Molecular Characterization and Tree Evolution of Rifampicine and Isoniazid-Resistance in Multi Drug Resistance Strains Isolated From Primary and Secondary Tuberculosis Diseases in Southern Endemic Border of Iran

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Abstract
Study Objective: The aim of this study was to investigate frequency and type of mutations in the katG and rpoB genes and provide tree evolution of M. tuberculosis isolates collected from patients in southern endemic region of Iran.
Setting and Design: 91 sputums were collected from suspected tuberculosis patients, 34 rifampicine isolates and 26 isoniazid-resistant were identified as M. tuberculosis. Drug susceptibility was determined by using CDC standard conventional proportional method.
Result: 60 mutations and 13 micro deletions were identified in 29 rifampicin resistant M. tuberculosis strains (85%). In 5 rifampicin resistant M. tuberculosis isolates (15%) no mutations were found in the core region of the rpoB gene. Most frequent mutations detected among Iranian strains were in codons 523(58.6%) and 526 (41%). Six alleles in codon 526 and 3 alleles in triplets in each codons 507(27%), 508 (20.5%), 513(10.2%) were found. In 21 isoniazid resistance isolates three types of mutations: AGC→ACC (Ser→Thr) (80%), AGC→AGG (Ser→Arg) (5%) and AGC→AAC (Ser→Asn) (15%) observed in codon 315. One type of mutation was detected in codons 311, 299, and 322. Two isolates revealed isoniazid resistance without demonstrating mutation in katG gene. In contract with tree evolution are 2 groups: group A- similar to the standard and Eastern strains (China, Taiwan) and group B- strains of another genotype.
Conclusion: This research demonstrates that codons 315 and 311 are more frequently observed agent indicating resistance to isoniazid and 523,526 for rifampicine resistance and gives a first overview of the M. tuberculosis strains circulating in Iran during the first survey of anti-tuberculosis drug-resistance.

Key words: Frequency, mutation katG and rpoB, tree of evolution, M. tuberculosis Iran

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INTRODUCTION

The World Health Organization (WHO) estimates one-third of the world's population or approximately 2 million people are infected with Mycobacterium tuberculosis. The key to control the spread of tuberculosis includes proper case finding, rapid diagnoses of tuberculosis and prompt initiation of effective chemotherapy [1-5]. Zabol is in Southern endemic region of Iran (Afghanistan border) with 10 to 13% MDR among 141 tuberculosis cases per 100.000 populations [1]. Several studies have indicated genomic region of MTB involved in development of resistance to isoniazid. Resistance to rifampicine is increasing because of widespread application and results in selection of mutants resistant to other components of short-course chemotherapy; 88% of hospitalized patients with drug resistant TB in Massih Daneshvari Hospital (Iran-Tehran) were resistant to at least isoniazid and rifampicine [1]. Drug resistance has been known since the discovery of the first anti-TB drug, streptomycin, in 1954 and the presence of resistant mutants in wild populations of mycobacteria has been well documented [5-7, 9]. Surveillance of the primary and secondary resistance patterns is important in assessing the quality of chemotherapy programs over several years and detecting errors in past treatments respectively [5, 10-13].

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 (RIF) resistance [2, 4, 7, 13-15]. Collectively, DNA sequencing studies have demonstrated that >95% of rifampicine-resistant M. tuberculosis strains have a mutation within the 81bp hot-spot region (codon 507 to 533) of the RNA polymerase beta-subunit (rpoB) gene [11, 12, 16- 18]. In contrast to several other pathogens with MDR phenotypes, plasmid or transposon-mediated mechanisms of resistance have not been reported in M. tuberculosis [13-17]. Since resistance to bacteriostatic in M. tuberculosis is exclusively due to genomic mutations, the bacterium would benefit from an increased mutation rate.

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M. tuberculosis is a member of the M. tuberculosis complex, a group of five closely related "sibling" species [M. tuberculosis sensu stricto (s.s.), M. africanum, M. microti, M. bovis, and M. canettii] that cause tuberculosis in humans and animals [6, 8, 10, 11, 19]. Study of 56 genes in several hundred M. tuberculosis complex strains suggested that there is about one synonymous nucleotide substitution per 10,000 nucleotide sites [17, 20-24]. Restricted allelic variation limits the utility of multilocus sequence analysis [5, 12, 13, 22, 23].

