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Molecular characteristics of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Russian Federation

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***Objectives*: Three *Mycobacterium tuberculosis* genetic loci—*rpoB* and *katG* genes and the *fabG1(mabA)–inhA* operon promoter region—were studied to reveal the mutations associated with rifampicin and isoniazid resistance.**

***Methods*: Four hundred and twelve isolates of *M. tuberculosis* from different regions of the Russian Federation were collected during 1997–2005. A matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF MS)-based minisequencing method was used for the detection of mutations.**

***Results*: Thirteen different variants of single mutations in codons 533, 531, 526, 516, 513 and 511 of the rifampicin resistance-determining region of the *rpoB* gene as well as the TTG insertion in the 514a position were found among the rifampicin-resistant isolates. Single nucleotide substitutions in codons 531, 526 and 516 (64.8%, 10.3% and 7.7%, respectively) were the most prevalent mutations. Codon 526 was shown to be the most variable of all. No mutations were detected in *rpoB* genes for 29 (10.7%) of the rifampicin-resistant isolates. 76.9% of the isoniazid-resistant isolates carried single mutations in codon 315 of the *katG* gene. For another 12.9% of them, double mutations in the *katG* gene and the *fabG1(mabA)–inhA* promoter region were revealed. No mutations were detected in 8.2% of the isoniazid-resistant isolates.**

***Conclusions*: Molecular analysis of the loci of *rpoB* and *katG* genes and the *inhA* promoter region of 412 *M. tuberculosis* clinical isolates from various parts of the Russian Federation was carried out. The new MALDI-TOF MS-based method may be used for rapid and accurate monitoring of the spread of drug resistance.**

Keywords: drug resistance, tuberculosis, SNPs, MALDI–TOF mass spectrometry

Introduction

Tuberculosis (TB) still represents a serious health problem in several parts of the world. In Russia, TB morbidity was about 80 cases per 100 000 of population in 2004. Although TB morbidity decreased slightly in recent years, the situation remains alarming due to a major increase in the incidence and prevalence of multidrug-resistant tuberculosis (MDR-TB). The MDR phenotype is defined as resistance at least to isoniazid and rifampicin— the two most effective drugs recommended by the WHO for the first-line treatment of TB.[1–3] The prevalence of primary resistance to both of them reaches up to 45% in several regions

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of the Russian Federation.[4] As rifampicin and isoniazid are the first-line drugs recommended by the WHO for the treatment of TB,[3] resistance to both medications substantially increases the cost and duration of treatment, while decreasing the efficacy.

Susceptibility testing of *Mycobacterium tuberculosis* isolates by phenotypic methods requires a minimum of 14 days from a primary specimen. This can be reduced significantly if molecular analysis is used.[5]

At present, drug resistance-associated point mutations, deletions or insertions have been described for 11 genes of *M. tuberculosis*.[6]

Numerous studies have determined that up to 95% of rifampicin-resistant *M. tuberculosis* strains have point mutations within a small 81 bp hot-spot region, named the rifampicin resistance-determining region (RRDR). This region is located between codons 507 and 533 of the RNA polymerase β subunit gene (*rpoB*). The most common mutations (up to 86%) in this region are observed in Ser-531, His-526 and Asp-516 codons.[7]

Unlike rifampicin, resistance to isoniazid is related to several genes. As a rule, the isoniazid-resistant strains have mutations in the catalase-peroxidase (*katG*) gene.

