobacterial agents for treatment and prophylaxis of tuberculosis, including rifampin, was reported by local practitioners [9]. Characterization of the *rpoB* gene (a region responsible for synthesis of beta-subunit of RNA polymerase) in *E. coli* demonstrated that Rifampin (RIF) specifically interacts with the subunit of RNA polymerase, thereby hindering transcription, and that mutations in the *rpo*B locus conferred conformational changes leading to defective binding of the drug and consequently resistance [10]. Subsequently, the *rpo*B locus from *M. tuberculosis* (MTB) was characterized and mutations conferring the resistant trait were identified. Most mutations were determined to be restricted to an 81-bp core region and are dominated by single nucleotide changes, resulting in single amino acid substitutions, although inframe deletions and insertions also occur at lower frequencies. The consistency of mutations in the *rpo*B locus and the RIFr phenotype (>95%) has marked clinical implications. Because it may act as a surrogate marker for MDRTB, RIF's resistance has prompted development of various diagnostic tests to improve the sensitivity of mutation detection. Although automated sequencing has been unambiguously applied to characterize mutations associated with RIF resistance, a number of other techniques such as Single-stranded conformational polymorphism (PCR-SSCP) [11-13], have been successfully applied to detect these mutations. PCR-SSCP analysis for the detection of mutations responsible for conferring drug-resistance is increasingly useful. In particular, the development of nonisotopic PCR-SSCP analysis has simplified the procedure, enhancing its utility in routine laboratories [11,12].

SSCP analysis, involves amplification of a segment of the gene encoding for the specific drug target and comparison of PCR products of drug-sensitive and drug-resistant strains by SSCP, in which mutations usually result in an altered pattern [4,5]. However, recent studies have questioned its sensitivity and specificity [15]. We investigated the usefulness of PCR-SSCP to detect mutations in the *rpo*B gene of *M. tuberculosis* strains and also determine the PCR-SSCP pattern of rifampin-resistant and rifampin-sensitive strains isolated in Isfahan from 2002 to 2004 to accordance with those have been reported from other geographic areas.

Material and Methods

Fifty three *M. tuberculosis* strains, which were isolated in Isfahan laboratories and TB center of Isfahan from 2002 to 2004, and also six strains from Pasteur Institute of Iran were used in this study. All isolates were characterized by conventional methods (Staining of smears by Ziehl Neelsen method, Growth rate in days, Pigment production in the colonies, Colony morphology of isolate, nitrate reduction test and niacin test) [17] and specific amplification of DR gene region(a region of gene which only present in *M. tuberculosis complex*) [16]. Also the drug-susceptible strain H37Rv DNA was included in this study.

The in-directed test was used for testing pure cultures of M.TB isolates. The number of CFU (Colony Forming Unit) on the rifampin-containing slant was compared with the number on control medium inoculated with similar dilutions. The breakpoint value of >1% was used to classify the organisms as resistant [17].

All isolates were cultured in 7H9 broth for about ten days and then were tested for resistance to rifampin by agar proportion method. Briefly, appropriate concentrations of RIF (4 μg/ml) were added to Lowenstein-Jenes (L.J) medium. Subsequently, L.J medium without and with rifampin were inoculated with 0.1 ml of 10^{-2} and 10^{-4} dilutions of a McFarland 1.0 concentration of a suspension of each isolate of *M. tuberculosis*. The inoculated plates were then incubated at 37°C for 3 weeks. The isolate was considered susceptible to rifampin if the number of colonies grown on the drug-containing plate was <1% of the number of colonies grown on the rifampin-free control. An isolate was considered resistant if 1% or more grew on the drug-containing plate.

Chromosomal DNA of 37 clinical isolates that were identified as belonging to *M. tuberculosis* was extracted by conventional methods [19,20]. Briefly Mycobacterial strains were cultured for three weeks at 37°C in 6 ml of Middlebrook 7H9 liquid medium with Dubos oleic albumen complex enrichment and 0.05% Tween 80. Cells were heat-killed at 80°C for 30 min, and then harvested by centrifugation at 9000 g for 30 min. To the cell pellet, added 550 µl lysozyme (10 mg/5.50 ml TE Buffer), 700 µl of 10% SDS and 60 µl proteinase K solution. Then 100 µl of 5M NaCl and 80 µl of CTAB/NaCl were added. The DNA was purified by chlorophorm/isoamylalcohol extraction and isopropanol precipitation. The pellet was washed twice in 70% ethanol, and then resuspended in TE buffer (50 mM Tris, 100 mM EDTA, pH 8.0). The concentration of extracted DNA was measured using spectrophotometere. 10 ng of extracted DNA was used for PCR amplifications.