Although many molecular methods have been used for categorizing M. tuberculosis strains, phylogenetic relationships among this group of organisms have not been resolved. The distribution of these nsSNPs provided evidence supporting the hypothesis that principal genetic group 1 is ancestral to group 2 and that group 2 is ancestral to group 3[10, 15, 16, 20, 25]. However, because the original genetic groupings are based on only two nsSNPs, the evolutionary hypothesis of M. tuberculosis outlined requires additional investigation. Moreover, the worldwide threat of M. tuberculosis to human health emphasizes the need to develop rapid methods to identify genetic relationships among all strains, especially among organisms responsible for large infection outbreaks, drug-resistant strains, and strains that cause severe clinical disease. The availability of genome sequences of two M. tuberculosis strains (http://www.tigr.org) and of partial genome sequences of a third M. tuberculosis strain (http://www.tigrblast.tigr.org) and an M. bovis strain (http://www.sanger.ac.uk /Projects/M_bovis) facilitated resolution of genetic relationships among M. tuberculosis complex organisms by large-scale sSNP analysis. Our results indicate that sSNP genotyping permits all strains of closely related pathogens to be assigned to lineages that are identical or related by descent and removes a critical barrier to population-based studies of the relationships between strain genotypes and patient phenotypes [11, 18, 26].

The polymorphism of the rpoB gene, which encodes the beta subunit of RNA polymerase, was used to differentiate mycobacteria through DNA hybridisation and DNA sequence comparison [1, 3, 9, 19, 22, 27]. 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 [22, 23, 25, 28].

The observation that most isoniazid-resistant M. tuberculosis strains did not have gross katG deletions suggested the need to more precisely analyze the structure of katG present in resistant organisms. About now resistant to at least one first-line or primary anti-tuberculosis drug (isoniazid{IHN}, rifampicine{RIF}, streptomycin{STR}, ethambutol{EMB}, and pyrazinamide {PZA}), and 3.2% are resistant to both isoniazid and rifampicine [17, 23, 24, 29]. Mutations in several genes and genomic regions of Mycobacterium tuberculosis are involved in the occurrence of resistance to isoniazid [10, 13, 22, 25, 30]. Another frequent target is the regulatory region of the inhA gene, where mutations have been reported in up to 32% of isoniazid-resistant isolates two enzymes involved in the biosynthesis of mycolic acids have been suggested to be the targets of KatG-activated isoniazid: the NADH-dependent enoyl-acyl carrier protein reductase (inhA) [10, 19] and the -ketoacyl acyl carrier protein synthase (designated KasA) [10, 13, 22]. The prevalence of the mutations determined so far varies for M. tuberculosis strains obtained from different countries. Multidrug-resistant M. tuberculosis is an emerging problem of great importance to public health of Iran.

The aims of the present study were to identify various types of mutations in katG region from 28 isolates and were to determine resistance-associated mutations in the 81 bp region of the rpo B gene MDR (Multi Drug Resistance) strains and give a first overview of the M. tuberculosis strains circulating in Belarus during the first survey of anti-tuberculosis drug-resistance isolated from sputum of tuberculosis patients in the southern endemic region of Iran (Afghanistan border, Zabol).

MATERIALS AND METHODS

Mycobacterial strains and Drug susceptibility testing:

Mycobacterial strains: 91 strains randomly isolated from sputum of patients with active pulmonary tuberculosis in different geographic regions of Afghanistan border from December 2004 to May 2005. All 91 tuberculosis patient cases had proven registration of clinical diagnostic exanimations, such as chest X-ray, PPD, Cough, weight loss, gender etc. Patient sputum samples was cultured on Lowenstein- Jensen solid medium and grown colonies were identified to the species level using TCH (2-thiophene carboxylic acid) and PN99B (paranitrobenzoic acid) selective media or by standard biochemical procedures. Four sensitive isolates was used as negative control [5].

Drug Susceptibility testing: Anti-Microbial drug Susceptibility Testing (AMST) was performed using CDC standard conventional proportional method (rifampicine (Rif) - 40 mg/L, isoniazid (INH) -0,2 mg/L, ethambutol (EMB) - 2 mg/L, ethionamide (ETH) - 20 mg/L, streptomycin (SM) -4 mg/L, and kanamycin (K) - 20 mg/L) [8], in addition to BACTEC system with drug concentration of isoniazid 0.1 μg/ml and rifampicine 2.0 μg/ml.

