Journal of Microbiology and Antimicrobials Vol. 1(1). pp. 001-008, October, 2009
Available online http://www.academicjournals.org/JMA

Full Length Research Paper

Multiple-mutations in the *katG* encoding catalase proxidase in isoniazid resistant *Mycobacterium tuberculosis* isolates correlate with high-level of resistance in patients with active pulmonary tuberculosis in Iran

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Accepted 22 September, 2009

The aim of this study was to investigate the significance of multiple-mutations in the *katG* gene, predominant nucleotide changes and its correlation with high level of resistance to isoniazid in *Mycobacterium tuberculosis* isolates that were randomly collected from sputa of 42 patients with primary and secondary active pulmonary tuberculosis from different geographic regions of Iran. Drug susceptibility testing was determined using the CDC standard conventional proportional method. DNA extraction, *katG* gene amplification and DNA sequencing analysis were performed. Thirty four (80%) isolates were found to have multiple-mutations (composed of 2 - 5 mutations) in the *katG* gene. Increased number of predominant mutations and nucleotide changes were demonstrated in codons 315 (AGC→ACC), 316 (GGC→AGC), 309 (GGT→GTT) with a higher frequency among patients bearing secondary tuberculosis infection with elevated levels of resistance to isoniazid (MIC µg/ml ≥ 5 - 10). Furthermore it was demonstrated that the combination of mutations with their predominant nucleotide changes were also observed in codons 315, 316 and 309 indicating higher frequencies of mutations among patients with secondary infection respectively. In this study 62% (n = 21) of multi-mutated isolates found to have combination of mutations with predominant nucleotide changes in codons 315 (AGC→ACC), 316 (GGC→GTT), 309 (GGT→GGT) and also demonstrated to be more frequent in isolates of patients with secondary infections, bearing higher level of resistance to isoniazid (≥ 5 – 10 µg/ml).

Key words: Predominant mutation, *Mycobacterium tuberculosis,* high level resistant to izoniazid, Iran.

INTRODUCTION

Isoniazid (INH) is a first-line chemotherapeutic drug used in tuberculosis (TB) therapy (Eltrigham et al., 1999; Mokrousov et al., 2002a,b; Herrera et al., 2004). Resistance to INH is associated with a variety of mutations affecting one or more genes such as those encoding catalase peroxidase *(katG)* (Abate et al., 2001; Bakonyte et al.,

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2005). The *katG* gene is the most commonly targeted region of the *Mycobacterium tuberculosis* genome with the majority of mutations occurring in codon 315 in 30 – 90% of ison INH-resistant strains depending on geographical distribution (Disk et al., 2000; Mokrousov et al., 2002a,b; Leung et al., 2003).

Resistance to INH is most frequently associated with a Single mutation in *katG* gene, a gene that encodes catalase proxidase enzyme in *M. tuberculosis* (Dobner et al., 1997).

Most INH-resistant *M. tuberculosis* strains have not been reported to have high proportions of *katG* deletions suggesting the need to more precisely analyze the structure of the *katG* gene in the resistant organisms. Further studies have revealed that *katG* gene deletions are very rare (Kiepiela et al., 2000; Miriam et al., 2001;) and this requires more detailed ana-lysis of the *katG* structure (Abate et al., 2001; Bakonyte et al., 2005;). Several groups have recently reported that many INH-resistant strains contain missense and other types of mutations (Abate et al., 2001; Silva et al., 2003). Mutations at the Ser315 codon of *katG* have been reported to be associated with high-level of resistance to INH (Van Soolingen et al., 2003). Resistance to INH has a second degree of magnitude in Iran (Zaker et al., 2006; Titov et al., 2006) and combinations of mutations conferring *M. tuberculosis* resistance to INH have been reported to be more common in the Multi-drug Resistance Tuberculosis (MDR-TB) than in mono-resistant *M. tuberculosis* isolates, suggesting that isolates develop resistance to INH by a stepwise accumulation of mutations (Bakonyte et al., 2003).

In this study, we investigate the significance of multiple-mutations in the *katG* gene, its correlation with predominant nucleotide changes and high level of resistance to INH in 42 isolates of *M. tuberculosis* collected from patients with primary and secondary active pulmonary tuberculosis from different geographic regions of Iran.

