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Detection and frequency of mutation in the *rpoB* gene of Mycobacterium tuberculosis isolates from patients with active Pulmonary Tuberculosis in different regions of Tehran City

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Abstract

The aim of this study was to investigate the frequency, location and type of *rpoB* mutations in Mycobacterium tuberculosis isolated from patients in Tehran City. 145 sputums were collected from suspected tuberculosis patients, 20 Rif-r isolates were identified as Mycobacterium tuberculosis. PCR Amplification and DNA sequencing methods were performed. 411 bp fragments of *rpoB* gene were sequenced and mutations in 81 bp regions were analyzed. 20 mutations were identified in 14 RIF-r MBT (70%). Missense mutations produced 20 types of amino acid substitutions. In 6 RIF-r MBT isolates (30%) no mutations were found in the core region of the *rpoB* gene. Most frequent mutations detected from Tehranian strains were in codons 531 and 515. Two alleles in codon 531 and one allele in all codons 526, 515, 510, 566, 490 and 476 were found. In the 6 isolates were identified 2 mutation in different codons and 8 strains harboured single mutations in codons. In this study, has been investigated the significance of mutations in the *rpoB* gene, its correlation with genotype and phenotype agents and high level of resistance to rifampicin in 14 isolates of M. tuberculosis collected from patients with active pulmonary tuberculosis from different geographic regions of Tehran.

Introduction

Mycobacterium tuberculosis is the most successful human pathogen worldwide, responsible for 3 million deaths each year and extensive morbidity and mortality (World Health Organization 1998). By the end of 2004, 199 (94%) of 211 countries notified 4.2 million new and relapsed cases, of which 1.9 million (44%) were new sputum smear-positive. A correlation between high mutation rate, antibiotic resistance and virulence in bacteria has been reported in several studies (Bostanabad et al., 2007). Correlations between mutation rates, geographical distribution of mutations, antibiotic resistance and virulence in bacteria have been reported in several studies (Makrousov et al., 2002; Bostanabad et al., 2007). Tehran is one of the oblast and capital of Iran (metropolis) that many people travel towards Tehran city from other countries and other cities of Iran (endemic region; Zabol [Afghanistan border]- Gorgan [Turkmenistan border] - Tabriz [Azerbaijan border] and Iraq border) with active pulmonary tuberculosis. Border of Iran is endemic region in Asia with 10-13 % multiple drug resistant (MDR) among 141 tuberculosis cases per 100,000 populations (Zakerbostanabad et al., 2008). Rifampicin is typically and first line drug used to treat Mycobacterium infections including tuberculosis with multi drug therapy used as the standard treatment. Rifampicin has proven to be an effective anti tuberculosis agent and its use has greatly shortened the duration of chemotherapy for the treatment of TB. Rifampicin resistance heralds higher rates of treatment failure and death for the patient and a poor outcome if the isolate is also resistant to isoniazid. The increasing incidence of multi drug-resistant tuberculosis (MDR-TB), defined as resistance to at least rifampicin and isoniazid, is a notable global health problem (Bakonyte *et al.,* 2005; Dvorska *et al.,* 2001; Garsia *et al.,* 2002; Leung *et al.,* 2003 Makorousev *et al.,* 2002; Bostanabad et al.,; 2007). The detection of resistant *M. tuberculosis* strains is generally perfo-