Standard PCR identification and katG gene amplification: DNA Extraction was done by: Fermentas kit (K512), and DNA purification by Fermentase kit (k513). DNA from Mycobacterium tuberculosi CDC1551 and Mycobacterium H37RV strains was purified and used as control. A 209 bp fragment of katG gene was amplified by PCR with the following synthetic oligonucleotide primers:

katG F 5-GAAACAGCGGCGCTGGATCGT-3, katG R 5-GTTGTCCCATTTCGTCGGGG-3 [24]. PCR amplification of 0.5 μl of chromosomal DNA was performed with a 30-μl mixture containing 50 mM KCL, 10mM Tris-HCL (pH 8.2), 1.5mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, and dTTP, 500 nM each primer, and 0.83 U of AmpliTaq DNA polymerase (Company DNA technology of Russia). The following thermocycler parameters were used: initial denaturation at 94°C for 5 min; 42 cycles of denaturation at 94°C for 1 min; primer annealing at 57°C for 1 min; extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The products was checked and purified on gel-electrophoresis and katG segment was amplified. The resultant DNA amplifications was used for sequencing.

Standard PCR identification and rpoB gene amplification: M. tuberculosis DNA extraction were purified using Fermentas kit's (K512). A 411-bp fragment of the rpoB gene was amplified by PCR with primers rpoB-F (5- TACG-GTCGGCGAGCTGATCC-3) and rpoB-R (5-TACG-GCGTTTCGATGAAC-3) [17]. 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 triphosphates (dNTPs), 1U Taq polymerase, 20 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 411-bp band after ethidium bromide staining. The DNA purification were performed on agaros using Sigma Kit (124K6083).

DNA sequencing katG fragment: Sequencing of 209-bp fragment of katG gene was amplified by PCR using forward and reveres primers; 33 cycles of denaturation at 94°C for 30sec; primer annealing at 48°C for 45 sec; extension at 60°C for 4 min. The katG gene fragments of tuberculosis isolates were sequenced using the Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits.

DNA Sequencing rpoB fragment: Sequencing of 411-bp fragment were done using the same forward and reveres primers; 33 cycles of denaturation at 94°C for 30min; primer annealing at 54°C for 30 sec; extension at 72°C for 90 sec, A 411-bp fragment of the rpoB gene extracted from tuberculosis strains were sequenced by 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).

Data analyzing of DNA sequencing: DNA sequences from rpoB and katG fragment were analyzed by "Blast" program (http://www.ncbi.nlm.nih.gov/BLAST/). In this manner, sequences of standard strains of H37RV, CDC1551 and M.B.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 analyzed and edited with DNAMAN programs.

Mutations spectrum and frequency analysis: DNA-MAN is a sequence analysis software package for IBM compatible computers with Microsoft Windows 95/98 or NT/2000 systems. This package provides effective and convenient tools for molecular biologists to deal with frequently used analyses in research. It contains text editor for fragments sequence alignment. The text editor is identical to WINDOWS WordPad. The editor allows to delete or paste any text or sequence to files.

Homologic dendrogram: This dendrogram is setup with the distance matrix using the UPGMA method (Sneath and Sokal, 1973, Numerical Taxonomy, San Francisco, USA). The matrix can be built up only with Observed Divergence method. Dendrogram shows related homologies between two sequences or groups.

Phylogenetic dendrogram: This dendrogram is setup with the distance matrix using the Neighbor-Joining method (Saitou and Nei, 1987, Mol. Biol. Ecol. 4:406-425). Phylogenetic tree shows related homologies between any two sequences in a multiple alignment.

RESULTS

Mycobacterial isolates and susceptibility: All samples were cultured and identified as M. tuberculosis by biochemical methods. All 34 isolates of rifampicine resistant examined were resistant to rifampicine, isoniazid (80%), streptomycin (90%) and 18 isolates (48%) were resistant to etambutol. In this study we found two strains Mono-resistance to rifampicine. All 26 isolates isoniazid resistant examined were resistant to isoniazid, rifampicine (65%) streptomycin (82%) and 8 isolates (28%) were resistant to etambutol. Mono-resistance to isoniazid was observed for 4 isolates (14%) in this study. 31 isolates were resistant to other drug or sensitive to drugs.