MATERIALS AND METHODS

Mycobacterial strains

One hundred sixty three *M. tuberculosis* were isolated from sputa of patients with active pulmonary tuberculosis collected from various geographic regions of Iran (Tehran, Zaboul, Isfehan, Mashad) from December, 2007 to May, 2008. Patients' history of tuberculosis, gender, clinical symptoms, radiography, tuberculin skin test (TST), etc was recorded before collection of specimen. All isolates were 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 using CDC standard biochemical procedures (Kent et al., 1985). Four sensitive isolates were selected and used as controls.

Susceptibility testing

Anti-microbial Drug Susceptibility Testing (AMST) was performed using the CDC standard conventional proportional method rifampicin (Rif) 40 µg/ml, isoniazid (INH) 2 µg/ml, ethambutol (EMB) 2 µg/ml, ethionamide (ETH) 20 µg/ml, streptomycin (SM) 4 µg/ml, and kanamycin (K) 20 µg/ml were used in slants and in addition to breakpoint concentrations for isoniazid 0.1 µg/ml, and rifampicin 2.0 µg/ml were also used in the BACTEC system (Kent et al., 1985). Four sensitive *M. tuberculosis* isolates and an H37Rv strain were used as negative controls. Mutations in the *katG* gene were identified on 42 isoniazid resistant isolates by sequencing methods and AMST was performed following sequencing to confirm resistance using different concentrations of isoniazid 2, 5 and 10 µg/ml in the slant proportional method (Kent et al., 1985).

Standard PCR identification and *katG* gene amplification

DNA extraction was done by Fermentas kit (Lithuania, Vilnius city, catalogue number 512) and DNA purification by Fermentas*e* kit (Lithuania, Vilnius city, catalogue number 513). DNA extracted from *M. tuberculosis CDC1551, Mycobacterium H37RV* strains and from four sensitive isolates of *M. tuberculosis* was used as negative controls. A 209 and 750 bp segment of the *katG* gene were amplified by PCR using the following synthetic oligonucleotide primers *katG F* 5'-GAAACAGCGGCGCTGGATCGT-3', *katG R*5'-GTTGTCCCATTTCGTCGGGG-3' for 209 bp and *katG* F 5' CGGGATCCGCTGGAGCAGATGGGC-3 and *katG* R 5'-CGGAATTCCAGGGTGCGAATGACCT-3' for the 750 bp fragment (Miriam et al., 2001; Telenti et al., 1997). PCR was carried out in 50 µl tube containing 2 µl KCl, 2 µl Tris (pH 8.0), 1.5 µl MgCl2, 5 µl dNTP, 1U*Taq* polymerase, 27 µl water (DDW molecular grade), 20 pmol of each primer and 6 - 10 µl of DNA template. The following thermocycling parameters were applied: initial denaturation at 95°C for 5 min; 36 cycles of denaturation at 94°C for 1 min; primer annealing at 56°C for 1 min; extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR product was amplified and purified again and controlled on the gel electrophoresis for getting purify segment. The final purified mycobacterial DNA obtained and was used for sequencing.

Analysis of DNA sequences

The 209 and 750 bp fragments of the *katG* gene were amplified by PCR using forward or reveres primers mentioned above; 33 cycles of denaturation at 94°C for 30 s; primer annealing at 48°C for 45 s; extension at 60°C for 4 min. *katG* gene fragments were sequenced by using an Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits (America). Purified DNA of the *katG* fragment obtained from *M. tuberculosis CDC1551, Mycobacterium H37RV* strains and from four sensitive isolates was used as negative controls.

Analyzing of DNA sequencing

Alignment of the DNA fragments *(katG)* were carried out using the MEGA and DNAMAN software (Gen bank_ PUBMED/BLAST) and was compared with the standard strains of CDC1551, H37RV and *M. tuberculosis 210*. The Blast 2 sequencing computer program was used for DNA sequence comparisons (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment of the DNA fragments (*katG*) was carried out with MEGA 3.1 software (www.megasoftware.net/mega3.1/) and obtained data were analyzed and edited with DNAMAN software.

