

Determination of principal genotypic groups among susceptible, MDR and XDR clinical isolates of *Mycobacterium tuberculosis* in Belarus and Iran

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ÖZET

Beyaz Rusya ve İran'da duyarlı, MDR ve XDR *Mycobacterium tuberculosis* klinik izolatlarında temel genotipik grupların belirlenmesi

Giriş: *Mycobacterium tuberculosis complex*'in tüm üyeleri KatG463/GyrA95 polimorfizmi temelinde üç temel genetik gruptan birinde yer alır.

Materyal ve Metod: Beyaz Rusya ve İran (Tahran ve Markazi)'in değişik bölgelerinden kültürle doğrulanmış tüberkülozlu hastalardan 50'si duyarlı, 121'i MDR (çoklu ilaç direnci) ve 31'i XDR (yaygın ilaç direnci) toplam 202 *M. tuberculosis* izolatı izole edildi. İzolatlar, sequencing ve PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) ile incelendi ve KatG463 GyrA95 kodonlarda polimorfizm ile Sreevatsan's patterni temelinde üç ana genetik gruba (PGG) ayrıldı.

Bulgular: Beyaz Rusya'dan MDR olarak tanımlanan 104 izolattan 57 (%54.8 ± 4.8)'si, 30 (%28.8 ± 4.4)'u ve 17 (%16.3 ± 3.6)'si sırasıyla PGG1, 2 ve 3'te yer alıyordu ($p < 0.05$). Beyaz Rusya'dan 31 XDR, 15 (%48.4)'i, 12 (%38.7)'si, 4 (%12.9)'ü sırasıyla PGG 1, 2 ve 3'te olmak üzere benzer bir patterne sahipti. İran örneklerinden, Markazi izolatları (ilaca duyarlı) 12 (%36.5), 15 (%45.5), 3 (%6), ve Tahran örnekleri (seçilmiş MDR): 9 (%53), 6 (%35.2), 2 (%11.8) (PGG 1, 2 ve 3, sırasıyla) patternine sahipti. Cezaevinde yatan tüberkülozlu hastalarda yapılan bir çalışmada, izoniazide direnç ile PGG arasında ilişki bulunmadı, ancak saptanan izolatların çoğu PGG 1'de yer alıyordu (%45.5 ± 10.9) ($p < 0.05$). Genel olarak, grup 1 izolatları, MDR ve XDR'de duyarlı türlere göre daha sıkı ve coğrafik bölge ile ilişki yoktu.

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Sonuç: Duyarlı türlerde dominant form grup 2 ve 3'te bulunuyordu. PGG grupları, coğrafik orijinden çok izolatlarn direnç durumu ile ilişkili gibi görünmektedir.

Anahtar Kelimeler: *Mycobacterium tuberculosis*, temel genetik gruplar, İran, Beyaz Rusya.

SUMMARY

Determination of principal genotypic groups among susceptible, MDR and XDR clinical isolates of *Mycobacterium tuberculosis* in Belarus and Iran

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Introduction: All members of the *Mycobacterium tuberculosis* complex were assigned to one of the three principle genetic groups based on *KatG463/GyrA95* polymorphism.

Materials and Methods: A total of 202 isolates of *M. tuberculosis* consisting of 50 susceptible, 121 MDR (multidrug resistant) and 31 XDR (extensively drug resistant) isolated from culture-confirmed tuberculosis patients in different regions of Belarus and Iran (Tehran and Markazi province). Isolates were screened by sequencing and polymerase chain reaction restriction fragment length polymorphism (RFLP) assay, and were further divided into three principal genetic groups (PGG), based on Sreevatsan's pattern as polymorphisms in *KatG463/GyrA95* codons.

Results: Among the 104 isolates, characterized as MDR from Belarus, 57 (54.8 ± 4.8%), 30 (28.8 ± 4.43%), 17 (16.3 ± 3.6), belonged to PGG 1, 2, and 3, respectively ($p < 0.05$). Thirty one XDR isolates from Belarus had a similar pattern as 15 (48.4%), 12 (38.7%), 4 (12.9%) PGG 1, 2, and 3, respectively. From Iranian samples, Markazi isolates (susceptible to drugs) had a pattern as 12 (36.5%), 15 (45.5%), 3 (6%), and Tehran samples were (selected MDR): 9 (53%), 6 (35.2%), 2 (11.8%) (PGG 1, 2, and 3, respectively). In a study of tuberculosis patients, who were in prison, no relation was found between PGG and resistance to isoniazid, but most of the identified isolates belonged to PGG 1 (45.5 ± 10.9%) ($p < 0.05$). Overall, the group 1 isolates showed more frequency in MDR and XDR rather than susceptible strains, and there aren't any relations to geographic region.

