Characterization of molecular evolution in multi-drug resistant *Mycobacterium tuberculosis* in patients with active pulmonary tuberculosis of different regions in Belarus

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Abstract
This is the first genetic biodiversity study of *M. tuberculosis* in Belarus. Thus, we investigated the genetic patterns of strains isolated in the first survey of anti-tuberculosis drug-resistance realized by *rpoB* gene as part of the Global Project of Anti-tuberculosis Drug Resistance Surveillance (BRIEM, Belarus). A 411-bp fragment of the *rpo*B gene, containing the sequence of the 81-bp *rpo*B fragment, was amplified by PCR and the *rpoB* gene fragments of tuberculosis strains were sequenced using the Amersham auto sequencer. This method uses the variability of nucleic sequences of genes such as beta subunit RNA-ase (*rpoB*) internal transcribed spacer and other genes. For analysing tree evolution used method UPGMA and Neighbour-Joining and analyzed by MEGA program. Clinical isolates (44/463) were analyzed by using sequencing gene *rpoB* and genotyped by program MEGA. The results were compared with the international database. MDR was 35% in never treated patients and 13.5% in previously treated patients. Mutations in *rpoB* gene and *katG* genes were detected in 95% and 84% of the MDR strains, respectively. Two clusters were found to be identical by the four different analysis methods, presumably representing cases of recent transmission of MDR tuberculosis. This study gives a first overview of the *M. tuberculosis* strains circulating in Belarus during the first survey of anti-tuberculosis drug-resistance. It may aid in the creation of a national database that will be a valuable support for further studies.

Keywords: Molecular evolution, MDR, M. tuberculosis, Belarusian isolates.

Introduction

Mycobacterium tuberculosis is the most successful human pathogen worldwide, responsible for 3 million deaths each year and extensive morbidity and mortality (World Health Organization 1998). By the end of 2004, 199 (94%) of 211 countries notified 4.4 million new and relapsed cases, of which 1.9 million (44%) were new sputum smear-positive. Among these notifications, 3.7 million were from DOTS (Directly Observed Treatment Short course) areas. Belarus covers an area of 207,600 km2 and has a population of around 10 million inhabitants, and has shown a marked decrease in the incidence of TB during the last years. The TB prevalence rate in 1994 was 35 per 100,000 inhabitants for mortality and 40.6 per 100,000

inhabitants for morbidity (Titov *et al.,* 2006; Zaker *et al.,* 2006). Since then, the incidence has decreased dramatically to reach 6734 cases (26 per 100,000 inhabitants), with most being confined within the 15 to 45 year age group (Titov *et al.,* 2006; Zaker *et al.,* 2006).

Nevertheless, in indigenous populations, TB incidence has remained very high (50.6 per 100,000 inhabitants) (Titov *et al.,* 2006; Zaker *et al.,* 2006). In 1982, the National Tuberculosis Control Program of Belarus (NTP) implemented the directly observed treatment and since 1994 has used a standardized six month treatment for all new TB cases with or without culture confirmation (Abate *et al.,* 2001; Douglas *et al.,* 2003; Dvorska *et al.,* 2001).

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 rifampicine resistance (Bakonyte *et al.,* 2005; Dvorska *et al.,* 2001; Makorousev *et al.,* 2002; Leung *et al.,* 2003; Garsia *et al.,* 2002). In contrast to several other pathogens with MDR phenotypes, plasmid or transposon-mediated mechanisms of resistance have not been reported in *M. tuberculosis* (Leung *et al.,* 2003; Marcia *et al.,* 2003; Miriam *et al.,* 2001). Since resistance to bacteriostatic in *M. tuberculosis* is exclusively due to genomic mutations, the bacterium would benefit from an increased mutation rate.

