Frequency and molecular characterization of rifampicin-resistance in *rpoB* region of multiple drug resistance (MDR) isolates from tuberculosis patients in southern endemic region of Iran

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Abstract
The aim of this study was to investigate the frequency, location and type of *rpoB* gene mutations in *Mycobacterium tuberculosis* (MTB) collected from patients in the southern endemic region of Iran. Drug susceptibility testing was determined by using the BACTEC system and the center for diseases control's (CDC) standard conventional proportional method. In 29 rifampicin-resistant MTB (85%) isolates, 60 mutations and 13 micro-deletions were identified. Missense mutations produced 23 types of amino acid substitutions. In five rifampicin-resistant MTB isolates (15%) no mutations were found in the core region of the *rpoB* gene. All silent mutations were localized in codon 507. Most frequent mutations detected in Iranian strains, were found in codons 523 and 526. Five alleles in codon 526 and three alleles occurring in triplets in each of the codons 507, 508, 513 were also found. Thus in Iran the highest frequency of common mutations shared between primary and secondary infections was found to occur in codons 523 and 526.
Keyword: *M. tuberculosis; rpoB* gene mutation; Rifampicin resistance; Tuberculosis; Iran.

INTRODUCTION

The World Health Organization (WHO) has estimated that one-third of the world's population, approximately 2 billion people have been infected with MTB. Five years ago in Iran, prevalence rate of tuberculosis (TB) was reported to be as high as 17 in 100,000 (Zaker *et al*., 2006). Recent worldwide surveillance has demonstrated that drug-resistant strains are now widespread and reaching alarmingly high levels in certain countries. Multiple drug resistance (MDR) TB is a potentially untreatable and transmissible disease associated with high mortality. Zabol is an endemic region in the south of Iran (Afghanistan border) with 10 to 13% MDR-TB among 141 TB cases per 100,000 populations (Zaker *et al*., 2006). Resistance to rifampicin is increasing because of its widespread application, resulting in the selection of mutants resistant to other components of short-course chemotherapy. For example, 88% of hospitalized patients with drug resistant TB admitted to Massih Daneshvari hospital (Tehran-Iran) proved to be resistant to at least isoniazid and rifampicin (Namaei *et al*., 2006).

In bacterial populations, the generation of antibiotic resistance depends on the rate of emergence of resistant mutants (Mokrousov *et al*., 2003). A correlation between high mutation rate, antibiotic resistance and virulence in bacteria has been reported in several studies (Valim *et al*., 2000). The detection of resistant MTB strains is generally performed by the conventional susceptibility method which requires culturing the bacilli in the presence of different drugs. The rapid detection of rifampicin-resistant strains is particularly important, since it also represents a valuable surrogate

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marker for MDR-TB resistance, which is a tremendous obstacle to TB therapy (Sajduda *et al*., 2004; Kapur *et al*., 1994). Collectively, DNA sequencing studies have demonstrated that more than 95% of rifampicin-resistant MTB strains have a mutation within the 81bp hotspot region (codons 507 to 533) of the RNA polymerase β-subunit (*rpoB*) gene (Sajduda *et al*., 2004). The prevalence of the mutations determined so far varies for MTB strains obtained from different countries. Recently, the molecular basis of rifampicin resistance in MTB was identified (Telenti *et al*., 1997). Thus, it is important to determine the distribution of resistance mutations in each country prior to molecular tests being introduced for routine diagnostics (Sajduda *et al*., 2004; Kim *et al*., 2004; Mani *et al*., 2001). Drug resistance has been known since the discovery of the first anti-TB drug, streptomycin in the mid 1940s and the presence of resistant mutants in wild populations of mycobacteria have been well documented (Lin *et al*., 2004; Mikhailovich *et al*., 2001; Musser *et al*., 1995; Williams *et al*., 1994).