Conclusion: In susceptible strains, dominant forms were belonged to groups 2 and 3. It seems that PGG typing is closely related to resistance status of isolates rather than geographic origin.

Key Words: *Mycobacterium tuberculosis*, principal genetic groups, Iran, Belarus.

INTRODUCTION

One-third of the world's population is infected with *Mycobacterium tuberculosis*, and 3 million human's deaths are annually attributed to the organism. Although there is a very large global pool of infected individuals and considerable chromosomal heterogeneity, based on restriction fragment length polymorphism (RFLP) patterns, generated by probing with mobile insertion elements, studies of drug resistance and pathogenesis have raised

the possibility that synonymous (silent) nucleotide substitutions in structural genes may be limited (1-5).

Isoniazid, a first-line anti-tuberculous drug, has a simple chemical structure consisting of a pyridine ring and a hydrazide group. The bifunctional bacterial enzyme catalase-peroxidase (*katG*) converts isoniazid to a range of oxygenated and organic toxic radicals that attack multiple targets in the mycobacterial cell. The best-characterized target of these radicals is the cell wall

mycolic acid, but DNA, carbohydrates, lipids, and DNA metabolism may be targeted as well (1,2).

The lack of neutral mutations in structural genes indicates that *M. tuberculosis* is evolutionarily young and has recently spread globally. Species diversity in *M. tuberculosis* is largely caused by rapidly evolving insertion sequences, which means that mobile element movement is a fundamental process generating genomic variation in this pathogen. *M. tuberculosis* is contagious, and spreads through the air; person with active tuberculosis infects, on average, 10-15 others every year. One in 10 people infected with *M. tuberculosis* bacilli will become sick with active tuberculosis in his or her lifetime. *M. tuberculosis* is responsible for more deaths than any other single infectious organism; there are more than 8 million new cases and 1.7 million deaths annually. European Union countries report 23% of all new cases, and Kazakhstan, Romania, the Russian Federation, Turkey, Ukraine and Uzbekistan account for 73% of the total number of cases (2,3,5,6).

Sreevatsan et al. studied 26 structural genes of 842 isolates and suggested a broad evolutionary scenario for mycobacteria organisms, characterized by *katG* codon 463 and *gyrA* codon 95, in which *M. tuberculosis* could be split into three principal genotypic groups (PGGs), inspection of the sequence data revealed that only the variants at *katG* codon 463 and *gyrA* codon 95 were present at high frequency. These two sites apparently do not participate in antibiotic resistance and, hence, they were used as genetic markers that record the history of organism divergence (7). All members of the *M. tuberculosis* complex were assigned to one of three distinct genotypic groups, based on the combination of polymorphisms located at these two sites (Table 1) (7): group 1 with KatG463 CTG (Leu), GyrA95 ACC (Thr); group 2 with KatG463 CGG (Arg), GyrA95 ACC (Thr); and group 3 with KatG463 CGG (Arg), GyrA95 AGC (Ser)(7).

M. tuberculosis organisms, belonging to group 1, have *katG* and *gyrA* sequences indistinguishable from those of *M. microti*, *M. africanum*, and *M. bovis*. One subgroup of genetic group 1, the Beijing/W lineage, has been widely studied because of its worldwide distribution and association with outbreaks (1).

Polymorphism located at *katG* codon 463 was identified by automated DNA sequencing, PCR-RFLP with restriction endonuclease *NciI* or *MspI*, dot-blot hybridization and also a real-time PCR assay based on the use of molecular beacons (6,8-11). Polymorphism occurring at *gyrA* codon 95 was indexed by automated DNA sequencing (7,11).

The value of this classification was further supported by typing according to the spoligotyping technique, and became a well-established and widely used scheme applied in the field of molecular epidemiology of tuberculosis (11-13).