PCR reactions (50 µl) contained target DNA (10 ng), 15 pmol primers, 2 mM dNTP (Pharmacia Biotech), 2.5 U Taq polymerase, 1.5 mM $MgCl_2$, and 5 µL 10X buffer. The reaction was performed in a thermal cycler from PCR Hybid (Omnigene).

The sequence of primers for the tuberculosis *rpo*B locus were as follows: upstream primer (*rpo* 105) 5′-CGTGGAGGCGATCACACCGCAGACGT-3′ and downstream primer (*rpo* 273) 5′-GACCTCCAGC CCGGCACGCTCACG-3′, which produce a 193-bp amplicons [20].

Samples were then subjected to one cycle of 96°C for 5 min, followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 30 sec, and one final cycle of 72°C for 10 min to complete the elongation of the PCR intermediate products. PCR products were then run on 2% agarose gels made up in 0.5X Tris-Borate-EDTA (TBE) buffer and examined for the presence of the 193-bp band after ethidium bromide staining [21,22].

The PCR products were SSCP analyzed by electrophoresis on 10% acrylamide gels. In brief, the SSCP gel was made by mixing 10 mL 40% acrylamide solution, 25.6 mL dH2O, 4 mL 10xTBE, 30 µL TEMED and 300 µL ammonium persulfate. About 6 µl of amplified product was mixed with 4 µl of loading buffer (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue) and the mixtures were boiled for 4 min, cooled on ice for 5 min, and then loaded on the gel at 1 W for 14-16 h in cold room. The gel was silver stained by using 10% gacial acetic acid as fix/stop solution; silver nitrate solution (2 g AgNO3 in 2 L ultrapure water and 3 mL 37% formaldyde) as stain solution and solution contains 60 g sodium carbonate, 2 L ultrapure water, and 3 mL 37% formaldyde and 400 µL sodium thiosulfate as developing solution.

Eight samples of 193-bp *rpoB* amplicons with different PCR-SSCP patterns from Rifr and one from Rifs were sequenced. The products were not purified, and were sequenced by using primer rpo105 with Applied Biosystems 377 automated sequence protocol (ABI PRISM^{TM} Dye Terminator, by an Italy Company). All post runs analysis was performed using Clustalw, version 1.82, and software, EMBL-EBI. Each sequence was compared both with the control strain sequence and with the published *rpoB* sequence (GenBank accession NC 000962 REGION: 759805..763323).

Results

All of the clinical isolates that were acid fast, slender, curved rods and red to pink colors with colony morphology rough, crumbly, waxy and non pigmented (cream colored) and were nitrate positive with the specific band of amplified DR gene (Fig. 1) considered as *M. tuberculosis*. [16,17].

Using 1% proportion method on Lewenstein-Jensen medium, the sensitivity or resistance of *M. tuberculosis* isolates was determined. Of 53 isolates of *M. tuberculosis*, 15 strains were determined as Rifampin resistant and with 16 strains of rifampin sensitivity used in this study (Table 1).

When PCR was performed with the amplification of a 193 bp rpoB gene, all strains had identical 193 bp rpoB gene products on 2% agarose gel electrophoresis, like to H37Rv strain (Fig. 2a-c).

SSCP assays were repeated at least two times with all isolates. Mutations were detected on a non-denaturated gel by analyzing migration patterns due to the conformational variability of single-stranded rpoB gene. Different PCR-SSCP patterns were obtained from rifampin-resistant strains and a susceptible control, H37Rv. On the basis of the SSCP results, the 21 RIFr isolates were grouped in seven groups. One main category: group one, 1 isolate (4.75%), group two, 2 isolates (9.5%), group three, 1 isolate (4.75%), group four, 9 isolates (42.8%), group five, 5 isolates (23.8%), group six, 1 isolate (4.75%), group seven, 2 isolates (9.5%). Six resistant strains from Pasteur Institute of Iran were grouped in four, 5 strains (83.3%) and seven, 1 strain (16.7%) (Table 1).

Some typical SSCP gel electrophoresis patterns corresponding to the *rpoB* amplicons are represented in Figures 4, 5, and 6. Of the 16 RIFs, 15 strains (93.8%) had a PCR-SSCP pattern identical to H37Rv; and 1 strains (6.2%) had different pattern. Assays were repeated two times for all isolates. On the basis of the SSCP results, the 21 RIFr isolates were grouped in seven main categories (Figs. 3, 4, and 5).

After SSCP analysis, 8 PCR product samples from Rifr and one from Rifs were sequenced. The results of sequencing were demonstrated in Table 2 and some Electropherograms of automatic DNA sequencing were shown in Figures 6 and 7.