The promoter of the *fabG1(mabA)–inhA* operon (*inhA* promoter) is the second significant region for isoniazid resistance. This operon includes two genes, *fabG1*(*mabA*) and *inhA*, coding for β-ketoacyl reductase and the fatty-acid enoyl–acyl carrier protein reductase involved in mycolic acid biosynthesis. Independent nucleotide substitutions in two positions of the promoter (−15T or −8C) lead to overexpression of the target for isoniazid active radical forms and, therefore, to isoniazid resistance. The role of the other genes, *kasA* and *ndh*, in isoniazid resistance is not completely resolved and needs further study.[6]

The aim of this study was to evaluate the prevalence of known mutations associated with resistance to rifampicin and isoniazid for 412 isolates of *M. tuberculosis*, collected in the Russian Federation during 1997–2005. Three genetic loci—*rpoB* and *katG* genes and the promoter region of the *fabG1(mabA)–inhA* operon—were analysed. We used a

Table 1. Internal oligonucleotide primers for primer extension reactions, conditions for reaction and identification of amino acid change by mass of reaction products

Gene	Primer name	Nucleotide sequence (5′–3′) MW (Da)	Mix dNTP/ ddNTP	Primer extension reaction program	Masses of reaction products (Da)
rpoB	Mt531f Mt533r	gacccacaagcgccgactg (5768) cagaccgccgggcccc (4814)	dATP, dTTP, dCTP, ddGTP	94°-20 s 59°-20 s 70 cycles 72°-20 s	6673, 5439 (Ser-531, Leu-533) 6688 (Ser-531→Leu) 6384 (Ser-531→Trp) 5126 (Leu-533→Pro)
	Mt526f Mt526r	gctgtcggggttgacc (4930) cgccgacagtcggcgcttg (5806)	dTTP, dGTP, ddATP, ddCTP	94°-20 s 53°-20 s 70 cycles 72°-20 s	5203, 7674 (His-526) 5531, 6407 (His-526→Tyr) 5227, 7649 (His-526→Asn) 5556, 6383 (His-526→Asp) 5203, 6079 (His-526→Arg) 5203, 7700 (His-526→Pro) 5203, 6102 (His-526→Leu)
	Mt516r1 Mt516r2 Mt516r3 Mt516r4	acagcgggttgttctg (4928) cccgacagcgggttgttctgc (6415) cccgacagcgggttgttctgcc (6704) cccgacagcgggttgttctgtc (6719)	dATP, dTTP, dGTP, ddCTP	94°-20 s 49°-20 s 70 cycles 72°-20 s	5834 (Asp-516) 5843 (Asp-516→Val) 5819 (Asp-516→Val) 5530 (Asp-516→Gly)
	Mt513f1 Mt513f2 Mt511f	ggcaccagccagctgagc (5494) ttcggcaccagccagctgagca (6705) gttcttcggcaccagccagc (6054)	dCTP, dTTP, dGTP, ddATP	94°-20 s 61°-20 s 70 cycles 72°-20 s	6080, 6984 (Gln-513, Leu511) 6369 (Gln-513→Pro) 6969 (Leu-511→Pro)
	Mt522f Mt514ins	atggaccagaacaacccgctgt (6714) ccagccagctgagccaattc (6047)	dATP, dCTP, ddTTP, ddGTP	94°-20 s 63°-20 s 70 cycles 72°-20 s	7315, 6648 (Ser-522, Phe-514) 6335 (514 ins Phe)
katG	kG315f1 kG315f2 kG315r	taaggacgcgatcacc (4876) ggtaaggacgcgattaccc (5823) catacgacctcgatgcct (5420)	dATP, dCTP, ddTTP, ddGTP	94°-20 s 50°-20 s 70 cycles 72°-20 s	5502, 5997 (Ser-315) 6104 (Ser-315→Asn) 5733 (Ser-315→Thr)
fabG1	MtfG15f MtfG8r	cccggccgcggcgaga (4849) gtggcagtcaccccgaca (5470)	dTTP, dGTP, ddATP, ddCTP	94°-20 s 56°-20 s 70 cycles 72°-20 s	5167, 5767 (−15C, −8T) 5824 (−15 C→T) 5849 (−8 T→C) 6047 (−8 T→A) 5743 (−8 T→G)

minisequencing reaction followed by matrix-assisted laser-desorption ionization–time-of-flight mass spectrometry (MALDI–TOF MS) of the reaction products for the detection of expected substitutions. This method was based on primer extension reaction to one, two or three nucleotides at the point of interest. Subsequent MALDI–TOF MS analysis of the reaction products allows determination of the nucleotide sequence in the investigated site. Previously, this approach was successfully used for hepatitis C virus genotyping[8] and for detection of fluoroquinolone resistance single nucleotide polymorphisms in *gyrA* and *parC* genes of *Neisseria gonorrhoeae*.[9]