Definitions

In this study: primary cases are referred to patients who did not

have a previous history of tuberculosis disease nor medical treatment. Secondary cases demonstrated a previous history of tuberculosis disease in their medical records.

RESULTS

Mycobacterial strains and suseptibility

All 163 tuberculosis patients' cases had proven registration of clinical diagnostic exanimations, such as chest X-ray, PPD, cough, weight loss, gender etc. Patients sputum samples was cultured on Lowenstein- Jensen medium. From 163 isolates, 42 were found to be resistant to INH (n = 42, 100%), rifampicin (*n = 38*, 90%), streptomycin (n = 38, 90%), and 8 (n = 8, 28%) were ethambutol resistant. Mono-resistance to INH was observed in 4 isolates (9.5%). In total, 42 isoniazid resistant and 121 sensitive isolates were identified. From 42 resistant isolates to INH, 31 isolates were identified secondary cases and 11 isolate primary cases.

Mutations were not detected for the four sensitive isolates to INH in 209 and 750 bp regions of *katG* gene. Mutations were observed in affected codons 305, 306, 307, 309, 314, 315, 316, 321, 328 in 209 bp fragment and in 357, 454 and 463 of the 750 bp fragment of *katG* gene. In 40 isolates four types of mutations were identified in codon 315: AGC→ACC (n = 36) 80%, AGC→AGG (n = 1) 2.3%, AGC→AAC (n = 2) 4.7% and AGC→GGC (*n* = 1) 2.3%. One type of mutation was found in codon 316: GGC→AGC (n = 18) 41.4%, and in 15 isolates four types of mutations were demonstrated in codon 309: GGT→GTT (n = 7)16.1%, GGT→GCT (n = 4)9.2%, GGT→GTC (n = 3)6.9%, GGT→GGG (n = 1)2.7% (Table 2).

Predominant nucleotide changes were observed in 40 isolates as 315 (AGC→ACC) indicated to evolve (*n* = 28) 77% from secondary and (*n* = 8) 23% from primary cases, 316 (GGC→AGC) in which 33.5% (*n* = 14) from secondary and 11% (*n* = 4) from primary and 309 (GGT→GTT) that 12.5% (*n* = 5) from secondary and 5.5% (*n* = 2) from primary cases respectively (Table 1). Of 105 mutations predominant nucleotide changes were seen in codon 315 AGC→ACC (Ser→Thr) 36% (*n* = 40), 316 GGC→AGC (Gly→Ser) 17.7% and in codon 309 GGT→GTT (Cys→Phe) 6.3% (*n* = 7) (Tables 1 and 2).

Six isolates 14% (*n* = 6) were identified from secondary cases with predominant mutations observed in three codons 315, 316 (100% each) and in codon 309 (67%, *n* = 4), including non predominant mutation observed in 2 isolates (33%) of codon 309 (Table 1).

Twenty-six isolates (62%) demonstrated multiple mutations in at least two of the three codons (309, 315, 316) with predominant nucleotide changes in which nucleotide combination 315 (AGC→ACC), 316 (GGC→AGC) n = 12 (46%) all differentiated from secondary cases and nucleotide combinations of 315 and 309 in which 315 (AGC→ACC) n = 9 (34.5%) was identified in 6 (23%) secondary and 3 (11.5%) primary cases, and a 309 mutation (GGT→GTT) found in secondary case (Table 1). Nucleotide combination in codon 316 and 309 were not found in primary or secondary cases (Table 1). Nucleotide combinations of 315 with others codons were observed in (n = 4) 15% of patient isolates including 3 secondary cases and 1 primary case (Table 1).

In two isolates 2 types of mutations were found in codon 357 GAC→CAC and GAC→AAC. In addition two mutations which were also observed in codons 463 CGG→CTG and 454 GAG→CGA were found in secondary cases and did not correspond to high level resistance to INH (MIC ≤ 2).

Isolates bearing a single mutation *n* = 9 (21%), double mutations *n* = 17 (40.46%), triple mutations *n* = 9 (21.42%), four mutations *n* = 4 (9.5%) and five mutations *n* = 4 (9.5%) were also observed among 42 resistant isolates (Table 2).