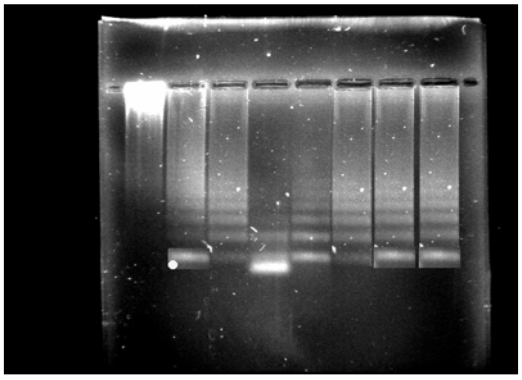

Figure 1. Agarose gel electrophoresis of PCR products of the *M. tuberculosis* DR gene shows ladder shape migration.

Table 1. Drug resistance strains *M. tuberculosis* used in this study

Strain no.	Region of birth	Resistance percentage	SSCP Pattern
MTBR5	IRAN	2/103	6(3 bands)
MTBR8	IRAN	25/105	4(4 bands)
MTBR13	IRAN	14/111	4(4 bands)
MTBR15	IRAN	21/112	5(4 bands)
MTBR18	IRAN	15/98	5(4 bands)
MTBR20	AFGANISTAN	28/110	1(4 bands)
MTBR23	IRAN	22/110	5(4 bands)
MTBR28	IRAN	5/97	2(5 bands)
MTBR30	IRAN	4/110	4(4 bands)
MTBR32	IRAN	30/103	5(4 bands)
MTBR33	AFGANISTAN	20/102	5(4 bands)
MTBR35	AFGANISTAN	3/102	2(5 bands)
MTBR39	IRAN	5/93	3(5 bands)
MTBR43	AFGANISTAN	18/107	4(4 bands)
MTBR48	IRAN	7/105	7(3 bands)
MTB5p	--	18/97	4(4 bands)
MTB9p	--	12/102	4(4 bands)
MTB15p	--	8/111	7(3 bands)
MTB21p	--	24/89	4(4 bands)
MTB104p	--	Nonviable	4(4 bands)
MTB105p	--	31/106	4(4 bands)

Eight RIFr isolates had four mutations that were in the core region of the *rpo*B gene; two mutations with no changes in amino acid contents (polymorphism) were found at the positions 511(CTG/TTG) (12.5%) and 523 (GGG/GGT) (12.5%), and two different missense

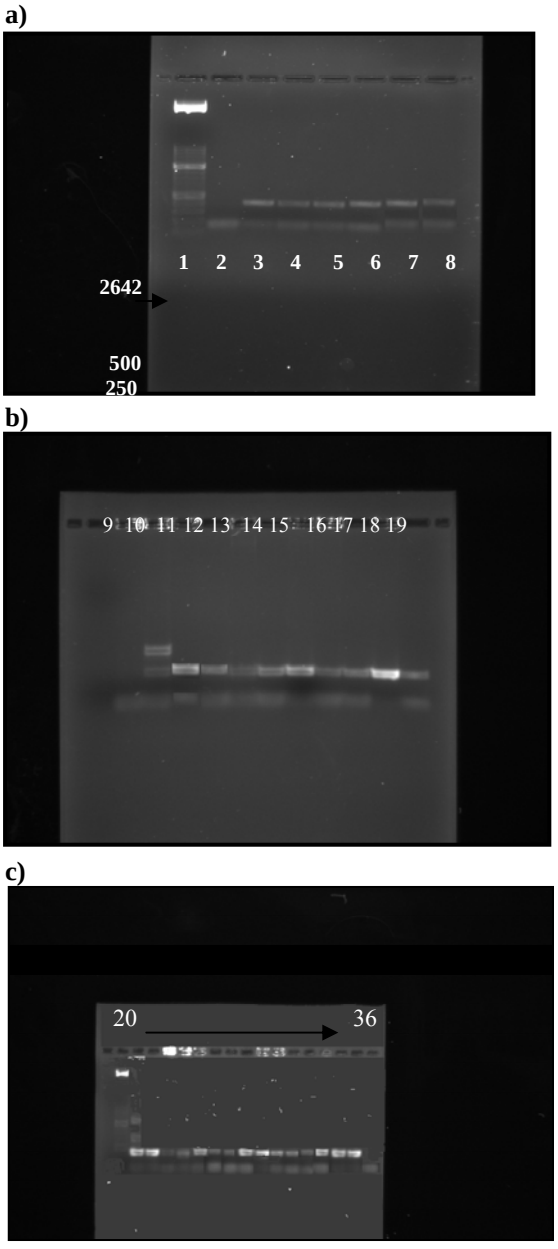

Figure 2. a, b, and c: Agarose gel electrophoresis of PCR products of the *M. tuberculosis rpo*B gene. Lanes 1 and 20 are ladder 50. Lanes 2, 9 and 36 are negative control. Lanes 3 to 8, 10 to 19, and 22 to 36 show PCR products of the *M. tuberculosis rpo*B gene (resistance and sensitive strains). Lane 10 show two bands, lane 21 shows PCR products of the BCG extracted DNA and lane 22 shows PCR products of *rpo*B gene of the H_{37}Rv extracted DNA.