PCR Amplification and DNA sequencing analysing for katG: All 26 samples were cultured and identified as M. tuberculosis by PCR method that reveals 49 mutations in all stains. From 26 isoniazid-resistance 2 strains showed no mutation and in 21 strains mutations were observed in codon 315, revealing three types of mutations consist of AGC→ACC (Ser →Thr) (80%),AGC→AGG(Ser→Arg) (5%) and AGC→AAC(Ser→Asn) (15%). One type of mutation obtained in codon 299 indicating GGC→AGC and changes in amino acid Gly→Ser. In codon 311(18%) (katG) only one base change were obtained Tyr→Tyr (GAC→TAC) in nine strains demonstrate a nonsense mutation (Table 3).

Furthermore only one mutation observed in codons 311,299 and 322. And in 12 strains one mutation in codon 315 (42.8 %), 7 strains 2 (25%), 5 strains 3 (17.8%) and 2 isolates 4 mutations (7.1 %) were obtained respectively (Table 3) (Figure 2).

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

Evolutionary analysis of relationships of M. tuberculosis strains prevalent on the territory of Iran

Sequencing of 81 bp fragment of rpoB gene was carried out for 29 strains. On the of the sequences obtained a dendrograms was constructed by UPGMA and Neighbour-Joining methods in DNAMAN software.

Homologic dendrogram was constructed by UPGMA method using distance matrix. According to the dendrogram Iranian strain 710 is closely relater to the strains from Taiwan, China and India (BLAST software). The other strains are supposed to have originated from this one.

Phylogenetic dendrogram. With the help of Neighbour-Joining method the data obtained confirm the results of Neighbour-Joining method for the Iranian strain 710 (Figure 3).

Table 1. Frequency of amino acid and nucleotide changes of different codons in rpoB gene of 29 rifampicin-resistant strains of M. Tuberculosis isolated in Iran

Codon and Amino acid chenge	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

Table 2. Data for rpoB mutations (single, double, triple, quartile and five) in Rifampicine-resistant M. tuberculosis strains isolated from Iran

Frequency of Mutation	Number of codon	Number of Isolates	Isolate number
Non mutation	-		23(2)-28-
			584,103,29
1 Mutation	526	3	
	510	3	3062,108,36
			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	2
	511-520-523	1	663
	511-523-526	1	303-281
			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

other authors who have reported different levels of high [8, 25, 27, 30] and low [6, 17, 31] resistance association with specific nucleotide replacements.

This is the first report describing the genetic characteristics of multidrug-resistant M. tuberculosis strains isolated from TB-patients in Iran. Rifampicin is the first line drug used in WHO TB treatment protocol of short course chemotherapy (6 months regimen) in Iran [1, 24].

Sequencing analysis of highly rifampicin-resistant isolates were found to have deletion mutations in codon 510 and point mutations in codons 526, 523 and 531, which were most frequent in our study population. Our result indicates prominent finding 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 (Table 1). Our finding of mutations is partially comparable and resemble to those reported strains from other countries [2, 4, 14, 21, 25, 27, 28]. CAG mutation of codon 510 (deletion or CTG or CAC or CAT) is very seldom detected in other countries [7, 12, 14, 21, 27, 28] or no changes in codon 510 [1, 4, 6, 30]. However in our study (Table 1) we found much more mutations deletion (9strains) in one base C (CAG). Mutation CAG→CAT was found in India, in Russia CAG→CAT, in Belarus CAG→GAG, TAG was also found in this codon, in Lithuania CAG→GAG and in Poland CAG→GAG [2, 7, 8, 10, 12, 21].