Silent mutations

Three silent mutations were identified in four isolates in codons 306 (CCG→CCC), 309 (GGT→GGG) and 314 (ACC→ACG). These silent mutations did not show an effect on the susceptibility testing pattern (Table 2).

DISCUSSION

The known genes related to INH-resistant are *katG, inhA, ahpC, kasA* (Marcia et al., 2003; Zheltokjva et al., 2005). Several investigators have reported *M. tuberculosis* resistance to INH corresponds to amino acid changes in codon 315 (Disk et al., 2000; Mokrousov et al., 2003; Van Soolingen et al., 2003; Herrera et al., 2005). Higher proportions of *katG* mutations are due to small insertions or point mutations (point mutations in two bases 944 and 945) (Zheltkova et al., 2002). In our study we have observed 95% of all INH resistant isolates (*n* = 40) showed mutations in codon 315. Whereas 40% of all mutations (*n* = 105) conferring different types of nucleotide changes were found to be in codon 315: AGC→ACC (Ser→Thr) as predominant nucleotide changes 36% and AGC→AGG (Ser→Arg) 0.9%, AGC→AAC (Ser→Asn) 1.8%, AGC→GGC (Ser→Gly) 0.9% were observed as non predominant. One type of mutation was found in codon 316: GGC→AGC (n=18) 41.4% and in 15 isolates four types of mutations were demonstrated in codon 309: GGT→GGT (n = 7)16.1%, GGT→GCT (n = 4)9.2%, GGT→GTC (n = 3)6.9%, GGT→GGG (n = 1)2.7% (Table 1).

Predominant mode of acquisition of resistance via *katG* alterations is the selection of particular mutations that decrease the catalase activity but that maintain a certain level of the peroxidase activity of the enzyme in viable INH-resistant (INHr) organisms. The above data correlate with our findings that such mutations were found in up to 85% of the INH-resistant clinical isolates with decreased catalase activity. These mutations appear to provide the

Table 1. Correlation between predominant mutations, nucleotide and amino-acid changes with high level resistance to isoniazid among 40 *M. tuberculosis* isolates collected from active primary and secondary pulmonary tuberculoses patient in Iran.

Codon	Predominant nucleotide changes in 40 patient isolates	Different type of mutations Total = 105	Number of isolates with multi-mutations				MIC µg/ml
			Mutation in codons 315, 316, 309 Sec (n = 6)	Combination of Mutations in Predominant codons (315, 316, 309) (n = 26) 62%			
				315, 316 Sec (n =12) 46%	315, 309 (n = 10) 38.5%	316, 309 (n = 0)	315 with Other Codons	≥ 5 - 10
315	AGC→ ACC (n = 36) 90% Sec (n = 28) 77% P (n = 8) 23%	36% (n = 40)	100%	34.5% (n = 9)	34.5% (n = 9) Sec (n = 6) 23% P (n = 3) 11.5%	–	15%(n = 4) Sec (n = 3) P (n = 1)	≥ 5 - 10
316	GGC → AGC (n = 18) 41.4% Sec (n = 14) 38% P (n = 4) 11%	41.4% (n = 18)	100%	46%(n = 12)	–	–	–	≥ 5 - 10
309	GGT→ GTT (n = 7) 17.5% Sec (n = 5) 13% P (n = 2) 5.5%	6.3% (n = 7)	67% (n = 4) 33% (n = 2) GGT, GTT	–	Sec (n = 4) 15%	–	–	≥ 5 - 10

Sec = Secondary infection, P = Primary infection.

Table 2. Frequency of multi-mutations with nucleotide and amino acid changes in *katG* gene among 42 isoniazid resistant isolates of *M. tuberculosis* collected from tuberculosis patients with active pulmonary infections in Iran

Isolate number	µl/ml MIC	Chang of nucleotide	Chang of amino acid	Codon	Frequency
1 Mutation (19%)					
94, 933, 984, 85, 849	≥ 5 - 10	AGC→ACC	Ser→Thr	315	8 (19%)
754		AGC→AAC	Ser→Asn		
644		AGC→GGC	Ser→Gly		
147		AGC→GGC	Ser→Arg		
2 Mutations (40.46%)					
141	≥ 5 - 10	GGC→GCC,AGC→ACC	Gly→Ala, Ser→Thr	305, 315	1 (2.38%)
554, 3221, 469	≥ 5 - 10	GGT→GTT, GTC, GCT, AGC→ACC	Gly→Cys, Phe, Ala, Ser→Thr	309, 315	3 (7.14%)
118	≥ 5 - 10	ACC→AAC, AGC→ACC	Thr→Asn , Ser→Thr	314, 315	1 (2.38%)