mutations were observed at the positions 531 (TCG/TTG) (50%) and 526(CAC/TAC) (25%). There were not seen any deletions or insertions or more than one mutation in the core region of the *rpo*B gene. No mutations were seen in the sequences of *rpo*B genes of one strain of Rifs isolates.

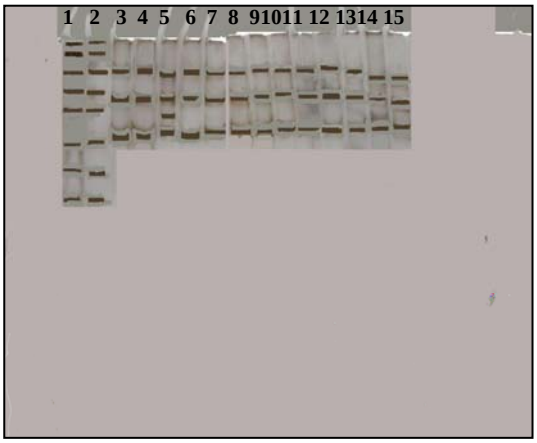

Figure 3. PCR-SSCP patterns of 13 sensitive *M. tuberculosis* strains: Lanes 1 and 2 are marker 50, Lane 3 H37Rv and Lanes 4, and 6-15 from rifampin sensitive strains are indistinguishable from 1(H37Rv) but lane 5 shows different pattern.

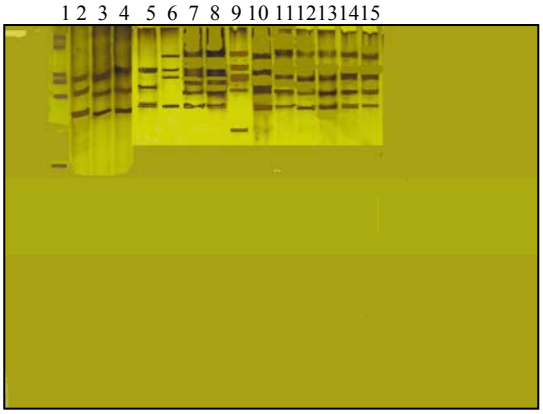

Figure 4. PCR-SSCP patterns of 3 sensitive and 10 rifampin resistant *M. tuberculosis* strains: Lane: 1: ladder 50, lane 2 is H37Rv, lanes 3,4, and 5 are sensitive strains and their patterns are indistinguishable from that of *M. tuberculosis* H37Rv (lane 2). Lanes 6 to 15 are rifampin-resistant strains and their patterns are distinguishable from that of *M. tuberculosis* H37Rv. Lane 6 is pattern 1 rifampin-resistant strains with 4 bands, Lanes 7 and 8, pattern 2 rifampin-resistant with 5 bands, Lane 9, pattern 3 rifampin-resistant with 5 bands different from 7 and 8, Lane 10, pattern 4 rifampin-resistant with 4 bands different from Lane 6 and 11 to 15, pattern 5 rifampin resistant with 4 similar bands. All strains show distinguishable patterns from that of *M. tuberculosis* H37Rv pattern.

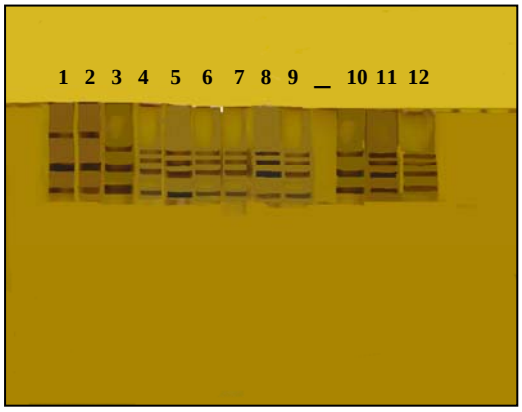

Figure 5. PCR-SSCP patterns of H37Rv and 11 rifampin resistant *M. tuberculosis* strains amplified *rpo*B gene (lanes 7 to 12, are Pasteur Institute of Iran strains). Lane 1 is *M. tuberculosis* H37Rv, lane 2, pattern 6 is similar to H37Rv, lane 3 and 10, pattern 7 are 3 bands and others have 4 bands similar to pattern 4 rifampin-resistant strains.