Materials and methods

Bacterial isolates

The 412 isolates of *M. tuberculosis* from sputum of epidemiologically unlinked TB patients from central Russia (Moscow and Moscow region; $n = 98$), Western Siberian (Novosibirsk; $n = 154$) and Ural regions (Yekaterinburg; $n = 160$) were collected in a non-randomized way from the reference laboratories. Species identification and drug susceptibility testing were performed according to the WHO recommendation-based[10] Ministry of Health of Russian Federation protocols.[11] Rifampicin and isoniazid susceptibility tests were carried out by the absolute concentration method on Löwenstein–Jensen medium containing 40 mg/L rifampicin or 1 mg/L isoniazid, respectively. All the drugs were manufactured by Sigma, USA. The results of microbiological tests were recorded 21 days after the inoculation. Isolates were considered resistant in the case when more than 20 colonies grew on the drug-containing medium.

DNA isolation

DNA of *M. tuberculosis* was isolated using the 'DNA-express' kit (Lytech Ltd, Russia) in accordance with the manufacturer's instructions.

Sample preparation

DNA extracts were dispensed into 96-well PCR plates (Simport, Canada) at 3 μL per well by means of an automatic workstation Biomec 2000 (Becman Coulter Inc., USA). Prepared plates were stored at −20°C.

PCR amplification

The standard PCR was carried out in 10 μL of reaction mixture. The reaction mixture contained 66 mM Tris–HCl (pH 9.0), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 250 μM of each dNTP, 1 U of *Taq* DNA polymerase (Promega, USA) and 10 pmol of each primer. The 321 bp fragment of the *rpoB* gene (including the RRDR), the 168 bp fragment of the *katG* gene and the 320 bp fragment of the *inhA* promoter region were amplified with the following primers sets: MtrpoBf (5′-GAGGCGATCACCGCAG AC-3′) and MtrpoBr (5′-GGTACGGCGTTTCGATGAAC-3′); MtkatGf (5′-ACCCGAGGCTGCTCCGCTGG-3′) and MtkatGr (5′-CAGCTCCCACTCGTAGCCGT-3′) and MtfabGf (5′-GCCTCG CTGGCCCAGAAAGG-3′) and MtfabGr (5′-CTCCGGATCCACGG TGGGT-3′). All primers were designed using Oligo_6.31 software (Molecular Biology Insights Inc., USA) and sequences of *M. tuberculosis* H37Rv strain (GenBank accession number NC000962).

A programmed thermocycler TETRAD DNA ENGINE (MJ Research, Inc.) was used. A universal amplification profile was worked out for all three loci: the initial heating step was at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 61°C for 15 s and 72°C for 20 s and a final step at 72°C for 5 min.

The dephosphorylation step was carried out in the same 96-well reaction plates. The 5 μL reaction mixture contained 66 mM Tris–HCl (pH 9.0), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ and 0.5 U of shrimp alkaline phosphatase (Fermentas, Lithuania) and was added to each PCR product and incubated at 37°C for 20 min to dephosphorylate the 5′-end phosphate groups of deoxynucleoside