Table 2. Contd

414, 384	≥ 5 - 10	GAC→TTC, TA, AGC→ACC	Asn→Phe, Tyr, Ser→Thr	311, 315	2 (4.76%)
24276, 23623, tub2,5532, 449, 171, 6432, 2323	≥ 5 - 10	AGC→ACC, AGG, GGC→AGC	Ser→Thr, Arg ,Gly→Ser	315, 316	8 (19.4%)
204	≥ 2	GAC→CAC, CGG→CTG	Asp→His, Arg→Leu	357, 463	1 (2.38%)
47	≥ 2	GAC→AAC, GAG→CGA	Asp→Asn , Glu→Arg	357, 454	1 (2.38%)
3 Mutations (21.42%)					
901	≥ 5 - 10	GGT→GCT, GAC→TTC AGC→ACC	Gly→Ala, Asn→Phe, Ser→Thr	309, 311 315	1 (2.38%)
2331-2	≥ 5 - 10	GGA→CGA,GGT→GGG AGC→ACC	Gly→Arg, Gly→Gly, Ser→Thr	307, 309 315	1 (2.38%)
766	≥ 5 - 10	GGC→GCC, AGC→ACC, TGG→TTG	Gly→Ala, Ser→Thr Trp→Leu	305, 315 321	1 (2.38%)
1614	≥ 5 - 10	GGC→GCC, GGT→GCT AGC→ACC	Gly→Ala, Gly→Ser, Ser→Thr	305, 309 315	1 (2.38%)
2387	≥ 5 – 10	AGC→ACC, GGC→AGC, TGG→TGT	Ser→Thr, Gly→Ser, Trp→Cys	315, 316 328	1 (2.38%)
704, 214	≥ 5 - 10	AGC→AAC, GGC→AGC, TGG→TCC, TAG	Ser→Thr, Gly→Ser, Trp→Ser, STOP	315, 316 321	2 (4.76%)
139.2,744	≥ 5 - 10	GGT→GTT, AGC→ACC, GGC→AGC	Gly→Cys, Ser→Thr, Gly→Ser	309, 315 316	2 (4.76%)
4 Mutations (9.52%)					
911, 730	≥ 5 - 10	Gly→Cys, Asp→Phe Ser→Thr, Gly→Ser	GGT→GTT, GAC→TTC, AGC→ACC,GGC→AGC	309, 311, 315, 316	2 (4.76%)
1712	≥ 5 - 10	Gly→Phe, Ser→Thr Gly→Ser, Trp→Cys	GGT→GTC, AGC→ACC GGC→AGC, TGG→TGT	309, 315, 316, 328	1 (2.38%)
355	≥ 5 - 10	Gly→Cys, Ser→Thr Trp→Leu, Trp→Cys	GGT→GTT, AGC→ACC, TGG→TTG, TGG→TGT	309, 315, 321, 328	1 (2.38%)
5 Mutations (9.52%)					
1527	≥ 5 - 10	Gly→Phe, Asp→Tyr, Ser→Thr Gly→Ser, Trp→STOP	GGT→GTC,GAC→TAC, AGC→ACC,GGC→AGCTGG →TAG	309,311,315, 316, 321	1 (2.38%)
884, 863	≥ 5 - 10	Gly→Ala, Gly→Ser, Asp→Tyr Thr→Thr, Ser→Thr	GGA→GCA,GGT→GCTGAC →TAC,ACC→ACG, AGC→ACC	307,309,311 314, 315	2 (4.76%)
1384	≥ 5 - 10	Pro→Pro, Ser→Thr, Gly→Ser Trp→Cys, Trp→Cys	CCG→CCC,AGC→ACC, GGC→AGC,TGG→TGT, TGG→TGT	306,315,316, 321, 328	1 (2.38%)

optimal balance between decreased catalase activity and a sufficiently high level of peroxidase activity in *KatG* (Ramaswami et al., 1998).