The susceptibility of *M. tuberculosis* to isoniazid and the Arg to Leu mutation at KatG463 are not associated (14).

With an evolutionary bottleneck approximately 15,000 to 20,000 years ago, possibly around the time of speciation of *M. tuberculosis*, PGG1 is thought to be evolutionarily older. Strain type of H37Rv is classified as a member of genetic group 3, and the Beijing is a subgroup of the genetic group 1 (7,15). As clustering is considered a marker of increased transmissibility, a higher virulence of group 1 and group 2 organisms compared to group 3 has been suggested (7,9).

Patients infected by principal genetic group 1 isolates were more likely to have extrathoracic involvement than those infected by group 2 isolates. However, this association was driven by the association of infection by the Beijing/W lineage isolates, a subgroup of group 1, with extrathoracic involvement. In addition, it was found that patients of Asian origin were the largest. Infections caused by isolates from group 1 are more likely to have

Table 1. Evolutionary chart for *Mycobacterium tuberculosis* complex organisms. The precursor of *M. tuberculosis* complex organisms was characterized by *katG* codon 463 (Leu) and *gyrA* codon 95 (Thr)* (7).

	Group 1		Group 2		Group 3	
<i>M. tuberculosis</i> complex precursor	KatG463 CTG (Leu)	GyrA95 ACC (Thr)	KatG463 CGG (Arg)	GyrA95 ACC (Thr)	KatG463 CGG (Arg)	GyrA95 AGC (Ser)
KatG463-CTG (Leu)	<i>M. tuberculosis</i>		<i>M. tuberculosis</i>		<i>M. tuberculosis</i>	
GyrA95-ACC (Thr)	(including Beijing)				(including H37Rv)	
	<i>M. bovis</i>					
	KatG463-CTG (Leu)					
	GyrA95-ACC (Thr)					

resulted from recent transmissions than infections caused by isolates belonging to the other two groups (1).

Aim of the work was the comparison of PGG situation in three distinct groups for evaluation of geographic parameter on *M. tuberculosis* isolates typing.

MATERIALS and METHODS

Research Material and *M. tuberculosis* Isolates

Fifty seven isolates from Iran and 35 isolates from Belarus were collected. The research material was sputum obtained from pulmonary tuberculosis patients from different regions of Belarus and Iran.

All the patients examined had clinically confirmed tuberculosis and proven registrations of clinical diagnostic examinations, such as chest X-ray, PPD, cough, weight loss etc. Patient sputum samples were cultured on Löwenstein-Jensen medium and grown colonies that were identified to the species level using TCH (2-thiophene carboxylic acid) and PN99B (paranitrobenzoic acid) selective media or by standard biochemical procedures.

Drug Susceptibility Testing

The antimicrobial drug susceptibility tests (AMST) were performed by World Health Organization (WHO) standard conventional proportional method preferably on Löwenstein-Jensen medium using the critical drug concentrations. Susceptibility testing was performed by the absolute concentration method. A microbial suspension containing 5×10^8 organisms/mL was prepared according to McFarland turbidity standards and was diluted 1/10; then, 0.2 mL of the dilution was added to Löwenstein-Jensen medium with or without a drug. The culture tubes were incubated at 37°C, and growth was monitored after three weeks of incubation and assessed as described WHO (2). All isolates were tested for susceptibility to first line drugs as rifampicin 40 µg/mL, isoniazid 1 µg/mL, ethambutol 2 µg/mL and streptomycin 10 µg/mL, on slants with H37Rv strain of *M. tuberculosis* as the positive control, using the BACTEC system in level III laboratory. An isolate was considered resistant to isoniazid when bacterial growth occurred in the presence of a concentration of 1 µg of isoniazid per mL. As recommendations of WHO those strains resistant to at least isoniazid and rifampicin (MDR) were tested for their susceptibility to any fluoroquinolone and at least one of three injectable second-line drugs (capreomycin, kanamycin, and amikacin) in the BACTEC system for detection of XDR isolates. This definition of XDR-TB was agreed by the WHO Global Task Force on XDR-TB in October 2006 (3).

DNA Purification and PCR

DNA purification from isolates performed by using of modified Chelex 100 method (15). In brief, it was per-

formed by solving of 3-4 colonies of fresh culture of isolate in 270 mL TAE buffer (1x) and heating in 95°C for 45 minutes, following by three 10-minutes centrifugations in 14.000 rpm for totally removal of Chelex 100 that would interferes to PCR reaction.