Genotypic assays which detect mutations within the *rpoB* regions are predictive of drug resistance and have the potential to provide a rapid method for detection of isoniazid-resistant isolates.The aim of this study was to determine resistance-associated mutations in the 81 bp region of the *rpoB* gene in 34 rifampicin-resistant MTB strains from Iranian TB patients.

MATERIALS AND METHODS

***Mycobacterium tuberculosis* (MTB) isolates:** From December 2005 to May 2006, MTB isolates were obtained from sputum samples of patients with active pulmonary tuberculosis (TB) in Zabol, the southern endemic region of Iran. All 91 TB patients had proven registration of clinical diagnostic examinations, such as chest X-ray, purified protein derivative (PPD), cough, weight loss, gender, *etc*. The isolates were cultured on Lowenstein-Jensen solid medium and the resulting colonies were identified at the species level using 2-thiophene carboxylic acid (TCH) and paranitrobenzoic acid (PN99B) selective media, or by standard biochemical procedures. Two sensitive isolates were used as negative controls.

Susceptibility testing: Anti-microbial drug susceptibility testing (AMST) was performed using the CDC standard conventional proportional method. This involved the use of rifampicin (Rif) 40 µg/ml, isoniazid (INH) 0.2 µg/ml, streptomycin (SM) 4 µg/ml, and kanamycin (K) 20 µg/ml in Lowen stein-Jensen medium. in addition, breakpoint concentrations of isoniazid 0.1 µg/ml, and rifampicin 2.0 µg/ml were also used in the BACTEC system. Mutations of the *rpoB* gene were identified in 34 rifampicin resistant isolates by DNA sequencing. AMST was performed following sequencing to confirm resistance using different concentrations of rifampicin (50, 75 and 100 µg/ml) in the Lowen stein-Jensen medium.

PCR amplification: DNA extraction was carried out using Fermentas kit's (K512). A 411-bp fragment of the *rpoB* gene was amplified by PCR with primers *rpoB*-F (5′-TACGGTCGGCGAGCTGATCC-3′) and *rpoB*-R (5′-TACGGCGTTTCGATGAACC-3′) (Miriam *et al*., 2001). PCR reaction was performed in a 50 µl reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM $MgCl_2$, 5 µM of deoxynucleoside triphosphates (dNTPs), 1U of *Taq* polymerase, 20 pmoles of each set of primers and 6 µM of chromosomal DNA. Samples were then subjected to one cycle of denaturation at 94ºC for 5 min, followed by 36 cycles at 94ºC for 1 min, 57ºC for 1 min, 72ºC for 1 min and a final cycle at 72ºC for 10 min to complete the elongation of the PCR intermediate products. PCR products were then run on 2% agarose gels and examined for the presence of the 411-bp band after ethidium bromide staining. The DNA purification was performed using a Sigma kit (USA).

DNA Sequencing: A 411bp fragment of the *rpoB* gene, containing the 81 bp *rpoB* fragment, was amplified by PCR using two primers: *rpoB*-F (5′-TACGGTCGGCGAGCTGATCC-3′) or *rpoB*-R (5′-TACGGCGTTTCGATGAACC-3′). PCR was carried out in a 8 µl reaction mixture containing 0.25 µl of DNA polymerase in 0.9 µl of buffer (PCR), 2 µl of a mixture of dNTP and dNNTP (dATP, dTTP, dCTP, dGTP), 0.5 µl of each primer (2.5 pmoles), 1 µl of DNA and 3.35 µl of H_2O (Molecular Biology grade). Amplification was carried out for 33 cycles, with the following programme: denaturation at 94ºC for 30 sec; primer annealing at 54ºC for 30 sec;

extension at 72ºC for 90 sec. A 411 bp fragment of the *rpoB* gene extracted from MTB strains was sequenced by the Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits. Alignment of the DNA fragments (*rpoB*) was performed with the help of the MEGA 3.1 software.