In this study nucleotide changes in codon 315: AGC→ACC (n = 36) 85%, 316: GGC → AGC (n = 18) 43% and 309: GGT→GTT (n = 7) 16.1% were more predominantly observed among isolates collected from secondary infection cases and correlating to a higher frequency level of resistance to INH MIC ≥ 5 - 10 µg/ml. This observation correlate with other studies reported that multi-drug resistance was found among 14% of the amino acid 315 mutants and 7% of the other INH-resistant strains (p > .05) (Van Soolingen et al., 2000) and reported that amino acid 315 mutants lead to secondary cases of tuberculosis as often as INH-susceptible strains (Van Soolingen et al., 2000). Distribution of INH resistance associated mutations reported by other investigators to be different in INH mono-resistant isolates when compared with multi-drug-resistant isolates, significantly fewer INH resistance mutations observed in the INH mono-resistant group and also mutations in *katG*315 were significantly more common in the multi-drug resistant isolates (Manzour et al., 2006).

Conversely, mutations in the *inhA* promoter were significantly more common in INH mono-resistant isolates (Manzour et al., 2006). It has been suggested that some drug resistance associated mutations occur at higher frequencies in MDR *M. tuberculosis* than in mono-isoniazd resistant clinical isolates (Manzour et al., 2006). Whereas our data demonstrate that only 9.5% (*n* = 4) mono-resistant, 90% (*n* = 38) multi-drug resistant and 26 (62%) of isolates with multiple mutation conferring high level of resistance to INH (MIC ≥ 5 - 10 µg/ml). Unfortunately we have not completely examined the role of *inhA* promoter among mono and multi-drug resistant conferring multiple mutations in this study.

Other studies reported that INH- resistant strain showed a mutation in the *katG* gene in codon 314 as ACC → CCC (Thr → Pro) which has not been previously defined (Elif Ozturl et al., 2005). However, in this study we found mutations in nearby similar segment of the *katG* gene in codons 309 and 316 which very seldom been reported and were associated with secondary MDR cases.

Unfortunately we do not have information concerning the patients reactivation or re-infection status, however it is very unlikely that such high frequency levels of predominant nucleotide changes correspond to secondary infection cases.

Of 32 isolates with multiple mutations including [single (*n* = 9) 19%, double (*n* = 17) 40.46%, triple (*n* = 9) 21.42%, quadruple (*n* = 4) 9.5% and five mutations (*n* = 4) 9.5%] all demonstrated having higher frequency levels of predominant nucleotide changes in codons 315, 316 and 309 among patient with secondary infection bearing higher level of resistance to INH (MIC ≥ 5 - 10 µg/ml) (Table 2).

Single mutations with higher frequency levels of predominant nucleotide changes were observed in codon 315 AGC→ACC *n* = 5 (11.9%) isolated from secondary infection cases and *n* = 3 (7.2%) non-predominant other types of nucleotide changes (Table 1 and 2).

Our findings are in agreement with similar data reported in Lithuania where 95% of strains displayed mutation in codon 315 of the *katG* gene with the predominance of codon substitution AGC→ACC (Ser→Thr) (90%) (Bakonyte et al., 2003). However, we could not identify the mutation of AGC→ACA (Ser→Thr) which has been reported in Lithuania (Bakonyte et al., 2003). In Poland 90% mutations are in the 315 AGC codon which corresponds to 5 types of mutations (ACC, ACT, ACA, AAC and ATC) and resemble similar pattern of changes with our data including nucleotide ACC and AAC. However contrary to the data from Poland we did not observe nucleotide changes of ACT, ACA and ATC in Iran (Sajduda et al., 2004). In Russia the highest proportion of nucleotide changes (70%) have been reported to be in the *katG* codon 315 AGC→ACC which is similar and in agreement with our data (Makrousov et al., 2002a,b). Mutations at the Ser315 codon of *katG* have been reported to be associated with high-level INH resistance (van Soolingen et al., 2003) which is similar to our findings in 8 (19%) isolates bearing a single mutation at codon 315 and conferring resistance to INH (MIC ≥ 5 - 10 µg/ml). This data suggests the alternative or complementary explanation that strains with mutations at codon 315 are more likely to gain increased resistance (van Soolingen et al., 2003).