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 [Herrera *et al.,* 2005; Eltrigham *et al.,* 1999; Kiepiela *et al.,* 2000; *Kim et al.,* 2004; Sajduda *et al.,* 2004]. 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 (Garsia *et al.,* 2002; Talenti *et al.,* 1997; Titov *et al.,* 2006; Zaker *et al.,* 2006). Restricted allelic variation limits the utility of multilocus sequence analysis (Zheltokjva et al., 2004; Leslie et al., 2003; Leung et al., 2003; Titov *et al.,* 2006; Zaker *et al.,* 2006).

Although many molecular methods have been used for categorizing *M. tuberculosis* strains (Miriam *et al.,* 2001; Mekonnen *et al.,* 2003; Telenti *et al.,* 1997), 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. However, because the original genetic groupings are based on only two nsSNPs, the evolutionary hypothesis of *M. tuberculosis* outlined by Sreevatsan *et al.* (1997) 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 (Kim *et al.,* 2004; Makrousov *et al.,* 2002)

(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.

The polymorphism of the *rpo*B gene, which encodes the beta subunit of RNA polymerase, was used to differentiate mycobacteria through DNA hybridization and DNA sequence comparison (Abate *et al.,* 2001; Douglas *et al.,* 2003; Kima *et al.,* 2003; Titov *et al.,* 2006). The variable region of *rpo*B in mycobacteria is suitable to be used in a PCR-REA assay. This variable region of the *rpo*B 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 *rpo*B region was amplified in 44 species of mycobacteria (Titov *et al.,* 2006; Zaker *et al.,* 2006).

In the paper of Kim *et al.* (1999), the *rpo*B gene was sequenced in 44 species of mycobacteria. Slowly and fast growing mycobacterial species were differentiated after comparison of the 306 bp nucleotide sequence. The pathogenic *M. kansasii* was easily distinguished from nonpathogenic *M. gastri*, which is not, for example, possible by sequencing of 16S rDNA. About 40 point mutations, deletions and insertions were discovered by sequencing *rpoB*. Point mutations occurred most frequently in the codon, which encodes Ser (531) and His (526). The point mutation Ser (531) to Leu dominated in approximately 70% of Rifampicin-resistant clinical isolates of *M. tuberculosis*.

The molecular characterization of the isolates collected during this study was not carried out and there is no information on the major circulating clades of *M. tuberculosis*. The mutations involved in drug resistance and the question of whether the Beijing family of the *M. tuberculosis* complex had spread into Belarus have not been studied until now. Molecular epidemiological studies have showed the widespread of this family and its association with drug-resistance (Herrera *et al.,* 2005;

Makorousev *et al.,* 2002; Ertrigham *et al.,* 1999; Kiepiela *et al.,* 2000).

In recent years, the genetic typing of *M. tuberculosis* complex (MTC) strains has been widely used to support conventional epidemiological investigations of TB outbreaks and as a tool for studying transmission dynamics.

Materials and Methods

Mycobacterial strains and drug susceptibility testing
From total 463 strains, 44 isolates were recovered from cultured sputum samples resistance to rifampicine and 42 isoniazid-resistant were isolated from sputum of patients with active pulmonary tuberculosis, from July to September 2006. All strains were cultured on Lowenstein-Jensen solid medium and identified to the species level using TCH (2-thiophene carboxylic acid) and PN99B (paranitrobenzoic acid) selective media or by standard biochemical procedures.

All isolates were resistant to at least one primary antituberculosis agent. The anti-microbial drug susceptibility tests (AMST) were performed using CDC standard conventional proportional method rifampicin (Rif) 40 mg/L, isoniazid (INH) 0,2 mg/L, ethambutol (EMB) 2 mg/L, ethionamide (ETH) 20 mg/L, streptomycin (SM) 4 mg/L, and kanamycin (K) 20 mg/L on slants with H37Rv strain of *M. tuberculosis* as the positive control, and using the BACTEC system. The breakpoints for INH were at 1µg/ml on Lowenstein-Jensen medium and 0.1 µg/ml on the BACTEC system; for RIF, 40.0 µg/ml on Lowenstein-Jensen medium and 2.0 µg/ml on the BACTEC system.