rmed by conventional susceptibility method which requires culturing the bacilli in presence of the different drugs. The rapid detection of rifampicin resistance is of particular importance, since it also represents a valuable surrogate marker for multi drug resistant (MDR) resistance, which is a tremendous obstacle to TB therapy .(Mc Cammon et al., 2005; Lin et al ., 2004; Titov, 2006; Zheltokjva et al., 2004). Collectively, DNA sequencing studies have demonstrated that >95% of Rif-resistant (Rif-r) *M. tuberculosis* strains have a mutation within the 81bp hot-spot region (codon 507 to 533) of the RNA polymerase beta-subunit (*rpoB*) gene (Titov et al., 2006). The prevalence of the mutations determined so far varies for *M. tuberculosis* strains obtained from different countries. Multi drug-resistant *M. tuberculosis* is an emerging problem of great importance to public health of Tehran.In *M. tuberculosis,* resistance to antibiotics occurs because of genomic mutations in certain genes, such as the *katG* gene for isoniazid (INH) resistance and the *rpoB* gene for rifampicin resistance (Bakonyte *et al.,* 2005; Dvorska *et al.,* 2001; Garsia *et al.,* 2002; Leung *et al.,* 2003 Makorousev *et al.,* 2002). In contrast to several other pathogens with MDR phenotypes, plasmid or transposon-mediated mechanisms of resistance have not been reported in *M. tuberculosis* (Leung *et al.,* 2003; Marcia *et al.,* 2003; Miriam *et al.,* 2001). Since resistance to bacteriostatic in *M. tuberculosis* is exclusively due to genomic mutations, the bacterium would benefit from an increased mutation rate. The polymorphism of the *rpoB* gene, which encodes the beta subunit of RNA polymerase, was used to differentiate mycobacteria through DNA hybridization and DNA sequence comparison (Abate *et al.,* 2001; Douglas *et al.,* 2003; Kima *et al.,* 2003; Titov *et al.,* 2006). The variable region of *rpoB* in mycobacteria is suitable to be used in a PCR-REA assay. This variable region of the *rpoB* gene is flanked by conserved sequences. They enable the amplification of the variable region using the same pair of primers for all mycobacterial species. The *rpoB* region was amplified in 44 species of mycobacteria (Titov *et al.,* 2006; Zakerbostanabad *et al.,* 2006). The aim of this study was to determine resistance-associated mutations in the 81 bp region of the *rpoB* gene in 20 rifampicin-resistances MBT among Tehranian isolates.

Materials and methods

M. tuberculosis isolates

From February to August 2009, 145 patients suspected of tuberculosis were referred to Mycobacteriology Department of Masoud Laboratory – Zarifi Laboratory and Pasteur Institute of Iran. All 145 tuberculosis patient cases had proven registration of clinical diagnostic examination, such as chest X-ray, PPD, cough, weight loss, gender, etc. All isolates were cultured on Lowenstein – Jensen solid medium and grown colonies were identified to the species level using 2- thiophene carboxylic acid (TCH) and paranitrobenzoic acid (PN99B) selective media or by standard biochemical procedures. Four sensitive isolates were used as negative control.

Drug susceptibility testing

The anti-microbial drug susceptibility tests (AMST) was performed using CDC standard conventional proportional method: rifampicin (RIF), 40 mg/L; isoniazid (INH), 0,2 mg/L; ethambutol (EMB), 2 mg/L; ethionamide (ETH), 20 mg/L; streptomycin (SM), mg/L, and kanamycin (K), 20 mg/L and also using BACTEC system (Becton Dickinson company in tuberculosis laboratory of Institute of pulmonology and tuberculosis in Minsk, Belarus (CDC, Kent et al.,1985).

PCR Amplification

DNA extraction was purified using DNA technology kit. 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). PCR was carried out in 50 µL of a reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM MgCl2, 5 µM of deoxynucleoside three phosphates (dNTPs), 1U *Taq* polymerase, 25 pmoles of each set of primers, and 6 µM of chromosomal DNA. Samples were then subjected to one cycle at 94°C for 5 min, followed by 36 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 1 min, and a final cycle at 72°C 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 (Fig.1). 20 cultures of Rif-r Mycobacterium spp. were isolated from 145 sputum specimens collected in Tehran city, 20 Rif-r isolates were identified as *M. tuberculosis.* The agarose DNA Extraction were performed by using DNATechnology Kit (124K6083). Sequencing reactions were performed with the DNA polymerase terminator cycle sequencing kit (Amersham) with 8, µl of PCR-amplified DNA as the template and 2.5 pmol of either the forward or the reverse primer.

DNA Sequencing

A 411-bp fragment of the rpo*B* gene, containing 81-bp rpo*B* fragment, was amplified by PCR using two primers: *rpoB*-F (5-TACGGTCGGCGAGCTGATCC-3) or *rpoB*-R (5-TACGGC-GTTTCGATGAACC-3). PCR was carried out in 8 µl containing(0.25 µl polymerase, 0.9 µl Buffer for DNA polymerase, 2 µl Mixture dNTP and dNNTP(dATP, dTTP, dCTP, dGTP), 0.5 µl primer (2.5 Pmol), 1 µl DNA and 3.35 µl H2O (molecular biology grade). Sequencing of the same primers with PCR parameters were used; 33 cycles of denaturation at 94°C for 30min; primer annealing at 54°C for 30 sec; extension at 72°C for 90 sec. The *rpoB* gene fragments of tuberculosis strains were sequenced using the Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits. Alignment of the DNA fragments (*rpoB*) was carried out with the help of MEGA software (Gen bank_ PUBMED/BLAST). The data were assembled and edited with MEGA and DNAMAN programs.