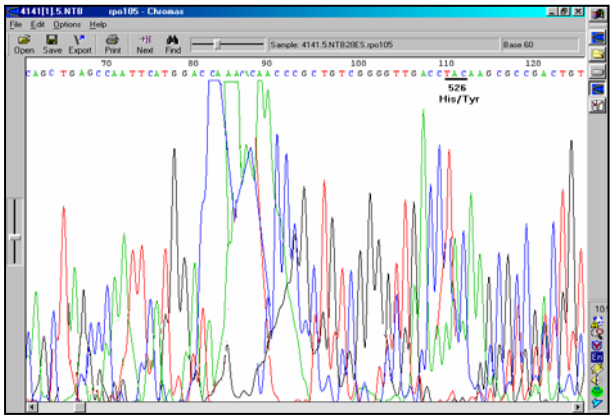

Figure 6. Electropherograms of automatic DNA sequencing after the amplification of *rpoB* DNA by PCR that were directly performed with the PCR product from sample MTB28ES. The PCR sequencing revealed a mutation at codon 526(CAC/TAC, His/Try). Resistance was confirmed by susceptibility testing (proportion method) and PCR-SSCP analysis.

Table 2. Specific mutations identified in 8 resistance strains of *M. tuberculosis* isolated from Isfahan

Mutated *rpo*B codon	Specific mutation	Strain no.
523	GGG/GGT (Gly/Gly)	1 resistant strain (mtbr35)
526	CAC/TAC (His/Tyr)	
531	TCG/TTG (Ser/Leu)	4 resistant strains (8,15, ,20 ,28,)
511	CTG/TTG (Leu/Leu)	1 resistant strain (mtbr48)

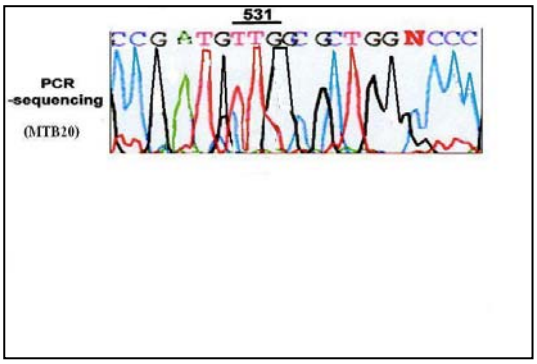

Figure 7. Electropherograms of automatic DNA sequencing after the amplification of *rpoB* DNA by PCR that were directly performed with the DNA from sample MTB20. The PCR DNA sequencing revealed a mutation at codon 531(TTG) in the *rpoB* DNA of *M. tuberculosis*. Resistance was confirmed by susceptibility testing and sequence analysis of culture isolate [TCG/TTG (Ser/Leu)].

Discussion

Because of its high bactericidal action, RIF, along with INH, forms the backbone of short-course chemotherapy [23] The RIF resistant strains arise by sequential acquisition of resistance-conferring mutations in *rpo*B gene as a consequence of antibiotic selection. This situation causes great concern worldwide because of the prolonged infectivity, which increases the risk of transmission.

Concerning mutation detection, molecular techniques is more rapid than rifampin susceptibility testing, which depends on culture and therefore requires an additional 4 to 6 weeks after the primary isolation to obtain the results. Although problems due to silent mutations were recently described [12], rifampin resistance in *M. tuberculosis* was successfully determined by PCR-SSCP [12,25,26] and PCR direct sequencing [27] when the DNA was prepared from cultures. The principle of PCR-SSCP is based on the fact that the two denatured strands of DNA, in this case PCR product, adopt stable intramolecular conformations, which may differ from the wild type upon mutation. This causes a change in the electrophoretic mobility of the strands.

In this study we present molecular genetic analysis of rifampin resistance in Isfahan (Iran) clinical isolates of *M. tuberculosis*. This study demonstrated that frequencies of particular PCR-SSCP patterns in RMP-resistant. *M. tuberculosis* isolates from Isfahan have some differences in comparison with those that have been reported for isolates of other geographic areas [15,21,23,25,26,31].

In spite of extensive and comprehensive standardization of the PCR-SSCP method, our data demonstrate that this procedure was highly specific and sensitive for detecting mutations in the *rpo*B gene in rifampin-resistant clinical isolates of *M. tuberculosis.* Our results are the same as those of Bahremand *et al.* finding [28] but are not exactly conform to Spindola de Miranda *et al.* although the method was fully reproducible, it was not sensitive enough to detect the mutations in *rpo*B region [20]. However, in this study, 1/21 (4.75%) of the resistant isolates had a PCR-SSCP pattern similar and 1/16 (6.3%) of the sensitive strains differ to that of the *M. tuberculosis* susceptible control strain H37Rv. These results may imply a mutation in another part of the rpoB gene or the existence of at least one additional gene that participates in rifampin resistance or the existence a mutation that produce any changes in normal conformation.

