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Multiple- mutations in the *katG* gene of *Mycobacterium tuberculosis* isolates correlate with high- level of resistance to isoniazid in patients with active pulmonary tuberculosis from Belarus

Zakerbostanabad S*[1,2], Molla Kazemi V[3], Rahimi MK[4], Shahidi SH[2], Pourazar SH[1], Massomi M[2], Nour Nemattolahi A[2], Abdolrahimi F[2], Surkova LK[5], Ghazanfari M[2] and Titov LP[1]

[1]Department of Clinical Microbiology, Belarusian Research Institute for Epidemiology and Microbiology, Minsk, Belarus.

[2]Deptartment of Mycobacteriology and Pulmonary Research; Pasteur Institute of Iran, Tehran, Iran.

[3] Department of Microbiology, Faculty of Medicine, Islamic Azad University of Tehran, Tehran, Iran.

[4] Cell Bank of Iran, Pasteur Institute of Iran,Tehran, Iran.

[5] Tuberculosis and Pulmonary Research Institute, Minsk, Belarus.

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ABSTRACT

Background and Objectives: 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 Belarus.

Materials and Methods: Drug susceptibility testing was determined using the CDC standard conventional proportional method. DNA extraction, *katG* amplification, and DNA sequencing analysis were performed.

Results: Thirty four (80%) isolates were found to have multiple-mutations (composed of 2-5 mutations) in the *katG*. 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.

Conclusion: 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 -10μg/ml).

Keywords: Predominant Mutations, *M. tuberculosis,* high level resistant, Izoniazid, Belarus.

INTRODUCTION

Isoniazide is a first-line chemotherapeutic drug used in tuberculosis (TB) therapy (1-6). Resistance to isoniazid is associated with a variety of mutations affecting one or more genes such as those encoding catalase-peroxidase (*katG*) (7- 8). Depending on geographical distribution the *katG* is the most commonly targeted region of the *M. tuberculosis* genome. With the majority of mutations occurring in codon 315 in 30 – 90% of isoniazid-resistant strains (9-12). Isoniazid resistance is most frequently associated with a single mutation in *katG* , a gene that encodes catalase-proxidase enzyme in *M. tuberculosis* (9). Most isoniazide- resistant *M. tuberculosis* strains

* Corresponding Author: Dr. Saeed Zakerbostanabad
Department of Mycobacteriology
Pasteur Institute of Iran, No.69, Pasteur Ave, 14131694351, Tehran, Iran.
Tel- Fax: + 98-21-66968853
E-mail: saeedzaker20@yahoo.com

have not been reported to have a 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 (13) and this requires more detailed analysis of the *katG* and its structure (5, 7, 8, 11). Several groups have recently reported that many isoniazide-resistant strains contain missense and other types of mutations (7). Mutations at the Ser 315 codon of *katG* have been reported to be associated with high- level of isoniazide resistance (14). Resistance to isoniazide has a second degree of magnitude in Belarus, and combinations of mutations conferring *M. tuberculosis* resistance to isoniazide 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 isoniazide by a stepwise accumulation of mutations (15).

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

MATERIAL AND METHODS

Mycobacterial strains. 163 isolates of *M. tuberculosis* isolated from sputa of patients with active pulmonary tuberculosis were collected from various geographic regions of Belarus (Minsk, Mogilev, Gomel, Grodho, Brest, Vitebsk) during December 2006 to May 2007. 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 (16). 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 rifampicine (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 rifampicine 2.0 µg/ml were also used in the BACTEC system (16). Four sensitive *M. tuberculosis* isolates and an H37Rv strain were used as negative controls. Mutations in the *katG* 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 (16).

Standard PCR identification and katG gene amplification. DNA extraction was done by Fermentas kit (K512), and DNA purification by Fermentas kit (k513 - Graiciuno8, Vilnius2028, Lithuania) (13). DNA extracted from *M. tuberculosis* CDC1551, *Mycobacterium* H37RV strains and from four sensitive isolates of M. tuberculosis was used as negative controls. A 209 bp and 750 bp segment of the katG 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 (17-19). PCR was carried out in 50 µl tube containing 2 µl KCl, 2 µl Tris (pH 8.0), 1.5 µl Mg Cl_2, 5 µl dNTP, *1UT*aq 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. The final purified mycobacterial DNA obtained and was used for sequencing.