PCR performed in 50 µL of a reaction mixture containing 50 mM KCl, 10 mM Tris (pH 8.0), 1.5 mM MgCl₂ (or Buffer of Amersham), 5 µM of deoxynucleoside triphosphates (dNTPs), 1U *Taq* polymerase, 20 pmoles of each set of primers as mentioned in Table 2, and 3-6 µM of chromosomal DNA. The thermocycler parameters run as requiring.

The products were checked on the gel electrophoresis and amplified *katG* and *gyrA* segments were purified. The resultant DNA amplifications would be used for sequencing.

PCR-RFLP

In this study, PCR-Restriction fragment length polymorphism analysis was done as described previously (5,6).

DNA Sequencing

Detection of mutation in GyrA95 and point mutations detected by PCR-RFLP, were verified by sequence method.

PCR reaction was performed with different primers and conditions as shown in Table 2. PCR products were detected by 1.5% agarose-ethidium bromide gel electrophoresis and extracted from agarose gel by DNA extraction kit (Fermentas, K0513) according to the manufacturer's instructions. Extracted DNA concentrations were measured by nucleic acid analyzer (DU 730, Life Science UV/Vis spectrophotometer).

837bp extracted fragment of *katG* gene (from nucleotide number of 571 to 1408) and 194 bp of *gyrA* gene were amplified in a Rotor-Gene (RG-3000, Corbett Research Inc.) by Thermo-Sequenase Cy5 Dye Terminator Sequencing Kit (GE Healthcare 27-2682-01). Amplification for sequence was performed by oligonucleotide primer that was designed as Table 2, from the *M. tuberculosis* H37Rv genome sequence, by help of server programs. Sequence was done directly using an automatic DNA sequencer (Amersham auto sequencer).

Detection of Principle Genetic Groups

The SNPs at KatG463 and GyrA95 were investigated by PCR assay and sequencing. Isolates were assigned to one of the three principal genetic groups based on the SNPs found in KatG463 and GyrA95 delineated by Sreevatsan et al., as group 1 (KatG463 CTG, GyrA95 ACC), group 2 (KatG463 CGG, GyrA95 ACC), or group 3 (KatG463 CGG, GyrA95 AGC).

Polymorphism at codon 95 of the *gyrA* gene was detected by PCR amplification of a 194 bp DNA fragment

Table 2. Primers were used in the study for PCR-RFLP and sequencing.

Reaction	Direction	Primer (5'-3')	Product size (bp)	Program
PCR-RFLP	F	AGCTCGTATGGCACCGGAAC	620	94C 60s
	R	TTGACCTCCCACCCGACTTG		56C 60s 72C 60s 40 cycles
PCR for Sequencing of <i>katG</i>	F	TTCGGCCGGGTCGACCAGT	975	94C 10S
	R	CGGAATTCAGGGTCCGATGACCT		62C 30S 72C 10S 43 Cycles
Amplification of <i>katG</i> in sequence	F	TTCGGCCGGGTCGACCAGT	837	95C 30S
	R	TGCGGTCCGAACTAGCTGTGA		56C 60S 72C 80S 33 Cycles
PCR for sequencing of <i>gyrA</i>	R	CCGGTGGGTCATTGCCTGGCG	194	95C 40S 68C 60S 72C 20S 40 Cycles
Amplification of <i>gyrA</i> in sequence	F	CGATTCCGGCTTCGCCCCGG	194	95C 40S
	R	CCGGTGGGTCATTGCCTGGCG		68C 60S 72C 20S 40 Cycles

with primers *gyrA* (Table 2). All amplicons were sequenced as above mentioned method by reverse primer of *gyrA* (Table 2).

RESULTS

From 140 Belarusian samples suffering active tuberculosis that have been referred, 70% were women and 30% men, and male/female ratio was 2.3. Furthermore, 42.5% of the patients suffering primary tuberculosis and 57.5% have secondary form of tuberculosis. Patients originated from different regions of Belarus (Brest, Magiliev, Gomel, Gorodna and Vitebsk) and different regions of I.R of Iran were admitted to the Reference Laboratory.