Different PCR-SSCP patterns were obtained from resistant strains (Figs. 4, 5, and 6). Most of resistant strains (95.2%) showed PCR-SSCP patterns different from that of H37Rv. Six strains (28.6%) had four bands, 5 strains (23.8%) had three bands that migrated more slowly or faster than those of H37Rv. Because 1 amplified *rpo*B in rifampin-resistant had identical pattern with H37Rv strain, PCR-SSCP did not detect the *rpoB* mutations in 1 out of 21 resistant strains (4.75%). Therefore, the overall sensitivity of the assay was 95.2%. Also we did not find any correlation between the percentage of resistance and the different PCR-SSCP patterns (P-value>0.05). These data are consistent with the PCR-SSCP analysis of this gene in Latvia that shows strand mobility differences between the resistant and susceptible *M. tuberculosis* for the D516V, H526D and D516Y plus P535S mutations but not for the S531L mutation [29]. Xiao-Yong Fan *et al.* demonstrated that among 39 rifampin-resistant, 36 strains had mutations in *rpo*B gene and three group mutations at different codons [30]. On the basis of the SSCP results of Miriam Bobadilla *et al.* [21], the 35 RIFr isolates were grouped in two main categories: group one, 24 isolates (68.6%) with an SSCP identical to that of the control strain H37Rv, and group two, 11 isolates (31.4%) with an SSCP different from that of H37Rv. They reported that the MICs were variable in-group one. In-group two, four polymorphisms were observed with different MICs. The 11 RIFs isolates showed an SSCP identical to that of H37Rv. Lee *et al.* [14] assessed the molecular mechanism of rifampin resistance in clinical strains of Mycobacterium tuberculosis. The molecular nature of a part of the rpoB gene in 77 *M. tuberculosis* clinical strains isolated in Korea was analyzed using PCR-SSCP and PCR-sequence analysis. Among 67 RMP-resistant isolates, 50 showed SSCP profiles different from that of an RMP-sensitive control strain, M. tuberculosis

H37Rv, indicating the possible existence of a sequence alteration in this region of the *rpo*B gene, while 17 resistant isolates displayed SSCP profiles indistinguishable from that of the sensitive control strain. Spindola de Miranda *et al.* demonstrated a clear association between *rpo*B mutations and the resistance profile [20]. In their studies mutations in susceptible strains were detected by this method. Their results showed that this procedure was highly specific but had poor sensitivity for detecting mutations in the *rpo*B gene in rifampin-resistant clinical isolates of *M. tuberculosis*, since two thirds of the resistant isolates had a PCR-SSCP pattern similar to that of the *M. tuberculosis* susceptible control strain H37Rv. Furthermore, in a recent study by Lee *et al.* in Korea, false-negative results were obtained in 17 (25.4%) out of 67 strains [15]. Our data indicate that sensitivity of this method was 95.2% with the specificity of 93.8%. Thus this method can be considered as a convenient method for detection of mutations responsible for conferring rifampin-resistance, and PCR-SSCP analysis is increasingly useful in particular, the development of nonisotopic PCR-SSCP analysis that has simplified the procedure and enhanced its utility in routine laboratories. Eight Rifr and 1 Rifs isolates were analysed for DNA sequences. No novel mutations or double or multiple mutations were exhibited beyond the 23 mutations described in earlier studies [32]. Two most prevalent mutations were missense mutations at the positions Ser-531 (TCG/TTG: Ser/Leu) and His-526 (CAC/TAC: His/Tyr). This finding is comparable to the results of early studies demonstrating the *rpoB* mutation frequencies in isolates from the United States [32,33], European and African countries [34], Japan [35,36], and Asian countries [37]. Of the 8 sequences of Rifr isolates, 6 strains (75%) showed a single mutation and two isolates (25%) had mutations caused polymorphism. In summary, our data on *rpoB* mutation frequencies in isolates from Iran supported the common notion that rifampin resistance genotypes with mutations at critical codons, *i.e.*, those encoding Ser-531 and His526 were the most frequently found in *M. tuberculosis* populations regardless of geographic origin.

Acknowledgments

This study was supported by grant from Isfahan University of Medical Sciences. We are thankful to Dr. Tazhibi for his helpful analysis of the data. Also we are greatly indebted to Dr. Ghazisaeedi from Tehran University and Dr. Karimi from Pasteur institute of Iran for scientific associations and providing mycobacterial strains.