DNA sequencing. The 209bp and 750bp fragments of the *katG* were amplified by PCR using forward or reveres primers mentioned above; 33 cycles of denaturation at 94˚C for 30sec; primer annealing at 48˚C for 45 sec; extension at 60˚C for 4 min. *katG* fragments were sequenced by using an Amersham auto sequencer and Amersham Pharmacia DYEnamic ET Terminator Cycle Sequencing Premix Kits.

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/mega 3.1/) 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. From 163 isolates, 42 were found to be resistant to isoniazid (100%), rifampicine (90%), streptomycin (90%), and 8 (28%) were ethambutol resistant. Mono-resistance to isoniazid was observed in 4 isolates (9.5%). In total, 42 isoniazid resistant and 121 sensitive isolates were identified.

Table1. 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 Belarus.

Codon	Predominant nucleotide changes in 40 patient isolates	Different type of mutations Total=105	Mutation in codons 315, 316, 309 Sec (n=6)	Number of isolates with multi-mutations: Combination of Mutations in Predominant codons (315, 316, 309) (n=26) 62%				MIC µg/ml
				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) 80% 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) 77% P (n=4) 23%	41.4% (n=18)	100%	46% (n=12)	–	–	–	≥5-10
309	GGT→ GTT (n=7) 16.1% Sec (n=5) 71% P (n=2) 29%	6.3% (n=7)	67% (n=4) 33% (n=2) GGT→ GTT	–	Sec (n=4) 15%	–	–	≥5-10

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 Belarus

Isolate Number	µl/ml	Chang of nucleotide	Chang of amino acid	Codon	Frequency
1 Mutation (19%)					
94, 932, 489, 85, 894	≥5-10	AGC→ACC	Ser→Thr	315	8 (19%)
457		AGC→AAC	Ser→Asn		
446		AGC→GGC	Ser→Gly		
471		AGC→GGC	Ser→Arg		
2 Mutations (40.46%)					
411	≥5-10	GGC→GCC,AGC→ACC	Gly→Ala, Ser→Thr	305, 315	1 (2.38%)
469 ,2331 ,455	≥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%)
414, 384	≥5-10	GAC→TTC, TA, AGC→ACC	Asn→Phe, Tyr, Ser→Thr	311, 315	2 (4.76%)
,23623 ,24276 tub2,3255, 443, 571, 3246, 2262	≥5-10	AGC→ACC, AGG, GGC→AGC	Ser→Thr, Arg ,Gly→Ser	315, 316	8 (19.4%)
402	≥2	GAC→CAC, CGG→CTG	Asp→His, Arg→Leu	357, 463	1 (2.38%)
74	2≤	GAC→AAC, GAG→CGA	Asp→Asn , Glu→Arg	357, 454	1 (2.38%)
3 Mutations (21.42%)					
139	≥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%)
7285	≥5-10	GGC→GCC, AGC→ACC TGG→TTG	Gly→Ala, Ser→Thr Trp→Leu	305, 315 321	1 (2.38%)
1416	≥5-10	GGC→GCC, GGT→GCT AGC→ACC	Gly→Ala, Gly→Ser Ser→Thr	305, 309 315	1 (2.38%)
2738	≥5-10	AGC→ACC, GGC→AGC TGG→TGT	Ser→Thr, Gly→Ser Trp→Cys	315, 316 328	1 (2.38%)

Table 2. Continued ...

407, 412	≥5-10	AGC→AAC, GGC→AGC TGG→TCC, TAG	Ser→Thr, Gly→Ser Trp→Ser, STOP	315, 316 321	2 (4.76%)
139.2,447	≥5-10	GGT→GTT, AGC→ACC GGC→AGC	Gly→Cys, Ser→Thr Gly→Ser	309, 315 316	2 (4.76%)
4 Mutations (9.52%)					
369, 370	≥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%)
1217	≥5-10	Gly→Phe, Ser→Thr Gly→Ser, Trp→Cys	GGT→GTC, AGC→ACC GGC→AGC, TGG→TGT	315 ,309 328 ,316	1 (2.38%)
453	≥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%)					
2715	≥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%)
388, 368	≥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%)
2831	≥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%)

Mutations were not detected for the four sensitive isolates to isoniazid in 209 bp and 750 bp regions of *kat G*. 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*. 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% (Fig.1) (Table.1).