DNA extraction and PCR amplification
DNA extraction was purified using Fermentas kit's (K512). A 411-bp fragment of the *rpo*B gene and 209-bp fragment of *katG* was amplified by PCR with primers rpoB-F (5-TACGGTCGGCGAGCTGATCC-3) and *rpoB*-R (5-TACGGCGTTTCGATGAACC-3) and *katG F* 5-GAAACAGCGGCGCTGGATCGT-3, *katG R* 5-GTTGTCCCATTTCGTCGGGG-3 (Titov *et al.,* 2006; Zaker *et al.,* 2006). 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 (Talenti

et al., 1997). For *rpoB* fragment 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 and for *katG* 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. PCR products were then run on 2% agarose gels and examined for the presence of the 411-bp and 210 bp band after ethidium bromide staining. The DNA purification were performed on agarose using Sigma Kit (124K6083) and the products were checked and purified on the gel electrophoresis and purified *rpoB* and *katG* segment were amplified. The resultant DNA amplifications were used for sequencing.

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).

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.

Data analyzing of DNA sequencing
DNA sequences from *rpoB* and *katG* gene were analyzed by "Blast" program (www.ncbi.cgi.htm). In this manner, sequences of standard strains of H37RV, CDC1551 and MBT 210 (http://www tigrblast.tigr.org) were used as control and compared with test strains. Comparison of all sequences was performed by applying "Mega"

and "DNAMAN" software. Alignment of the DNA fragments (*rpoB* and *katG*) was carried out with the help of MEGA 3.1 software (www.megasoftware.net/mega3.1/) and data obtained were analyzed and edited with DNAMAN software.

Mutations spectrum and frequency analysis
DNAMAN 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 deleting or pasting any text or sequence to files.

Cluster analysis by Neighbor-Joining method
This dendrogram is setup with the distance matrix using the Neighbor-Joining method (Saitou and Nei, 1987). Phylogenetic tree shows related homologies between any two sequences in a multiple alignment. With the help of nucleotide sequencing methods precise description of small gene fragments can be obtained; with the help of other methods of molecular strain typing larger genome fragments can be compared, but finally the comparison should be fulfilled by means of similarity and difference evaluation. For this purpose a distance matrix should be constructed by Neighbour-Joining method. An assumption is made about independent development of elements. In the cause of bootstrapping other analysis functions can be used.

The basic unit of data used in Neighbor-Joining (Nearest Neighbor) cluster analysis is the similarity coefficient derived from any one of the similarity analysis formula. A similarity matrix derived from sequential pairwise comparisons of two most similar operable taxonomic units (OTU) or clusters of OTUs is used to construct a phenogram.

Unweighted pair-group method using arithmetic averages
Unweighted pair-group method using arithmetic averages (UPGMA) is one of the most frequently used cluster analysis methods an is widely applied in the construction of dendrograms based on electrophoresis fingerprint patterns, hybridization matrices, and nucleic acid sequences. With UPGMA, an OTU, or every OTU in a cluster of OTUs is joined to a known

cluster on the basis of the average (mean) distance between all combinations of pairs of OTUs. Thus, UPGMA calculates the average distance from one cluster of OTUs to a comparison cluster, where each OTU in the cluster is given equal weight. This dendrogram is setup with the distance matrix using the UPGMA method (Sneath and Sokal, 1973). The matrix can be built up only with Observed Divergence method. Dendrogram shows related homologies between two sequences or groups.

Results and Discussion

Study of population and bacterial strains
A total of 86 patients with sputum smear-positive pulmonary TB was included in the survey to determine the initial prevalence and acquired resistance to the principal anti-TB drugs. Of these, 59 (68%) had never been treated for tuberculosis and the other 27(32%) had previously received treatment. Of the 463 isolates, 377 were sensitive to all four evaluated drugs and 44 (52%) showed resistance to rifampicine with other drugs or isoniazid (42) with other drugs.