Analyze of sequence data

DNA sequences from *rpoB* gene were analyzed by "Blast" program. In this manner, sequences of standard strains of H37RV, CDC1551 and M.T.210 (W Beijing) were used as control and compared with test strains. Comparison of all sequences, mutations was performed, by applying "Mega" and "DNA MAN" program. Alignment of the DNA fragments (*rpoB)* was carried out with the help of MEGA software (Gen bank_ PUBMED/BLAST). The obtained data were assembled and edited with DNAMAN programs.

Table 1. Frequency of amino acid and nucleotide changes in different codons of *rpoB* gene of *M. tuberculosis* strains isolated from Teranian isolates

Codon	Frequency	Chang of amino acid	Chang of nucleotide	Isolates
1 Mutations				
531	6	Ser→Leu	TCG→TTG	8, 21, 23,29, 44, 49
531	1	Ser→Trp	TCG→TGG	6
515	1	Met→Ile	ATG→ATA	36
490	1	Gln→His	CAG→CAT	12
476	1	Arg→Gly	CGG→GGG	41
2 Mutations				
531, 566	1	Ser→Leu, Gly→Arg	TCG→TTG, GGG→GGC	15
510, 526	2	Gln→ Glu, His→Asp	CAG→GAG, CAC→GAC	16, 32
510, 515	1	Gln→ Glu, Met→Ile	CAG→GAG, ATG→ATA	26
515, 531	2	Met→Ile, Ser→Leu	ATG→ATA, TCG→TTG	29, 49
Non Mutation				
-	6	-	-	1, 5, 34, 39, 42, 50

Results

Bacterial strains

All specimens were cultured and identified as *M. tuberculosis* by PCR method. 20 Rif-r *M tuberculosis* clinical isolates (including MDR strains) were subjected to DNA sequencing analysis of the hyper variable (hot-spot) *rpoB* region.

Drug susceptibility

All 20 isolates examined were resistant to rifampicin (100%), isoniazid (45%), streptomycin (80%) and 4 isolates (20%) were resistant to ethambutol. In this study we found five strains Mono-resistance to rifampicin.

PCR Amplification and DNA sequencing analysis

All 20 samples were cultured and identified as *M. tuberculosis* by PCR method which revealed 20 mutations in all stains (Table1, Fig. 1). No mutations were found in the core region of the *rpoB* gene in 6 RIF-r M. tuberculosis (30%) (Table.1). In the all isolates not found silent mutations and 20 mutations were missense. All mutations were localized in codons 531,526,515,510,566, 476 and 490 missense mutations revealed 20 types of amino acid substitutions. Most frequent mutated codons in Tehranian strains were 531 TCG/TTG, TCG/TGG and 515 ATG/ATA. (Three types of mutations, Table1, Fig 2). Mutations in codons 531, 515, 510 were observed in 40% (n=8), 20% (n=4), 15% (n=3) of isolates correspondingly and Mutations in codon 531 resulted in Ser→ Leu and Ser→ Trp replacement and in 515 Met→ Ile. We have identified nucleotide change in codons 566 GGG→ GGC, 476 CGG→GGG and 490 CAG→CAT in the 3 isolates (Table 1). In the 6 isolates two mutations in the beta-subunit and in 8 isolates single mutation has been detected (Table 2).