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 77% (n=14) from secondary and 23% (n=4) from primary, and 309 (GGT→GTT) that 71% (n=5) from secondary and 29% (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) (Table 1-2) .

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 one 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 isoniazid (MIC≤2).

Isolates bearing a single mutation n=9 (19%), 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 related to isoniazid- resistant are *katG*, *inhA*, *ahpC*, *kasA* (9- 13, 20- 21). Several investigators have reported *M. tuberculosis* resistance to isoniazid corresponds to amino acid changes in codon 315 (6, 21-23). Higher proportions of *katG* mutations are due to small insertions or point mutations (point mutations in two bases 944 and 945). In our study we have observed 95% of all isoniazid 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%) (Fig.1) (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 INHr clinical isolates with decreased catalase activity. These mutations appear to provide the optimal balance between decreased catalase activity and a sufficiently high level of peroxidase activity in *katG* (24).

In this study nucleotide changes in codon 315: AGC→ACC (n=36) 80%, 316:GGC→ AGC (n=18,41.4%) 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 isoniazid (MIC ≥ 5-10 µg/ml). This observation correlate with other studies that reported multi-drug resistance was found among 14% of the amino acid 315 mutants and 7% of the other INH-resistant strains (P >.05) (14) , and reported that amino acid 315 mutants lead to secondary cases of tuberculosis as often as INH-susceptible strains (14). Distribution of isoniazid resistance associated mutations reported by other investigators to be different in isoniazid mono-resistant isolates when compared with multi-drug- resistant isolates, significantly fewer isoniazid resistance mutations observed in the isoniazid mono-resistant group and also mutations in *katG* 315 were significantly more common in the multi-drug resistant isolates (25). Conversely, mutations in the *inhA* promoter were significantly more common in isoniazid mono-resistant isolates (25). 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 (25). 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 isoniazid (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 research.

Other studies reported that INHr strain showed a mutation in the *katG* gene in codon 314 as ACC → CCC (Thr → Pro) which has not been previously defined (10). 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 isoniazid (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 1-2).

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%) (8). However, we could not identify the mutation of AGC→ACA (Ser→Thr) which has been reported in Lithuania (8). 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 Belarus (21, 22). 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 (6). Mutations at the Ser315 codon of *katG* have been reported to be associated with high-level isoniazid resistance (14) which is similar to our findings in 8 (19%) isolates bearing a single mutation at codon 315 and conferring resistance to isoniazid (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 (14).

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 isoniazid (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 Belarus 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 isoniazid 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 (25). Higher frequency of combination of multiple mutations in *katG* (codon 315, 309 and 316) has not been previously reported in patients with secondary infection (Table.1-2). One explanation can be postulated that high population rate of alcoholism and incomplete treatment can be the cause of mutations. The other logical reason could be explained that high rate of immigrant transits of patients from high TB incidence areas like India, Afghanistan and China to Europe via center of eastern Europe (Belarus) 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 isoniazid 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.

We have re-sequenced, re-sub cultured and repeated AMST for all 42 isolates in order to assure isoniazid resistance, reproducibility, kept all isolates, and DNA in our strain bank for future collaborative investigation with other research settings.

Phylogenetic molecular analyses of these 42 isolates demonstrated and differentiated them into three genetically linked groups. Sixteen isolates showed genetic relation with strains from Russia, China and India, 11 isolates linked genetic relation with Belarusian and 12 with European isolates (Data is not shown).