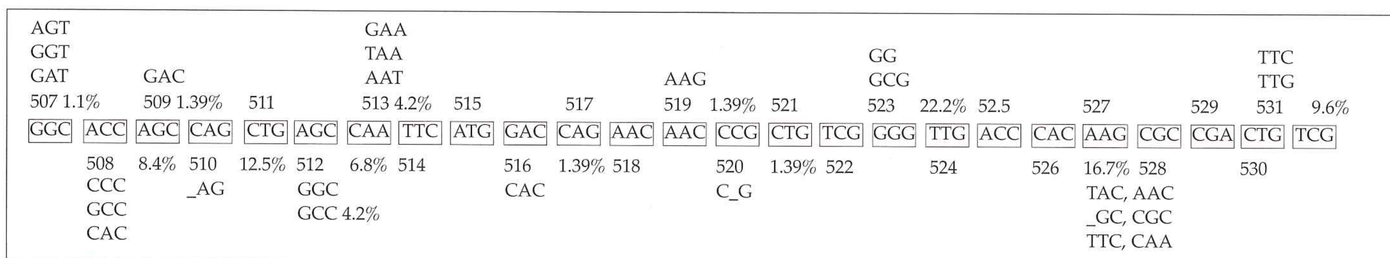

Figure 1. Percentages of mutation in different codons of the rpoB gene from 29 rifampicine resistance strains M. tuberculosis isolated in Iran

DISCUSSION

Mutations in rpoB indicate resistance to the rifampicine class of drugs, and is indeed associated with resistance to other classes of drug notably INH [1, 4-6, 13]. The rpoB gene codons 531 (TCG→TTG), 526 (CAC→TAC), 516 and 511 are the most frequently mutated sites worldwide [1, 2, 4, 7, 12, 13], although variations in the relative frequencies of mutations in these codons have been described for isolates from different geographic locations [1, 13, 18, 24-26]. Other studies also indicated that these mutations are the most prevalent worldwide [2, 17, 19, 27-29].

These differences reflect the complex and crucial interaction between the drug and its target at the molecular level, where the position of the affected polymorphism seems to be variable. This finding is not in agreement with

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 526 (in India CAC→CTC, TAC, GAC, CGC or ACC in Russia CAC→CTC, GAC, CAA, CAG, TGC, AAC, CGC or CCC, in China CAC→TAC, in Japan CAC→TAC, in Taiwan CAC→TAC and CGC, in Korea CAC→TAC) [2, 8, 14, 21, 27, 28]. We observed two polymorphisms in codon 531 that previously were not described: of 7 strains, five isolates (6.78%) TCG→TTG (Ser→Leu) and two were (2.78%) TCG→TTC (Ser→Phe). And CAC→TAC change in codon 526 was detected in four strains; in contrast, we could not find GAC, CTC change in all isolates studied. While in

Table 3. Frequency of amino acid and nucleotide changes of different codons in katG gene of 26 isoniazid-resistant strains of M. Tuberculosis isolated in Iran

Codon	Frequency		Amino acid change	Nucleotide change	Isolates
			1 Mutation		
315	12	10	Ser→Thr	AGC→ACC	3542, 600, 90, 10, 19, 441, 28, 3708, 29, 3548
		1	Ser→Arg	AGC→AGG	98(1383)
		1	Ser→Asn	AGC→AAC	1619(MAC)
			2 Mutations		
309	2	2	Gly→Val	GGT→GTT	633, 12
315	2		Ser→Asn	AGC→AAC	
315	1	1	Ser→Thr	Asn→Ile	633
322			Asn→ Ile	AAC→ATC	
315	2	2	Ser→Thr	Pro→Ala	
324			Prof→Ala	CCG→GCG	108, 303(281)
299	1	1	Gly→Ser	GGC→AGC	411
311			Tyr→Tyr	GAC→TAC	
311	1	1	Tyr→Tyr	GAC→TAC	610
315			Ser→Thr	AGC→ACC	
			3 Mutations		
299	1	1	Gly→Ser	GGC_AGC	159 (MDR)
311			Tyr→Tyr	GAC→TAC	
315			Ser→Thr	AGC→ACC	
299	3	3	Gly→Ser	GGC→AGC	163, 167,
311			Tyr→Tyr	GAC→TAC	165(MDR)
322			Asn→Ile	AAC→ATC	
299	1	3	Gly→Ser	GGC→AGC	161(MDR)
311			Tyr→Tyr	GAC→TAC	
324			Pro→Ala	CCG→GCG	
			4 Mutation		
299	2	2	Gly→Ser	GGC→AGC	290 and 710 (MDR)
311			Tyr→Tyr	GAC→TAC	
315			Ser→Thr	AGC→ACC	
322			Asn→Ile	AAC→ATC	