The rate of MDR was 38% (34) and 11% (10) in strains of never treated and previously treated patients respectively. Total resistance to INH and RIF in never treated patients was 100% (42 patients) and 100% (44 patients), respectively. Most of the patients with drug-resistant isolates (74/ 90-82%) were aged between 15 and 54 years.

Drug susceptibility
All 44 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 42 strains examined were resistant to isoniazid, streptomycin (90% of all isolates that were resistant to rifampicine and isoniazid were too resistant to streptomycin) and 16 strains (43%) were resistant to etambutol. Only 40% of 42 isolates that were resistant to isoniazid were mono-resistant to isoniazid. So from 163 isolates, 4 isolates were mono-sensitive to isoniazid.

PCR amplification and DNA sequencing analysis for rpoB
Belarusian patients and comparison with the initial gene allowed revealing 16 different types

of mutations. Of 16 different types of mutations 14 ones in four *M. tuberculosis* isolates in codons 520 (*CCG→CCC*)-2, 521 (*CTG→TTG*), 508 (*ACC→ACG*)-2, 523 (*GGG→GGA*), (*GGG→GGC*)-4 и 510 (*CAG→TAG*)-4 were referred to the silent mutations. They comprise 16.8% of all mutations. All mutations detected were point ones except the one in codon 531 which is a substitution *TCG→TGC*. The most frequently mutated codons were 510, 526 and 523 with corresponding frequencies 47.7%, 40.9% and 38.6%. Of forty five mutated isolates eleven (27.7%) had one mutation, twenty three (52%) – two mutations, seven (16%) – three mutations, four (7%) – four ones. In Belarusian *M. tuberculosis* strains 16 types of mutations were revealed. In *rpoB* gene the most affected codons were 511, 516, 526 and 531. Of 44 isolates collected from the patients with primary tuberculosis 11 strains (29.5%) had the following mutations: 5 isolates had four *TCG→TTG* mutations in codon 531 and one *CAG→GAG* mutation in codon 510. Among the strains mentioned double mutations were detected of which three in codons 531 (*TCG→TTG*) and 510 (*CAG→GAG*). Double mutation was also observed in codons 531 (*TCG→TTG*), 523 (*GGC→GCG*), 512 (*AGC→GCC*) and 507 (*GGC→GCC*). One strain had thee silent mutations in codons 520 (*CCG→CCC*), 507 (*GGC→AGC*) and 510 (*CAG→A*AG). Eight patients (72%) with primary tuberculosis had prevailing mutations in codons 531 and 510; patients with secondary tuberculosis had mutations in codons 526, 523 and 510. The research accomplished showed that one mutation was more frequently observed in codons 526, 531 (36%, 36%); two ones – in codons 526, 510 (52%, 52%). Three or four mutations were detected in codons 510 (70%). To study the prevalence of mutations in the Republic of Belarus comparison was made depending on the region of residence. According to the result in all regions of Belarus two mutations are found with the same frequency. Four mutations in *rpoB* gene were only detected in Minsk (2.27%) and Minsk region (4.54%). It was noted that the majority of mutations were missense ones and were assisted by coding amino acid substitution.

PCR amplification and DNA sequencing analysing for katG
In the four isolates of all isolates that were sensitive to isoniazid any mutation were not detected in region 210 bp of katG gene.