In 2 isolates mutations were not detected in the 209 bp fragment. Therefore, we sequenced the larger 750 bp fragment of *katG* 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 Belarus when compared with neighboring countries (6, 7, 18, 21). Other investigators have reported no silent mutations detected in the *katG* (13, 26- 27). Whereas, in our strain set of 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 Belarus clearly differed from the lower prevalence of double mutations in other studies (2, 16, 19, 20). 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* 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 isoniazid (≥5-10µg/ml) in isolates collected from *M. tuberculosis* patients with active pulmonary tuberculosis in Belarus.

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REFERENCES

1. Bobadilla-del-Valle M, Ponce-de-Leaon A, Arenas-Huertero C, Vargas-Alarcon G, Kato-Maeda M, Small PM, Couary P, Ruiz-Palacios GM, Sifuentes-Osornio J. RpoB Gene Mutations in Rifampin-Resistant *Mycobacterium tuberculosis* Identified by Polymerase Chain Reaction Single - Stranded Conformational Polymorphism. *Emerg Infect Dis* 2001; 7:1010-1013.

2. Eltrigham IJ, Drobniewski FA, Mangan JA, Butcher PD and Wilson SM. Evaluation of reverse Transcription-PCR and a bacteriophage- Based assey for rapid phenotypic detection of rifampin resistance in clinical isolates of *Mycobacterium tuberculosis*. *J Clin Micribiol* 1999;11:3524-3527.

3. Garcia M, Vargas JA, Castejon R, Navas E, Durantez A. Flow-cytometric assessment of lymphocyte cytokine production in tuberculosis. *Tuberculosis* 2002; 82:37-41.

4. Hall L, Doerr KA, Wohlfiel SL, Roberts GD. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. *J Clin Micribiol* 2003; 41:1447-1453.

5. Herrera-Leon L, Molina T, Saiz P, Saez-Nieto JA, Jimenez MS. New multiplex PCR for rapid detection of isoniazid-resistant *Mycobacterium tuberculosis clinical* isolates. *Antimicrob Agents Chemother* 2005; 49:144-147.

6. Mokrousov I., Narvskaya O, Otten T, Limeschenko E, Steklova L, Vyshnevskiy B. High prevalence of KatG Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001. *Antimicrob Agents Chemother* 2002; 46:1417- 1424.

7. Abate G, Hoffner SE, Thomsen VO, Miörner H. Characterization of isoniazid- resistant strains of *Mycobacterium tuberculosis* on the basis of phenotypic

properties and mutations in katG. *Eur J Clin Microbiol Infect Dis* 2001; 20:329-333.

8. Bakonyte D, Baranauskaite A, Cicenaite J, Sosnovskaja A, Stakenas P. Molecular Characterization of Isoniazid-Resistant *Mycobacterium tuberculosis* Clinical Isolates in Lithuania. *Antimicrob Agents Chemother* 2003; 4:2009–2011.

9. Dobner PS, Rusch-Gerdes, Bretzel G, Feldmann K, Rifai M, Loscher T, and Rinder H. Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes katG and inhA for the prediction of isoniazid resistance. *Int J Tuberc Lung Dis* 1997; 1:365-369.

10. Elif Ozturl C, Ahmet Sanic, Demet Kaya and Ismail Ceyhan. Molecular analysis of isoniazid, rifampin and and streptomycin resistance in mycobacterium isolates from patients with tuberculosis in Duzce Turkey. *Jpn J Infect Dis* 2005, 58: 309-312.

11. Leung ET, Kam KM, Chiu A, Ho PL, Seto WH, Yuen KY, Yam WC. Detection of katG Ser315Thr substitution in respiratory specimens from patients with isoniazid-resistant *Mycobacterium tuberculosis* using PCR-RFLP. *J Med Microbiol* 2003; 52:999-1003.

12. Mokrousov I, Otten T, Filipenko M, Vyazovaya A, Chrapov E, Limeschenko E, Steklova L, Vyshnevskiy L, Narvskaya O. Detection of Isoniazid-Resistant *Mycobacterium tuberculosis* Strains by a Multiplex Allele-Specific PCR Assay Targeting katG Codon 315 Variation. *J Clin Microbiol* 2002; 5:2509-2512.