Data analysis of DNA sequences: Alignment of the DNA fragments (*rpoB*) was carried out with the help of MEGA 3.1 and DNAMAN softwares and compared with standard strains CDC1551, H37RV and MTB strain 210. The BLAST 2 sequences program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov /BLAST/).

Table 1. Frequency of amino acid and nucleotide changes in different codons of the *rpoB* gene of 34 rifampicin-resistant strains of MTB solated in Iran.

Codon and amino acid change	Nucleotide change	Frequency	Isolates*
531 Ser→Leu	TCG→TTG	5(6.78%)	3708, 441, 163(2), 29(2), 710
531 Ser→Phe	TCG→TTC	2(2.78%)	159, 163
526 His→Tyr	CAC→TAC	4(5.5%)	3062, 108, 36, 159
526 His→Asn	CAC→AAC	1(1.39%)	167
526 His→deletion	CAC→_GC	1(1.39%)	165
526 His→Arg	CAC→CGC	3(4.2%)	663, 600, 710
526 His→Phe	CAC→TTC	2(2.78%)	36asli, 161
526 His→Gln	CAC →CAA	1(1.39%)	163
510Gln→deletion	CAG→_AG	9(12.51%)	90,633,411, 73,23,3708, 441,163(2),29(2)
507 Gly→Ser	GGC→AGT	1(1.39%)	3542
507 Gly→Gly	GGC→GGT	6(8.3%)	19,10,33,10(2),163,710
507 Gly→Asp	GGC→GAT	1(1.39%)	159
508 Thr→Ala	ACC→GCC	1(1.39%)	290
508 Thr→Pro	ACC→CCC	3(4.2%)	3548,3542,663
508 Thr→His	ACC→CAC	2(2.78%)	710,163
509 Cys→Asp	AGC→GAC	1(1.39%)	600
511 Leu→Ser	CTG→CCG	2(2.78%)	303-281, 165
511 Leu→Val	CTG→GTG	1(1.39%)	600
512 Ser→Tyr	AGC→GGC	2(2.78%)	36asli,710
512 Ser→Gly	AGC→GCC	1(1.39%)	159
513 Gln→Asn	CAA→AAT	1(1.39%)	36asli
513 Gln→Stop	CAA→TAA	1(1.39%)	159
513 Gln→Glu	CAA→GAA	1(1.39%)	600
516 Asp→His	GAC→CAC	1(1.39%)	663
519 Asn→Lys	AAC→AAG	1(1.39%)	600
520 Leu→deletion	CCG→C_G	1(1.39%)	303-281
523 Gly→Ala	GGG→GCG	16(22.24%)	167,161,290,3548,173,23,19,10,33,10(2),3708, 441,163(2), 303-281,165,710
523 Gly→deletion	GGG→GG_	1(1.39%)	29(2)
527 Lys→deletion	AAG→deletion	1(1.39%)	36asli

*Number of isolates that collected in different regions of Iran.

RESULTS

Bacterial strains and drug susceptibility assay: All samples were cultured and identified as MTB by the PCR method. All 34 isolates examined were resistant to rifampicin. But 11 (34%), 28 (90%) and 10 (31%) of the isolates were found to be resistant to isoniazid, streptomycin and etambutol, respectively. In this study we found four strains to be mono-resistant to rifampicin. From 34 rifampicin resistant isolates, 12 (35%) were isolated from sputum of patients with primary infection and 22 (65%) isolates were obtained from secondary infections.

Definitions in this study: primary infection is referred to a patient who does not have a previous history of TB disease nor medical treatment. Secondary infection demonstrates a previos history of TB disease in the patient's medical records.