DNA extraction by Chelex 100 was the best material for purification and maintenance DNA for long time. As shown in Table 1, PCR method using primers for amplification of 620 bp amplicon, could further detect all resistant and susceptible isolates that identified by conventional methods.

DNA sequencing of the *katG* gene from randomly selected isolates verified 100% sequence accuracy of the point mutations, detected by PCR-RFLP.

All of XDR and MDR isolates, suffered mutation in nucleotide 944 as G944C (AGC → ACC) in aminoacid Ser315Thr. Standard strain H37Rv has non mutated codon. In *M. bovis* both of sites were mutated.

In this study, *M. tuberculosis* isolates were classified in three genotypic groups on the basis of the presence of

single nucleotide polymorphisms in codon 463 of the *katG* gene (KatG463) and codon 95 of the *gyrA* gene (GyrA95) (Table 3) (7).

From the 104 MDR isolates from Belarus, dominating the first version of the genetic groups (PGG 1) was 57 (54.8 ± 4.8%). The second genetic group frequency was 30 (28.8 ± 4.43%), isolates and 17 (16.3 ± 3.6), isolates belonged to group 3 (p < 0.05).

Tehran samples that were selected MDR isolates had a pattern as: 9 (53%), 6 (35.2%), 2 (11.8%) (PGG 1, 2, and 3, respectively) .

Among the 31 XDR-isolates, 15 (48.4 ± 9.1%) belonged to the first group, 12 (38.7 ± 8.9%) and 4 (12.90 ± 6.0%) respectively belonged to the 2nd and 3rd groups (p < 0.05).

These data (Table 3) indicated that susceptible isolates of Belarus representatives different types of genetic groups (with low number of samples): PGG 1-3 (33.3 ± 15.7%), PGG 2-4 (44.4 ± 16.5%), and PGG 3-2 (22.2 ± 13.8%) (p > 0.05). Thirty eight samples from Markazi providence of Iran and Tehran were susceptible to drugs and PGG 3 was dominant form: PGG 1-13 (34.2%), PGG 2-10 (26.3%), and PGG 3-15 (39.4%) (p > 0.05).

From 22 strains isolated from patients residing in prisons, 10 (45.5 ± 10.9%) isolates belonged to genetic group 1, 8 (36.4 ± 10.5%) group of 2 and 4 (18.2 ±

Table 3. Principle genetic groups analysis of *M. tuberculosis* isolates from different geographic regions.

Type of isolates	Geographic regions	Number	Principle genetic group (PGG)		
			PGG 1	PGG 2	PGG 3
Standard strains	H37Rv, Academic and <i>M. bovis</i>	3	1 (33.33 ± 27.3%)	0%	2 (66.66 ± 27.2%)
Susceptible	Belarusian	9	3 (33.3 ± 15.7%)	4 (44.4 ± 16.5%)	2 (22.2 ± 13.8%)
	Iranian- Markazi and Tehran	38	13 (34.2 ± 15.7%)	10 (26.3 ± 13.7%)	15 (39.4 ± 11.2%)
MDR isolates	Minsk 1,2	12	9 (75.0 ± 12.5%)	2 (16.6 ± 10.7%)	1 (8.3 ± 7.9%)
	Minsk Region	18	10 (55.5 ± 11.7%)	5 (27.7 ± 10.5%)	3 (16.6 ± 8.7%)
	Brest	4	1 (25 ± 10.2%)	2 (50 ± 25.0%)	1 (25 ± 10.2%)
	Grodna Region	10	5 (50 ± 15.8%)	3 (30 ± 14.5%)	2 (20 ± 12.6%)
	Vitebsk	7	4 (57.1 ± 18.6%)	2 (28.5 ± 17.0%)	1 (14.3 ± 13.2%)
	Vitebsk Region	11	4 (36.3 ± 14.5%)	4 (36.3 ± 14.5%)	3 (27.2 ± 13.4%)
	Mogilief	9	8 (88.9 ± 1.1%)	1 (11.1 ± 10.4%)	0%
	Gomel	11	6 (54.5 ± 15.0%)	3 (22.3 ± 13.4%)	2 (18.2 ± 11.6%)
	Prison	22	10 (45.5 ± 10.6%)	8 (36.3 ± 10.26%)	4 (18.0 ± 8.2%)
	Iran-Tehran	17	9 (53.0 ± 21.9%)	6 (35.2 ± 21.9%)	2 (11.8 ± 17.8%)
	Total MDR*		121	66 (54.8 ± 4.8%)	36 (29.7 ± 4.43%)
XDR isolates	Belarus	31	15 (48.4 ± 9.1%)	12 (38.7 ± 8.9%)	4 (12.90 ± 6.0%)
Total *		202	98 (48.5 ± 4.05%)	62 (30.7 ± 3.76%)	42 (20.8 ± 3.09%)

* Statistically significant between three PGG groups ($p > 0.05$).