Mutations were observed in affected codons of PCR product segment 209 bp of *katG* gene, 305, 306, 307, 314, 316, 321, 328, 315, 316,357, 454,463 and 309. Four types of mutations were identified in codon 315: AGC→ACC (n=36)36%, AGC→AGG (n=1)0.9%, AGC→AAC (n=2)1.8%, AGC→GGC (n=1)0.9%. One type of mutation was found in codon 316: GGC→AGC (n=16)%, four types of mutations were detected in codon 309: GGT→GGT (n=7)%, GGT→GCT (n=4)%, GGT→GTC (n=3)%, GGT→GGG (n=1)%. Mutations in codon 309 revealed 34%, in codon 316 – 37%, other types of mutations 29% of all detected mutations. Among all strains (n=40) 95% of mutation were detected in codon 315 (four type mutation). For two isolates 2 types of mutations were seen in codon 357 GAC→CAC and GAC→AAC, in addition two mutations were also observed in codon 463 CGG→CTG and 454 GAG→CGA. In all 42 strains, nine one mutation 21.5%, 16 – two mutations38%, 9 - three mutations21.5%, four – 4 mutations9.5% and 4 – five mutations9.5% were determined. Two isolates have not any mutations in kat G gene. Three silent mutations were detected in codons: 306 (CCG→CCC), 309 (GGT→GGG) and 314 (ACC→ACG) these silent mutations revealed no effect on the susceptibility pattern tested strains.

Phylogenetic analysis of evolutionary relationships of *M. tuberculosis* strains prevalent on the territory of Belarus
RpoB gene sequences were used to construct a dendrogram with the help of MEGA and DNAMAN softwares, and the strains were divided into clonal groups.

Phylogenetic dendrogram
With the help of nucleotide sequencing methods precise description of small gene fragments can be obtained; with the help of other methods of molecular strain typing larger genome fragments can be compared, but finally the comparison should be fulfilled by means of similarity and difference evaluation. For this purpose a distance matrix should be constructed by Neighbour-Joining method. An assumption is made about independent development of elements. In the cause of bootstrapping other analysis functions can be used. The data obtained with the help of this software confirm the results of Neighbour-Joining method for the strain 408 isolated from the patient from Mogilev region.

With the help of dendrogram constructed by Neighbour-Joining method the relation of strains isolated from the patients from different regions of Belarus and their classification by phenotypic factors were obtained (Fig. 2).

Strains isolated from the patients from different regions of Belarus were divided into three genetic groups. The first one is closer to European strains (from Italy, France, Finland), the second one – to Central Asiatic (from Russia, China, Taiwan), the third one – Belarusian residential mycobacteria (Fig. 2).

According to the dendrogram the strain isolated from Mogilev patients is essentially older (44 years old). Within this period it was not considerably changed after its appearance in Belarus. The other strains with the help of bioinformatics analysis were divided into three main genetic groups:

1. Strains of the first group are more similar to the standard European and Eastern ones (From China and Taiwan) which diverged in the last 10 years (evolution rate and time), i.e. they are relatively new ones, and that is confirmed by the mutations (Fig. 1-2).

2. Strains of the second group diverged earlier; they are older than the strains of the first group (16 years old- time and rate of evolution) (Fig. 2).

3. Strains of the third group are similar to European strains and only circulate in Brest region.

All the other strains are very similar to each other and located on the same branch (Fig. 3). With the help of bioinformatics software general classification of *M. tuberculosis* isolated from the patients from different regions of Belarus was obtained. In the dendrogram constructed by Minimum method all strains are divided into five groups, and in each group there are neighbouring strains (Fig. 3).

According to the dendrogram constructed strain 408 from Mogilev region is closer in its primary structure to the strains from Russia and China (BLAST software). Strains 3255, 23632, 1217 and 24276 from Brest region originate from Europe (Fig. 2, 4).

According to unweighted pair group's method with use of arithmetic average (UPGMA) it is obvious that the strain from Mogilev is older, and the other ones associated with each other depending on the homology level of the fragments examined (Fig. 4). According to the dendrogram constructed by UPGMA method, all

the strains are divided into three genetic groups. It is evident that the Brest strains differ from the rest ones and probably originate from Western Europe and represent a separate cluster. One Mogilev strain also differs in its age and genotype (Fig. 4).

BLAST software allowed clarifying that one isolate from Mogilev region belongs to ancient representatives of *M. tuberculosis* since it did not contain mutations in codons 526, 531. Twenty isolates (45%) of different resistant Belarusian strains are combined into one phylogenetic group.