PCR amplification and DNA sequencing: In 29 rifampicin-resistant MTB strains (85%), 60 mutations and 13 micro-deletions were identified. In 5 (15%) rifampicin resistant MTB isolates, no mutations were found in the core region of the *rpoB* gene. Of 60 mutations identified, 6 were silent (8.3%) and 54 (91.7%) were missense. Most of detected deletions were located in codons 510 GAG/_AG (12.5%). All silent mutations were localized in codon 507, while missense mutations revealed 23 types of amino acid substitutions. Most frequent mutated codons in the Iranian strains were codon 523 (GGG→GG_, GGG→GCG) and codon 526 (CAC→TAC, CAC→CGC, CAC→AAC, CAC→TTC, CAC→CAA, CAC→_GC) indicating six types of mutations (Tables 1 and 2). Mutations in codons 510, 507 and 531 were observed in 27%, 24% and 21% of isolates, respectively. Correspondingly, mutations in codon 523 resulted in Gly523Ala replacement and in codon 531, Ser531Leu and Ser531Phe. Six alleles were observed in codon 526, and 3 alleles in triplets 507, 508 and 513. In 6 strains (18%) single mutations were located in codons 526 and 510, while isolates with multiple mutations revealed double (34%), triple (22%) and quadruple (3%) mutations. 12% of the strains harboured 5 muta-

Table 2. Data for *rpoB* mutations (single, double, triple, quartile and five) in rifampicin-resistant *M. tuberculosis* strains isolated from Iran (Accession number EF628338-EF628369 in Genbank).

Frequency of mutation	Codon number	Number of isolates	Isolate number *
No mutation	-		23(2)-28-584,103,29
1 Mutation	526	3	3062,108,36
	510	3	90,633,411
2 Mutations	523-526	2	167,161
	508-523	2	290,3548
	510-523	2	173,23
	507-508	1	3542
	507-523	4	19,10,33, 10(2)
3 Mutations	510-523-531	4	3708,441,163(2),29(2)
	508-516-526	1	663
	511-520-523	1	303-281
	511-523-526	1	165
4 Mutations	507-508-526-531	1	163
5 Mutations	512-513-526-527-531	1	36 asli
	507-508-512-523-526	1	710
	507-512-513-526-531	1	159
	509-511-513-519-526	1	600

*Number of isolates that collected in different regions of iran.

tions (Tables 1 and 2).

We detected deletion mutations in codons 510, 520, 523, 526 and 527, stop mutation in codon 513 and silent mutation in codon 507 (Table 1).