In our study, four types of mutations were detected in codon 309: GGT→GTT (Cys→Phe) 6.3%, GGT→GCT (Cys→Ser) (3.6%), GGT→GTC (Cys→Phe) (2.7%), GGT→GGG (Cys→Thr) (0.9%). Additionally, we identified a mutation in codon 316 GGC→AGC (Gly→Ser) (14.4%) which has not been reported previously. In this study seventy-five percent of all isolates resistant to INH (*n* = 42) demonstrated multiple types of mutations in codons 309 (*n* = 15, 34%) and 316 (*n* = 18, 41.4%) which might represent a second importance of mutations present in isolates of patients bearing secondary infection in Iran which has not been reported previously. In six isolates (14%) bearing a combination of multiple mutations in three codons (309,315 and 316) and in 26 (61.9%) isolates that demonstrated having combinations of multiple mutations (in at least two of the three mentioned codons) were found to be MDR isolates having high frequency levels of resistance to INH MIC ≥ 5 - 10 µg/ml.

These finding indicate correlation of high level resistance due to mutation in codon 315 which has been shown by other authors (Manzour et al., 2006). Higher frequency of combination of multiple mutations in *katG* gene (codon 315, 309 and 316) has not been previously reported in patients with secondary infection (Tables 1 and 2). The other logical reason could be explained that high rate of immigrant transits of patients from high TB in-

cidence areas like India, Afghanistan and China to Europe via center of eastern Europe (Iran) which can cause distribution, circulation and interaction of numerous different molecular types of tuberculosis cases might lead to such combination of rare mutations.

Other researchers have suggested that isolates develop resistance to INH by a stepwise accumulation of mutations, which may be important for achieving the higher level of resistance or maintaining virulence in a human host. Inadequate prolonged treatment results in an accumulation of mutations, ultimately leading to *katG* and/or *inhA* mutations in virtually all strains. This finding is in agreement with our data regarding higher frequency of predominant nucleotide changes among secondary case infections. In contrast to our findings other investigators have not reported the association of multiple mutations and predominant nucleotide changes with high level resistance among patients with secondary infection cases.

In two isolates mutations were not detected in the 209 bp fragment, therefore we sequenced the larger 750 bp fragment of *katG* gene for all isolates and have identified mutations in codons 463, 357 and 454, 357, which may indicate that this type of mutation is non-predominant colon in Iran when compared with neighboring countries (Talenti et al., 1997; Abate et al., 2001; Makrousov et al., 2002a,b; Zaker et al., 2006). Other investigators have reported no silent mutations detected in the *katG* gene (Kiepiela et al., 2000; Kim et al., 2004). Whereas, in our strain set three silent mutations (2.7%) in codons 306 (CCG→CCC), 309 (GGT→GGG) and 314 (ACC→ACG) were demonstrated which had no effect on the susceptibility testing pattern. The high percentage of double mutations found among the isolates of Iran clearly differed from the lower prevalence of double mutations in other studies (Mokrousov et al., 2002a, b; Bakonyte et al., 2003; Sajduda et al., 2004). A prominent finding of this study was the high frequency of double (40.47%), triple (21.42%), quadruple (9.5%) and five nucleotide mutations (9.5%) occurring in separate codons indicating predominant nucleotide changes in codons 315, 316 and 309 to be more prevalent among secondary cases (Table 2).

In conclusion this study demonstrates an association between the multiple mutations of the *katG* gene and their correlation with predominant nucleotide changes in codon 309, 315 and 316. These mutations may have a possible role among secondary case infections bearing a high level of resistance to INH (≥ 5 – 10 µg/ml) in isolates collected from *M. tuberculosis* patients with active pulmonary tuberculosis in Iran.

ACKNOWLEDGMENTS

We thank our colleagues in Iranian Institutes of Pulmonology and Tuberculosis for samples collected. This study was a joint research project with Islamic Azad University and Belarusian Research Institute for Epidemiology and Microbiology, Minsk.

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