Five isolates made up a separate branch from the first one: one strain from Mogilev region, another – from Vitebsk region. Sequences of three isolates are close to the ones of the strains from Italy, India, and Taiwan. Sequences of four strains correspond to the ones of the standard strains H37RV и CDC1551. One isolate from the city of Minsk completely corresponds to the standard strains. One isolate from Grodno region corresponds to the strain 1237P from India, two isolates from Minsk correlate with the strains 2224 AY155357 from India. Three isolates from Vitebsk region and the city of Minsk correlate with the strains TCGVH22 from Taiwan, 1928 from China, 2455 from India; five isolates correlate with RM from Italy and MDR 119 from Taiwan (figures 2-4). Thus, the highest frequency of mutations in *rpoB* gene of *M. tuberculosis* isolated from Belarusian patients is observed in codon 510. In the strains isolated from the patients with primary tuberculosis mutations were identified in codons 531 (72%), 510 (45.4%), from the patients with secondary tuberculosis – in codons 526 (60.6%), 523 (48.4%), 510 (39.3%).

Thus, the highest frequency of mutations in *katG* gene of *M. tuberculosis* isolated from Belarusian patients is observed in codon 315. In the strains isolated from the patients with primary tuberculosis mutations were identified in codons 315 (23.8%), 316 (11.9%), 309 (21.4%), from the patients with secondary tuberculosis – in codons 315 (49.2%), 523 (19%), 309 (11.1%).

With the help of Neighbour-Joining method evolutionary dendrograms were obtained for Belarusian strains which were divided into three genetic groups: A – strains more similar to Eastern ones (Taiwan, China); B – Belarusian strains; C – European strains (Fig. 1, 2).

According to the dendrograms obtained by UPGMA method strains were divided into three genetic groups: A – closely related to

Eastern strains (Taiwan, China); B – Belarusian residential strains; C – related to European strains (Fig. 4).

Evolutionary dendrograms constructed for the first time for *M. tuberculosis* strains collected in Belarus show that strain 408 of *rpoB* gene collected from the patient from Mogilev region is more related to the strains circulating in Russia and China. The other strains are divided into three genetic groups: group A – strains closely related to Eastern ones (Taiwan, China) (appeared about 10 years ago- rate and time of evolution); group B – Belarusian residential mycobacteria diverged about 16 years ago; group C – related to European strains (appeared about 9 years ago- rate and time of evolution) (Fig. 1-4).

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

In conclusion, this study gives a first overview of the *M. tuberculosis* strains circulating in Belarus during the first survey of anti-tuberculosis drug-resistance. It may aid in the creation of a national database that will be a valuable support for further studies and according to the dendrograms obtained by UPGMA method strains were divided into three genetic groups: A – closely related to Eastern strains (Taiwan, China); B – Belarusian residential strains; C – related to European strains.

Acknowledgment

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Figures follow…..

Mogilev – 1 strain D and 7E; Gomel – 1 strain E; Vitebsk – 4 strains D, 1E and 3F; Minsk – 6 strains D, 5E, 3F and 3G; Hrodno – 2 strains E; Brest – 1 strain D, 5E and 2F