DISCUSSION

The *rpoB* codons 531, 526, 516 and 511 are the most frequently mutated sites, observed worldwide. However, variations in the relative frequencies of mutations in these codons have been described for isolates from different geographic locations (Bakonyte *et al*., 2005; Marin *et al*., 2004; Matsiota *et al*., 1998). Other studies have also indicated that these mutations are the most prevalent worldwide (Namaei *et al*., 2006; Kapur *et al*., 1994; Bakonyte *et al*., 2005). These differences reflect the complex and crucial interactions between the drug and its target at the molecular level where the position of the affected allele seems to be variable. This finding is not in agreement with other authors who have reported different levels of high (Pozzi *et al*., 1999; Hirano *et al*., 1999; Sifuentes *et al*., 1995) and low (Namaei *et al*., 2006; Hirano *et al*., 1999) resistance associated with specific nucleotide replacements. All rifampicin resistant isolates studied had mutations of different types in the *rpoB* region, however the relationship between the combination of specific types of mutations and rifampicin resistance is unclear. This is the first report describing the genetic characteristics of multidrug-resistant MTB strains isolated from TB-patients in Iran. The finding of mutations is partially comparable and resembles those strains reported in other countries (India, Russia, China, USA and Lithuania). CAG mutation of codon 510 (deletion or CTG or CAC or CAT) is very seldom detected in other countries. However in this study (Table 1) a higher number of deletion mutations (9 strains) with respect to one base C (_AG) were found. On the other hand, in other countries no changes in codon 510 (1, 5, 15) have been observed. Mutation CAG→CAT has been found in India (Mani *et al*., 2001), –CAG→CAT in Russia, and CAG→GAG, TAG in Belarus, CAG→GAG in Lithuania and CAG→GAG in Poland, for the same codon (Namaei *et al*., 2006; Bakonyte *et al*., 2005; Mokrousov *et al*., 2003; Telenti *et al*., 1997). The important findings of this study revealed that codons 510 (12.51%), 523 (23.6%) and 526 (16.6%) had the most frequent occurrence of mutation bearing sites. Infact mutations in codons 531 and 526 occur most frequently in the world (TCG→TTG for codon 531, and CAC→TAC for 526) (Namaei *et al*., 2006; Bakonyte *et al*., 2005; Lishi *et al*., 2002; Sifuentes-Osornio *et al*., 1995). The data of this study are very closely related to those observed in Asia (60%). Comparison of our data with other countries indicate fewer mutations in codon 531 (TCG→TTG) (Ruiz *et al*., 2004; Lilly *et al*., 1999; Yuen *et al*., 1999) and more mutations in codon 526 (CAC→TAC, CAC→_GC, CAC→CGC, CAC→AAC, CAC→TTC and CAC→CAA) (Valim *et al*., 2000; Huang *et al*., 2002; Matsiota-Bernard *et al*., 1998; Williams *et al*., 1998). Mutations in codon 526 (CAC→CAG) and codon 516 (GAC→GTC) not often seen in Iran are usually observed in Poland and USA (Sajduda *et al*., 2004; Kim *et al*., 2004; McCammon *et al*., 2005., Yun *et al*., 2005). In this investigation mutations in codon 511, representing one of the frequent mutations worldwide (Tables 1 and 2) were also observed. The high percentage of double mutations found among Iranian strains (32%) differed clearly with the lower prevalence of double mutations in other studies (Namaei *et al*., 2006; Williams *et al*., 1994; Lishi *et al*., 2002; Barfai *et al*., 2001). Noticeable findings of this study indicate the high frequency of double (32%), triple (20%) and quadruple (2.9%) mutations occurring in separate codons. It should be noted that five phenotypic rifampicin resistance strains revealed no mutations. The combination of two single point mutations has been described previously for rifampicin-resistant isolates of MTB (Pozzi *et al*., 1999; Kapur *et al*., 1994). Silent mutations 6 (17.6%) were also detected in 6 different isolates which demonstrated an absence of drug resistance to rifampicin. These 6 isolates consisted of quadruple mutations amongst which one silent mutation was detected in codon 507. Twenty-two isolates (65%) were collected from secondary cases (data not shown).

Of 34 rifampicin resistant isolates, 12 (35%) obtained from the sputum of patients with primary infection, consisted of 14 different types of mutations. predominant mutations were demonstrated by 4 isolates (28.5%) in codon 526, 3 (21.4%) in codon 523, 2 (14.2%) in codon 510, and the remaining codons showed no significant frequency of mutations (Tables 1 and 2). The 22 (65%) isolates obtained from secondary infection contained 59 different types of mutations. From these 8 isolates (13.5%) showed mutations in

codon 526, 14 (23.7%) in codon 523 and 7 (11.8%) in codon 510.

This study indicates multiple mutations in codon 523 and codon 526 among the MDR strains of MTB collected from sputum of patients bearing secondary infections. It also demonstrates that the highest frequency of mutations in codons 523 and 526, is observed in both primary and secondary infections, in the southern endemic border of Iran. Although different mutations have been reported for the *rpoB* gene of MTB by PCR-SSCP, but it may not be a reliable tool for the detection of resistance to rifampicin in this strain (Miriam *et al*., 2001). However, if a strong correlation between specific mutations and the level of resistance is confirmed in other settings, the level of rifampicin-resistance may be predictable by DNA sequence-based resistance detection methods (Miriam *et al*., 2001). In this study the detection of deletion mutations (in codons 510, 520, 523, 526 and 527), stop mutation (in codon 513) and silent mutation (in codon 507) (Table 1) confirmed results by Van Der Zanden *et al*. (2003).

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