References

1. Bloom B.R. and Murray C.J.L. Tuberculosis: Commentary on a reemergent killer. *Science*, **257**: 1055-64 (1992).
2. Rattan A., Kalia A., and Ahmad N. Multidrug-Resistant Mycobacterium tuberculosis: Molecular Perspectives. *Emerging Infectious Disease*, vol. 4, no. 2, April-June (1998).
3. Frieden T.R., Sterling T., Pablos-Mendez A., Kilburn J.O., Cauthen J.O., and Dooley S.W. The emergence of drug-resistant tuberculosis in New York City. *N. Engl. J. Med.*, **328**: 521-526 (1993).
4. Iseman M. Treatment of multidrug-resistant tuberculosis. *Ibid*., **329**: 784-791 (1993).
5. Snider D.E., Jr., and Roper W.L. The new tuberculosis. *Ibid*., **326**: 703-705 (1992).
6. Kato-Maeda M., Sifuentes-Osornio J., Bobadilla-del-Valle M., Ruiz-Palacios G.M., and Ponce-de-Leon A. Drug resistance among acid-fast bacilli. *Lancet*, **353**: 1709 (1999).
7. Garcia-Garcia M.L., Jimenez-Corona M.E., Ponce-de-Leon A., Jimenez-Corona A., Palacios-Martinez M., Balandrano-Campos S., *et al.* Mycobacterium tuberculosis drug resistance in a suburban community in southern Mexico. *Int. J. Tuberc. Lung Dis.*, **4**(Suppl. 2): 168-70 (2000).
8. Granich R.M., Balandrano S., Santaella A.J., Binkin N.J., Castro K.G., Marquez-Fiol A., *et al.* Survey of drug resistance of Mycobacterium tuberculosis in 3 Mexican states, 1997. *Arch. Intern. Med.*, **160**: 639-44 (2000).
9. Singh S. and Kaur M. High rate of primary and acquired drug resistance in India isolated *Mycobacterium tuberculosis. International Journal of Infectious Disease*, Vol. 8, supplement 1 (March 2004).
10. Jin D. and Gross C. Mapping and sequencing of mutations in the Escherichia coli *rpo*B gene that leads to rifampin resistance. *J. Mol. Biol.*, **202**: 45-58 (1988).
11. Pretorius G.S., Van Helden P.D., Sergel F., Eisenach K.D., Victor T.C., *et al.* Mutations in katG gene sequences in isoniazid-resistant clinical isolates of Mycobacterium tuberculosis are rare. *Antimicrob Agents Chemother*, **39**: 2276-81 (1995).
12. Kalia A., Ahmad N., and Rattan A. Diagnosis of multi-drug resistant tuberculosis: comparison of traditional, radiometric and molecular methods [abstract]. In: Abstracts of the 20th International Congress of Chemotherapy; Sydney, Australia. Sydney: International Society of Chemotherapy; 1997. p. 211; 29 Jun-3 Jul (1997).
13. Morris S.L., Bai Gh., Suffys P., Portillo-Gomez L., Fairchok M., and Rouse D. Molecular mechanisms of multidrug resistance in clinical isolates of Mycobacterium tuberculosis. *J. Infect. Dis.*, **171**: 954-60 (1995).
14. Telenti A., Imboden P., Marchesi F., Lowrie D., Cole S., Colston M., *et al.* Detection of rifampin-resistance mutations in Mycobacterium tuberculosis. *Lancet*, **341**: 647-50 (1993).
15. Lee H., Cho S.N., Bang H.E., Lee J.H., Bae G.H., Kim S.J., *et al.* Molecular analysis of rifampin-resistant Mycobacterium tuberculosis isolated from Korea by