Fig. 1 – Association of genetic groups of *M. tuberculosis* with regions of the Republic of Belarus.
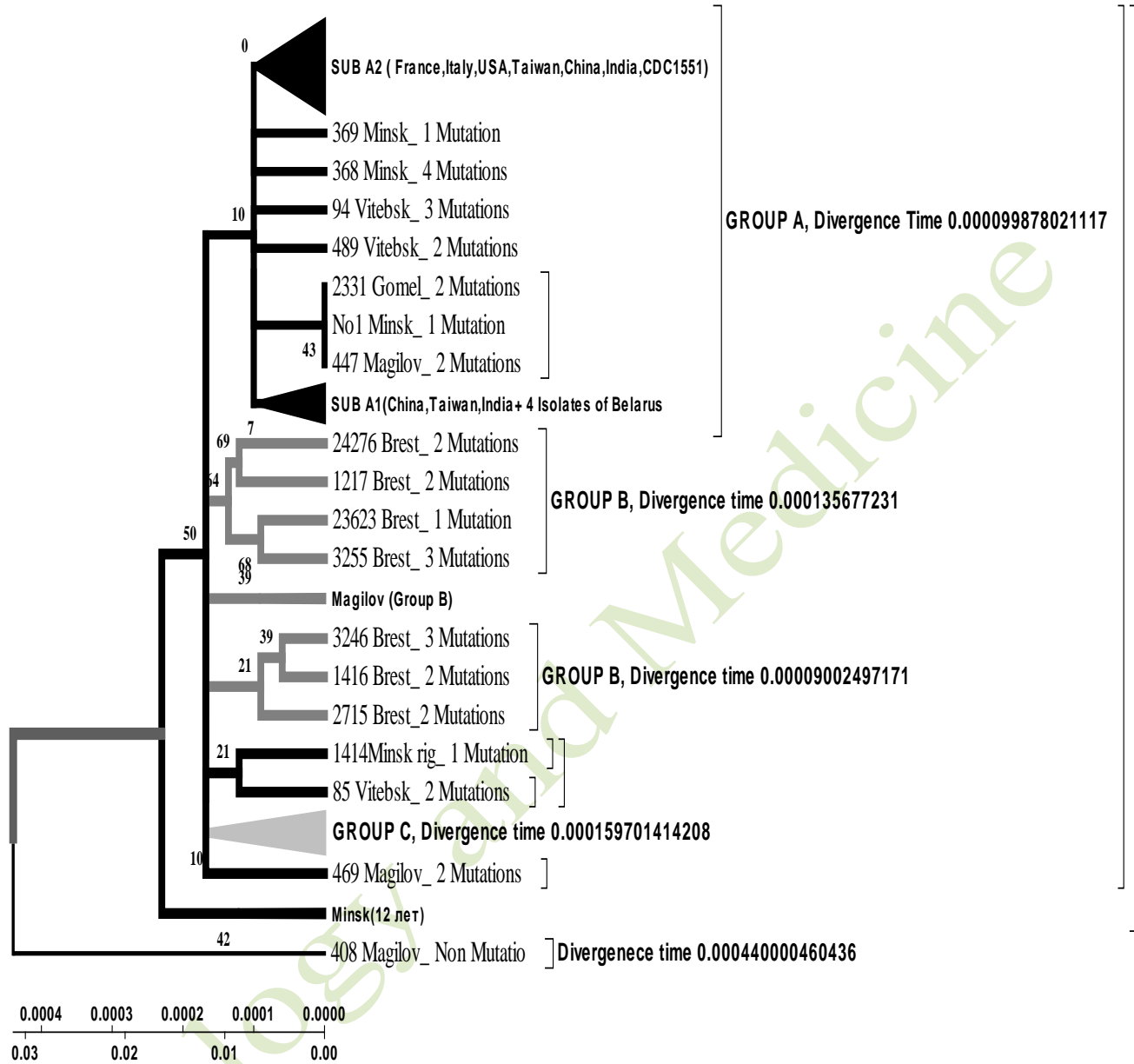
Fig. 2 – Evolutionary dendrogram of 44 Belarusian *M. tuberculosis* strains constructed by Neighbour-Joining method. The tree was generated using the Neighbor-Joining method with 1000 bootstrap replicates and distance calculation using the number of different sSNP loci (http://www.megasoftware.net/).

Fig. 3 – Dendrogram constructed by Minimum method with the use of Belarusian strains sequences. The phylogenetic tree, generated using the minimum method with 1000 bootstrap replicates and distance calculated using the number of different sSNP loci (http://www.megasoftware.net/).

Fig. 4 – Dendrogram and classification of *M. tuberculosis* isolates by UPGMA method. The phylogenetic tree, generated using the UPGMA method with 1000 bootstrap replicates and distance calculated using the number of different sSNP loci (http://www.megasoftware.net/).