Discussion

Mutations in the *rpoB* gene indicate resistance to rifampicin and are associated with resistance to other classes of drugs, most notably isoniazid (Bostanabad *et al*., 2008; kapur *et al*., 1994). Codons 531 (TCG→TTG) , 526 (CAC→ TAC), 510 (CAG→ _AG), 516 and 511 have been reported as the *rpoB* sites with the most frequent mutations worldwide (Bakonyte *et al*., 2005; Bostanabad *et al*., 2007; Makrousev *et al*., 2003; Sajduda *et al*., 2004; Titov *et al*., 2006), although variations in the relative frequencies of the mutations in these codons have been described for *M. tuberculosis* isolates from different geographic locations (Bostanabad *et al*., 2008; Williams *et al*., 1994). These differences reflect the complex and crucial interaction between rifampicin and targets at the molecular level, where the position of the affected nucleotide changes seems variable. Other investigators have reported different levels of high and low resistance associated with specific nucleotide replacements (Bostanabad *et al*., 2007; Huang *et al*., 2002; Pozzi *et al*., 1999; Titov *et al*., 2006). Sequencing analysis of highly rifampicin-resistant (>100 µg/mL) isolates were found to have point mutations in 531 and 515, which were most frequent in our study population. Other studies also indicated that these mutations are the most prevalent worldwide (Abate *et al*.,2001; Garcia *et al*.,2002; Herrera-Leon *et al*.,2005; Kima *et al*.,2003; Zheltokjva *et al* .,2004). Additionally, we observed one alleles in the codon 515 that previously had not been reported. Mutations associated with nucleotide replacements in codons 510, 526, and 523 were associated with high-level of rifampicin resistance (>100 mg/L), whereas mutations in codon 516 were observed in low-level rifampicin resistance ($p<0.005$) . This finding is not in agreement with other authors who have reported different levels of high (Deepa *et al*., 2005; Mokrousov *et al*., 2002; Shin *et al*., 2005; Sreevatsan *et al*.,1997) and low (Garcia *et al*., 2002) resistance association with specific nucleotide replacements. As *rpoB* is an essential gene for the survival of the cell, then in principle the single nucleotide change or deletion should result in a frame shift and the formation of a truncated and non-functional protein. Although we have re-subcultured and re-sequenced all isolates, we have no explanation of how and what type of truncated protein might have been produced which could not kill the cell (Bostanabad *et al*., 2008; McCammon *et al*., 2005; Ruiz *et al*., 2004). This is the first report describing the genetic characteristics of multi-drug resistant *M. tuberculosis* strains isolated from TB-patients in the different region of Tehran city. Our finding of mutations is partially comparable and resemble to those reported strains from other countries (Abate *et al*.,2001; Dvorská *et al*., 2001; Garcia *et al*., 2002; Kiepiela *et al*., 2000; Zheltokjva *et al*., 2004). Higher frequencies of mutation bearing sites found in our data, in codon 531, 515,

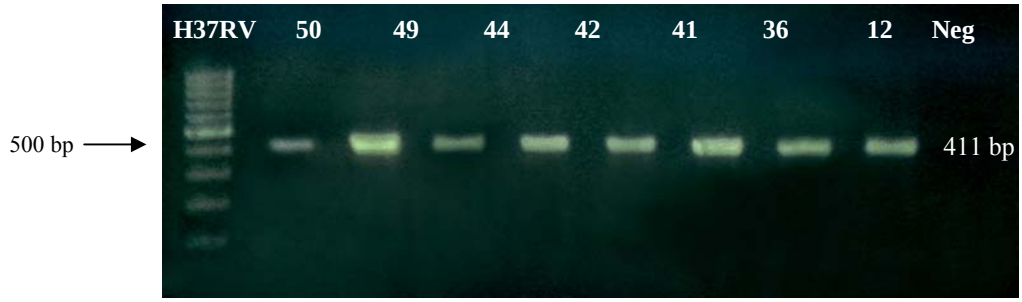

Fig1. PCR amplification genome *rpoB* fragment 411bp from DNA extracted from Tehran's patients