8.4%) group of 3. There was no relation between PGG and resistance to isoniazid, but most of the identified isolates belonged to PGG 1 ($p < 0.05$).

In susceptible group, there was not any significant difference between three genetic groups. Frequency of principle genetic groups among MDR isolates of Iran-Tehran was like to Belarusian isolates.

Overall, as shown in Table 3, resistant isolates in this study, from both regions of Belarus and Iran, had similar situation about PGG analysis (dominant PGG 1) but susceptible strains belonged to PGG 2 and 3. There is no relation between PGG typing and geographic region, but it seems that PGG status is related to drug resistance.

DISCUSSION

The group 1 isolates showed more frequency in MDR and XDR (54.8 and 48.4%, respectively) rather than susceptible and standard strains (34 and 33%, respectively). Dominance of PGG 1 organisms provides an additional support for the hypothesis that the group is evolutionarily older than groups 2 and 3, and therefore has more time to accumulate divergence and resistance (16).

Due to the fact that highly resistant genetic variant Beijing, belonging to the first genetic group, spreads rapidly, and it is currently a public health threat in many

countries, it can be assumed to spread on the territory of Belarus. The majority of the Iranian MDR isolates were belonged to group 1 (53.0 ± 21.9%). Supporting this provision is incidence of isolates in first genetic group, which dominates among the analyzed MDR and XDR isolates in our study ($p < 0.05$).

The Beijing genotype of *M. tuberculosis* is a virulent strain that has originated out of East Asia, and has disseminated around the world. One potential explanation for the increased virulence in Beijing genotype is the production of phenolglycolipid (PGL), a surface antigen that suppresses the Th1 response. PGL is produced in Beijing strains from principle genetic group 1, but it is not produced by members of the other PGGs (2 and 3), such as *M. tuberculosis* H37Rv (17).

The Beijing genotype is strongly associated with drug resistance and outbreaks, including multidrug resistance (MDRTB) and extensive-drug resistance (XDR-TB) (1). This finding accords with our results in this work.

Among isolates from patients living in the city of Minsk and Mogilev, they constitute the majority (75% and 88% respectively). However, the structure of PGG isolates from patients with tuberculosis from prisons is no different from those isolates throughout the country.

Overall, the results indicate that *M. tuberculosis* strains belonging to group 2 and group 3 are predominant among susceptible isolates.

This work describes the development of a simple and specific PCR-based typing method that differentiates subspecies of the *M. tuberculosis* and segregates them from various clinically important mycobacteria other than tuberculosis (MOTT) species. If confirmed, the discovery of three distinct *M. tuberculosis* lineages with variable epidemiologic and clinical manifestations would have important implications for public health control strategies, studies of bacterial virulence, and mathematical modeling of tuberculosis epidemiology.

To our knowledge, this is the first study describing the distribution of Iranian and Belarusian *M. tuberculosis* isolates among the three groups delineated by the *katG* and *gyrA* polymorphisms.

Infections caused by isolates from group 1 are more likely to have resulted from recent transmissions than infections caused by isolates belonging to the other two groups. Public health approaches and tuberculosis transmission models may benefit from data in the context about the three genetic groups (7).

From point of application, the test has three benefits:

Detection of principle genetic groups, fast detection of *M. tuberculosis* in sample and differentiation from MOTT by determination of 620 bp fragment of *katG* gene, and simultaneous detection of mutation in KatG315 that determinates resistance to isoniazid [with a high percentage of precision (5)].

It seems that PGG typing is closely related to resistance status of isolates rather than geographic origin. Supporting this provision is predominance of PGG 1 in resistant isolates that was significantly higher than other groups in our study ($p < 0.05$). The PGG typing is a good and fast tool for detection of more important types based on clinical aspects.

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