299	309		311				315							322		324
GGC	AAG	GAC	GCG	ATC	ACC	AGC	AGC	ATC	GAG	GTC	GTA	TGG	ACG	AAC	ACC	CCG
AGC 6.3%	GTT 4%		TAC 18%				ACC 34% AGG 2.04 % AAC 6.1%							ATC 12.4%		GCG 6%

Figure 2. Percentages of mutation in different codons of the katG gene from 26 isoniazid resistance strains M. tuberculosis isolated in Iran

other reports frequency of mutation (40.1%) in codon 526 (CAC to GAC) occurred in Italian [6] and 17.6% in Greece isolates [13]. CAC to TAC (27.9%) was more prevalent in codon 526 in American isolates [4, 6]. Our data have very close relation (60%) to those observed in Asia, while in contrast to Italian and Greece isolates 40.1% (His-526-Asp) which we have not found such mutation of His-526-Asp among all our strains studied [2, 4, 6, 8, 17, 19, 22]. Comparison of our data with other countries indicate fewer mutations in codon 531 (TCG→TTG) [2, 4, 6, 11, 17, 19], and more mutations in codon 526 (CAC→TAC, CAC→CGC, CAC→CGC, CAC→AAC, CAC→TTC and CAC→CAA) are found in Iran. Mutations in codon 526 (CAC→CAG) and mutations in codon 516 (GAC→GTC) are not often seen in Iran, Poland and USA [4, 6, 12].

We detected two polymorphisms in codon 511 (CTG→GTG, CCG) which is one of frequent mutations worldwide [2, 4, 14, 21, 28]. These findings demonstrate that the frequencies of particular mutations in rifampicin-resistant M. tuberculosis isolates from Iran are different

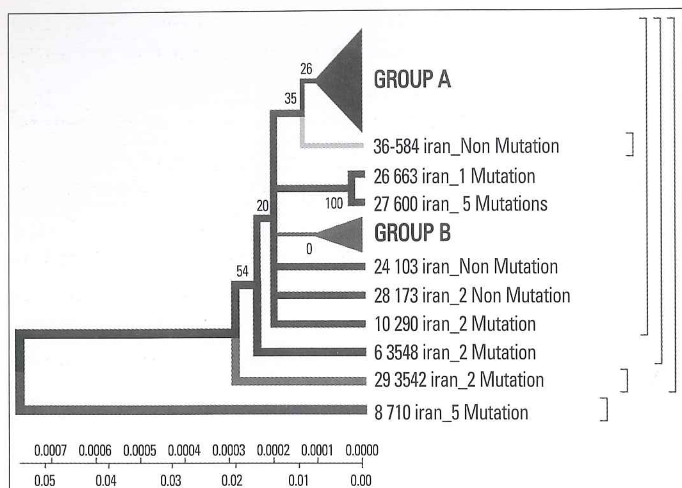

Figure 3. Phylogenetic dendrograms of M. tuberculosis strains isolated from Iranian patients within the time period of 70 years by Neighbour-Joining method. The phylogenetic tree, generated using the neighbor-joining method with 1000 bootstrap replicates and distance calculated using the number of different sSNP loci (http://www.megasoftware. net/).

from those that have been reported from other parts of the world. Although the combination of two single point mutations has been described previously for rifampicin-resistant M. tuberculosis strains [2, 4, 6, 13, 15, 19].

In our study an extraordinary 12 mutations deletions result in a frame shift upon deletion of one base pares mutation in a codon which did not affect the viability of cells upon 3 consecutive sub-cultures and re-sequencing, which might indicate gene duplication phenomena that we don't have solid evidence to prove this extraordinary observation. Our finding about deletion mutations (in codons of 510,520,523,526,527), stop mutation (in codon 513) and silent mutation (in codon 507) (Table 1) is similar to result obtained by Van Der Zanden [19] and corresponds to stop codons and multi-mutations observed in critical region of M. tuberculosis rpoB gene in 44 rifampicine-resistant isolates in Belarus [32].

The high percentage of double mutations found among Iranian strains (32%) clearly differed with other studies [2, 4, 6, 12, 21, 27, 28, 30]. In this study high frequency of triple (20%) and quadruple (2.9%) occurring in separate codons were observed (Table 2). We also found five mutations in each of four strains that have not been previously reported. However we don't have any knowledge of which combination of specific types of mutations are associated with rifampicin resistance.