526, and 510, can be partially compared with those isolates reported from other investigators in the countries (Abate *et al*., 2001; Bakonyte *et al*.,2005; Douglas *et al*., 2003; Dvorská *et al*.,2001; Leung *et al*., 2003; Makorousev *et al*., 2002). However, we identified higher point mutation (n=8, 40%) in one cysteine nucleic acid base (TCG → TTG, TGG). There are other changes found in codons 531 (in India – TCG→TGG, TTG, in Russia – TCG→TGG, CAG or TGT, in China – TCG→ TTG, in Japan- TCG→ TTG, in Korea- TCG→ TTG, in Taiwan- TCG→ TTG and Ser→ Gln). CAG mutation of codon 510 (deletion or CTG or CAC or CAT) is very seldom detected in other countries (Bobadilla-del-Valle *et al*., 2001; Mohammad *et al*., 2006). However in our study we found much more mutation deletion (3 strains) in one base C (GAG). On the other hand, in other countries there are no changes in codon 510 (Abate *et al*., 2001; Garcia *et al*., 2002; Titov *et al*., 2006). Mutation CAG→CAT was found in India (Bakonyte *et al*., 2005), in Russia – CAG→CAT, in Belarus CAG→GAG, TAG was also found in this codon, in Lithuania CAG→GAG and in Poland CAG→GAG (Abate *et al*., 2001; Garcia *et al*., 2002; Kima *et al*., 2003; McCammon *et al*., 2005; Spindola de Miranda *et al*., 2001; Titov *et al*., 2002). Our result indicate Prominent findings which is in contrast with other reported investigations on codons 510 (12.51%), 523 (23.6%) and 526 (16.6%) which are the most frequent mutations bearing sites. In this study, we observed one nucleotide polymorphism change among 4 isolates in the codon 515 that has been changed (515 ATG→ATA), 566 (GGG→ GGC), 476 (CGG→ GGG) and 490 (CAG→CAT). This change nucleotide has not been previously reported by other investigators. In addition, predominant mutations in codon 526: CAC→ GAC (n=2, 10%) was also found to be more prevalent in patient isolates bearing secondary cases. However, in other reports the frequency of mutations in codon 526 (CAC to GAC) among Italian isolates was 40.1% (Ohno *et al*., 1996), in Greece isolates (CAC to GAC) 17.6% (Matsiota-Bernard *et al*., 1998) and CAC to GAC were reported to be more prevalent among American isolates 27.9% (Kapur *et al*., 1994). Our data reveals a similar mutation frequency (58%) as others have observed in Asia, while significantly lower than Italian and Greece isolates (40%). In this study, we found six rifampicin resistant strains revealing no mutation. This finding is in agreement with other reports indicating geographical variations in nature, including mutations outside the 81-bp segment of *rpoB* gene or additional molecular mechanism that may be involved in rifampicin resistance of *M. tuberculosis*. Mechanism such as a permeability barrier or membrane proteins acting as drug efflux pumps may also confer resistance to rifampicin (Bostanabad *et al*., 2008; Hirano *et al*., 1999; Ohno *et al*., 1996). In this research, we did not attempt to characterize any gene other than β-

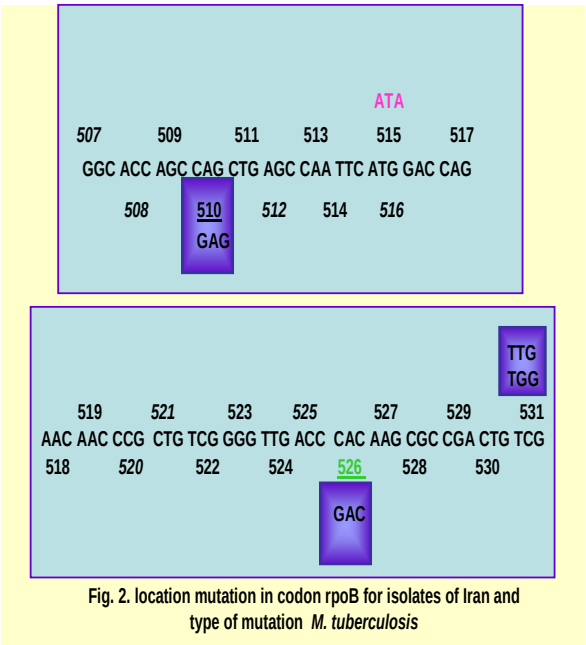

Fig 2. Location of mutation in codon *rpoB* for isolates of Tehran and type of mutation *M. tuberculosis*

subunit region and a second formal possibility to account for the lack of detected *rpoB* mutations in these six rifampicin – resistant isolates which confer resistance without revealing any mutations that changes might have occurred in one or more genes whose products participate in antibiotic permeability or metabolism (Bostanabad *et al*., 2008). This study was performed in a unique limited geographical endemic area with poor border control on population movement due to economical and tribal refuge camp communications, in which inadequate chemotherapy can be a major factor for mutation. Other explanation could be possibly that a subset of the mutations detected was actually naturally occurring polymorphisms or silent mutation that do not change the amino acid sequence present in isolates circulation in this endemic region. These polymorphisms may not be correlated with rifampicin resistant (in our data has not been identified). Although the presence of a mutagenic agent (chemical or physical) can be a major factor causing mutations, we have used Fermentas kits. In conclusion, this study demonstrates an association between the mutation of

rpoB gene and their correlation with the predominant nucleotide change in codons 531, 515, 510 and 526. Notably, mutations in codon 531 were more commonly identified from secondary infection cases with a high level resistance (MIC≥ 100 to 400 mg/l) in the isolates from Tehran city. The high proportion of mutations in the 81-bp fragment hot-stop region of *rpoB* was observed to be located in a segment of the *rpoB* gene. This result has been identified distribution mutants in the different regions of Tehran city.

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