13. Kiepiela P, Bishop KS, Smith AN, Roux L and York DF. Genomic mutations in the *katG*, *inhA*, and *ahpC* genes are useful for the prediction of isoniazid resistance in *Mycobacterium tuberculosis* isolates from Kwazulu Natal, South Africa. *Int J Tuber Lung Dis* 2000; 80:47-56.

14. Van Soolingen, D, de Hass PE, van Doom HR, Kuijper E, Rinder H and Brogdorff MW. Mutations at amino acid position 315 of the katG Gene are Associated with High-level Resistance to isoniazid, Other Drug resistance, and successful transmission *Mycobacterium tuberculosis* in the Netherlands. *J Infect Dis* 2000; 182:1788-1790.

15. Titov LP, Zakerbostanabad S, Slizen V, Surkova L, Taghikhani M, Bahrmand A. Molecular characterization of rpoB gene mutations in rifampicine-resistant *Mycobacterium tuberculosis* isolates from tuberculosis patients in Belarus. *Biotechnol J* 2006; 24:1447-1452.

16. Kent PT and Kubica GP. Public health mycobacteriology, a guide for the level III laboratory, CDC, U S. Department of Health and Human Service Publication no. (CDC) 86-216546, Atlanta 1985; 21-30.

17. Kurabachew M, Enger Ø, Sandaa RA, Lemma E, Bjorvatn B. Amplified ribosomal DNA restriction analysis in the differentiation of related species of mycobacteria. *J Microbiol Methods* 2003; 55:83-90.

18. Telenti A, Honore N, Bernasconi C, March J, Ortega A, Heym B, Takiff HE, Cole ST. Genotyping assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. *J Clin Microbiol* 1997; 35:719-723.

19. Zakerbostanabad S, Titov LP, Bahrmand AR, Taghikhani M. Genetic mechanism of *Mycobacterium tuberculosis* resistance to antituberculsis drugs, isoniazid and rifampicine. *J Daklady of National Akademia Science Belarus* 2006; 50(6), 92-98.

20. Silva MS, Senna SG, Ribeiro MO, Valim AR, Telles MA, Kritski A, Morlock GP, Cooksey RC, Zaha A, Rossetti ML. Mutations in katG, inhA, and ahpC Genes of Brazilian Isoniazid-Resistant Isolates *Mycobacterium tuberculosis*. *J Clin Microbiol* 2003; 9:4471-44.

21. Zheltkova EA, Chernousova LN, Smirnova TJ, Andreevskaya SN, Yates M, Drobniewskii F. Molecular genetic typing *Mycobacterium tuberculosis* strains isolated from patents in the samara region by the restriction DNA fragment length polymorphism. *Zh Microbial* (Moscow) 2004; 5:39-43.

22. Sajduda A, Brzostek A, Popławska M, Augustynowicz-Kopec E, Zwolska Z, Niemann S, Dziadek J, Hillemann D, Molecular Characterization of Rifampine- and Isoniazid-Resistant *Mycobacterium tuberculosis* Strains Isolated in Poland. *J Clin. Microbiol* 2004; 42:2425–2431.

23. van Doorn HR, Claas EC, Templeton KE, van der Zanden AG, te Koppele Vije A, Jong MD, Dankert J, Kuijper EJ. Detection of a Point Mutation Associated with High-Level Isoniazid Resistance in *Mycobacterium tuberculosis* by Using Real-Time PCR Technology with 3'-Minor Groove Binder-DNA Probes. *J Clin Microbiol* 2003; 41:4630-4635.

24. Ramaswami S, and Musser JM. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis. Update.*Tuberc Lung* Dis 1998; 79: 3-29.

25. Hernando Hazbo M, Brimacombe M, Bobadilla del Valle M et al. Population Genetics Study of Isoniazid Resistance Mutations and Evolution of Multidrug-Resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2002; 50: 2640–2649.

26. Kim BJ, Hong SK, Lee KH, YJ Yun, Kim EC, Park YG, Bai GH, Kook YH. Differential Identification of *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria by duplex PCR assay using the RNA Polymerase gene (rpoB). *J Clin Microbiol* 2004; 42:1308-1312.

27. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, Colston MJ, Matter L, Schopfer K, Bodmer T. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993; 341:647-650.