Of 34 rifampicine resistance isolates 12(35%) from sputum of patients with primary infection were consist of 14 different types of mutations with predominant mutations in 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, 2). From 22 (65%) isolates of secondary infection which were consist of 59 different types of mutations in 8 isolates (13.5%) in codon 526, 14(23.7%) in codon 523 and 7 (11.8%) in codon 510 (Table 1, 2) (Figure 1).

The majority of hot mutations of Mycobacterium tuberculosis in katG gene have been reported in codon 315 (Ser→Thr) and less in other codons [16, 26, 31]. Unlike most resistance-confering mutations in codon 315 (Ser→Thr) was found to result on near-normal catalase-proxidase activities and some levels of virulence which also conferring resistance to isoniazid. This mechanism of isoniazid resistance is not usually associated with a large reduction in virulence and is an exception to the rule that antibiotic-resistance conferring mutation carry a significant fitness cost [4, 31]. In this research most mutations in 209-bp fragment was observed in codon 315, indicating three types of mutations AGC→ACC, AGC→AGG, AGC→AAC in 21 isolates, confers 75% of all of isolates (Fig.1). Other authors reported the following nucleotide changes in codon 315 in other countries, consist of (AGC→ACC, ACA, ACT, ATC, AAC) [4, 16, 17, 27, 31], Contrary to our data higher nucleotide changes observed as AGC→ACC. In addition we observed one type of mutation as AGC→AGG, which has not been reported.

Nucleotide changes observed in codon 322 AAC→ATC (n=6) and 299 GGC→AGC (n=8) resulted in amino acid changes of Asn→Ile and Gly→Ser respectively (Figure 1), which also have not been reported and may play a role in emergence of resistance due to changes in cell wall or preplasmic protein. In addition to mutations in catalase peroxidase gene (katG) in codon 315 which is 100% specific for isoniazid resistant isolates from respiratory specimens, since no susceptible isolates exhibited Thr315 [16, 21, 31]. Other mutations have also been reported in several countries, codon 279 (Poland), 88 and 155 (Russia) [16, 22], which have not been demonstrated in this study. However, Lower number of mutations was detected in codon 299, 311, 322 (Table 1). These results may indicate that different types of mutations observed in codons 315, 299 and 311 may reflect geographic and epidemiologic position in the southern endemic regions of Iran (Afghanistan border, Zabol) (Figure.1).

Among isoniazid resistance of primary infection cases two isolates demonstrated no mutation in katG gene. Other studies showed there are additional genes responsible for isoniazid resistance, such as inhA, ahpC,oxyR and kasA [17, 23], that we have not studied in this research. Absence of mutation in 209 bp fragment of the above two isolates does not exclude the absence of katG alteration, since any frame shift would result in loss of

katG activity (entire katG was not sequenced in this study) therefore we can not clarify the nature of these two isonioazid-resistant isolates.

High percentage of single mutations found among Iranian isolates considerably differed from the lower prevalence of double mutations in other studies [1]. A characteristic prominent finding of this study was the high frequency of single (42.8%), double (25%) and triple (17.8%) mutations were found in separate codons (Table 1, Figure 1).

In this research all isoniazid resistant isolates demonstrated different types of mutations in katG gene; however we don't have any data of combinations of specific type of mutations associated with isoniazid resistance.

Among 28 isoniazid-resistant isolates 9 (32.2%) were from sputum of patients with primary infection in which 2 isolates {n=2, 7.1%} revealed no mutation and 7 found to have different types, consist of one quartile and one triple mutation, and five isolates demonstrated single mutations in codon 315. Predominant mutations in codons 315 (63.6%), 311 (0.9%) and 299 (0.9%) were observed in 7 isolates from primary infection cases (25%). We could not find MDR isolates among 9 primary infection cases (Table 1).

Among 19 (67.8%) isolates of secondary infection cases in patients with history of tuberculosis not responding to standard WHO short course chemotherapy, "six months treatment regimen", (we don't have data on reactivation cases) 14 (36.8%) were found to have mutations in codons 315, 7(18.4%) in 299 and 8 (21%) in 311 (Table 1, Fig. 1). Interestingly four isolates conferring triple (17.8%) mutations and five MDR isolates were all found in sputum samples taken from patients with secondary infection (Table 3) (Figure 2).