polymerase chain reaction-single strand conformation polymorphism sequence analysis. *Int. J. Tuberc. Lung Dis.*, **2**: 585-9 (1998).
16. Tavakkoli A., *et al.* Unreported results (2004).
17. Bahremand A. Laboratory procedures for the isolation and identification of Mycobacteria. Iran Pastor Institute, Edited (1992).
18. Mitchell W.C., Marjorie Kidd Keating. Antibiotics Laboratoy Medicine. Copyright Williams @ Wilkins., pp. 127-175 (1999).
19. Cooper C., Ph.D., Revised by Bob Jovell M.S. Isolation and quantification of genomic DNA from Mycobacterium tuberculosis, www.Wadsworth.Org/rflp/tutorials/ DNAisolation.html, January 14 (1999).
20. Spindola S., *et al.* Mutations in the rpoB gene of rifampin resistant Mycobacterium tuberculosis strains isolated in Brazil and France. *Mem. Inst. Oswaldo Cruz*, vol. 96, n. 2, Rio de Janeiro (Feb. 2001)
21. Bobadilla-del-Valle M., Ponce-de-Leon A., Arenas-Huertero C., Vargas-Alarcon G., Kato-Maeda M., Small P.M., Couary P., Ruiz-Palacios G.M., and Sifuentes-Osornio J. rpoB gene mutations in rifampin-resistant *Mycobacterium tuberculosis* identified by Polymerase Chain Reaction Single-Stranded Conformational Polymorphism. *Emerging Infectious Disease*, Past Issue Vol. 7, No. 6 (Nov-Dec 2001).
22. Glenn T. Step-by-Step SSCP. Laboratory of Molecular Systematic Smithsonian Institution, Washington, 20560. E-mail: Glenn@onyx.si.edu
23. Kim B.J., Kim S.Y., Park B.H., Lyu M.A., Park I.K., Bai G.H., Kim S.J., Cha C.Y., and Kook Y.H. Mutations in the *rpoB* gene of *Mycobacterium tuberculosis* that interfere with PCR-single-strand-conformation polymorphism analysis for rifampin susceptibility testing. *J. Clin. Microbiol.*, **35**: 492-494 (1997).
24. Matsiota-Bernard P., Vrioni G., and Marinis E. Characterization of *rpoB* mutations in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates from Greece. *Ibid.*, **36**: 20-23 (1998).
25. Kochi A., Vareldzis B., and Styblo K. Multi-Drug resistant tuberculosis and control. *Res. Microbiol.*, **144**: 104-10 (1993).
26. Telenti A., Imboden P., Marchesi F., Schmidheini T., and Bodmer T. Direct, automated detection of rifampin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single-strand conformation polymorphism analysis. *Antimicrob. Agents Chemother.*, **37**: 2054-2 (1993).
27. Kim B.J., Lee S.H., Lyu M.A., Kim S.J., Bai G.H., Kim S.J., Chae G.T., Kim E.C., Cha C.Y., and Kook Y.H. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). *J. Clin. Microbiol.*, **37**: 1714-1720 (1999).
28. Bahremand A.R., Velayati A.A., Feizabadi M.M., Sanami A., Babaei M.R., and Bakayev V.V. Rapid detection of resistance to rifampin in *Mycobacterium tuberculosis* by single strand conformation polymorphism. *Arch. Irn. Med.*, Vol. 1, No. 1 (October 1998).
29. Tracevskr T., Jansone I., Broka L., Marga O., and Baumanis V. Mutation in *rpo*B and *kat*G genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia. *J. Clin. Microbiol.*, **40**(10): 3789-3792 (Oct. 2002).
30. Fan X.-Y., Hu Z.-Y., Xu F.-H., Yan Z.-Q., Guo S.-Q., and Li Z.-M. Rapid detection of *rpo*B gene in *Mycobacterium tuberculosis* isolated in Shanghai by using the amplification refractory mutation system. *Ibid.*, **41**(3): 993-997 (March 2003).
31. Telenti A., Imboden P., Marchesi F., Schmidheini T., and Bodmer T. Direct, automated detection of rifampin-resistant Mycobacterium tuberculosis by PCR and single-strand conformation polymorphism analysis. *Agents Chemother.*, **37**: 2054-2058 (1993).
32. Williams D.L., Spring L., Collins L., Miller L.P., Heifets L.B., Gangadharam P.R.J., and Gillis T.P. Contribution of *rpoB* mutations to development of rifamycin cross-resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.*, **42**: 1853-1857 (1998).
33. Kapur V., *et al.* Characterization by automated DNA sequencing of mutations in the gene *rpo*B encoding the RNA Polymerase B subunit in rifampin resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *J. Clin. Microbiol.*, **32**: 1095-1098 (1994).
34. Telenti A., Imboden P., Marchesi F., Lowrie D., Cole S., Colston M., *et al.* Detection of rifampin-resistance mutations in Mycobacterium tuberculosis. *Lancet*, **341**: 647-50 (1993).
35. Ohno H., Koga H., Kohno S., Tashiro T., and Hara K. Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tuberculosis* strains isolated in Japan. *Antimicrob. Agents Chemother.*, **40**: 1053-1056 (1996).
36. Suzuki Y., Katsukawa C., Inoue K., Yin Y.P., Tasaka H., Ueda N., and Makino M. Mutations in *rpoB* gene of rifampicin resistant clinical isolates of *Mycobacterium tuberculosis* in Japan. *J. Jpn. Assoc. Infect. Dis.*, **69**: 413-419 (1995).
37. Hirano K., Abe C., and Takahashi M. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. *J. Clin. Microbiol.*, **37**: 2663-2666 (1999).