In this study we found multiple mutations in codon 315 and 299 that previously has not been reported. Five isolates with 3 (17.8 %) and 2 isolates with 4 mutations (7.1%) were found which might demonstrate MDR isolates from sputum of patients with secondary infection in adjacent areas of southern endemic region of Iran (Zabol).

Absence of mutation in 209 bp fragment of the above two isolates does not exclude the absence of katG alteration, since any frame shift would result in loss of katG activity (entire katG was not sequenced in this study) therefore we can not clarify the nature of these two isonioazid-resistant isolates.

In this study we found multiple mutations in codon 315 and 299 which have not been reported previously. Five isolates with 3 (17.8 %) and 2 isolates with 4 mutations (7.1%) were detected conferring MDR isolates collected from sputum of patient with secondary infection, which might indicate presence of mutations among isolates form secondary infection in adjacent areas in southern endemic region of Iran (Zabol).

In addition to mutation in catalase peroxidase gene (katG) in the codon 315, 463 other mutations have also been reported in codon 279 (Poland) 88 and 155 (Russia) [16, 21], which have not been demonstrated in our study. These results may indicate that different types of mutations observed on codons 315, 299 and 311 may reflect geographic and epidemiologic position in the southern endemic regions of Iran (Afghanistan border, Zabol). Changes in codon 315 and 311 is also associated with isoniazid-resistance in Iran .Other studies showed that there are additional genes responsible for isoniazid resistance ,such as inhA, ahpC,oxyR and kasA genes [9, 16], that we have not been studied in this research. Finally the question that any changes in other genes above that may affect isoniazid resistant must be further investigated.

According to the dendrogram one strain from the town of Zabol is older than the rest of the strains (it is about 77 years old - evolution rate), and it was considerably changed after its appearance in Iran and was resistant to anti tuberculosis drugs (Figure 2). This strain contains five mutations. When it appeared in Iran it possessed drug resistance. The other strains similar to each other are located on the same branch of the dendrogram (Figure 2). The other strains are divided into 2 groups: group A - similar to the standard and Eastern strains (China, Taiwan), they are about 10 years old. These strains were changed in Iran and acquired mutations (Figure 2); group B - strains of another genotype. They are grouped separately on the dendrogram and became prevalent in Iran (they are called Iranian residential strains).

According to the dendrogram constructed by UPGMA method all strains are divided into two genetic groups. Some of the strains differ in genotype and divergence time and are located separately (Figure 2). 60% of Iranian strains diverged as a result of mutations from the same M. tuberculosis strain 710 similar to the strains from China, Taiwan and India. 20% of the strains had the highest similarity with the ones from Taiwan. One isolate (from Zabol region) 710 is related evolutionary to ancestral M. tuberculosis family. In this sample there are no mutations in codon 531. Strain 163 located separately and the number of strains combined in one group originated from it. Four isolates belong to the standard M. tuberculosis strains (H37RV, CDC1551), and on of these four is similar to Indian strain 3062 Iran (Figure 3). According to the dendrograms constructed for rpoB gene the strain 710 from the town of Zabol is more closely related to the Chinese strains. The other strains are

supposed to have diverged from this strain. Iranian strain 710 has the greatest similarity with the strains from China, Taiwan and India.

Using Neighbour-Joining method evolutionary dendrograms were obtained for Iranian strains which were divided into two genetic groups: group A - strains more related to Eastern ones (Taiwan, China, India, Japan, Korea); group B - Iranian residential strains.

Concluding that in Iran the highest frequency of common mutations shearing in between primary and secondary infections among isolated rifampicin resistance strains found in this study is occurring in codons 523 and 526 and highest frequency of common mutations shearing in between primary and secondary infections observed in this research was occurring in codons 315 and 299 that might characterize wide spectrum of important mutations belong to epidemic widespread clones. So all strains analysed collected from Iranian patients are divided into 2 genetic groups: group A - similar to Eastern strains (Taiwan, China, India, Japan, Korea) and group B - Iranian residential strains.

Construction of evolutionary dendrograms using sequences of M. tuberculosis resistance genes collected in Iran and bioinformatics analysis are recommended to study epidemic process of tuberculosis.

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