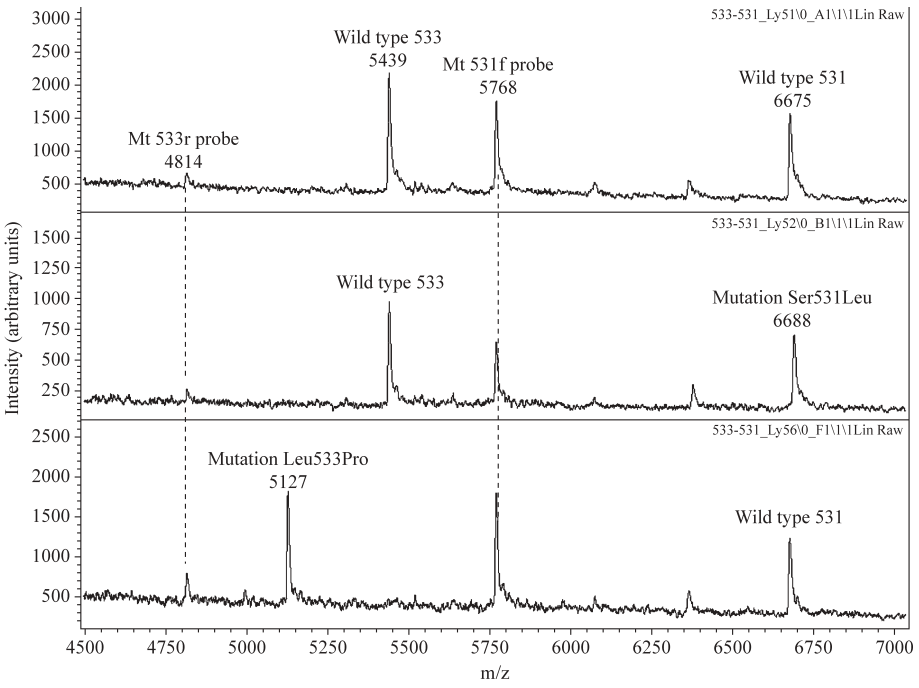

Figure 1. Mass spectra of products of the primer extension reaction. Analysis of the polymorphisms of codons 531 and 533.

triphosphates. At the end, the enzyme was deactivated by heating at 85°C for 10 min.

Primer extension reaction (minisequencing)

Primer extension reactions were carried out in the same 96-well PCR plate. The 7 μL of the reaction mixture contained 66 mM Tris–HCl (pH 9.0), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ and 2 U of TermiPol DNA polymerase (Solis Biodyne, Estonia), 20 pmol of oligonucleotide primer, 0.2 mM dNTP and 0.2 mM ddNTP, (in accordance with Table 1) were added to PCR products of *rpoB*, *katG* and *fabG1* genes. Thermocycling protocols were developed to optimize the output of the extended primers: generally, the protocol was 70 cycles of 94°C for 20 s, T_a for 20 s and 72°C for 15 s. Here, T_a is a temperature that is optimal for the working of all oligonucleotide primers in the reaction mixture (Table 1).

Products of the primer extension reaction were purified on a cation-exchange resin (SpectroClean Resin, Sequenom, USA). Resin (6–8 mg) was suspended in 15 μL of ultrapure water (Merck, Germany) and added into the reaction volume. Then, the contents of the test tubes were thoroughly mixed and incubated at room temperature for at least 1 h. After centrifugation for 5 min at 1000 rpm, the supernatant was used for MS analysis.

MS analysis

MS analysis was performed as described previously.[8] Briefly, the sample aliquot (0.2–1 μL) was spotted onto a matrix, which was preliminarily dried on the AnchorChip™ (Bruker Daltonics, Germany) 400 μm targets. The matrix employed was a saturated solution of 3-hydroxypicolinic acid (Fluka, Germany) in a 1:1 acetonitrile/water mixture (Merck) mixed with 0.4 M dibasic ammonium citrate (Fluka) in a 9:1 (v/v) ratio. All the solvents utilized were MS grade. Mass spectra were collected using the Reflex IV MALDI–TOF MS (Bruker Daltonics), which was operated in the linear mode. A 337 nm nitrogen laser with 9 Hz pulse frequency and mode of positive ions was used. MS parameters were optimized for the range of *m/z* values from 1000 to 10 000, using the mass spectra of peptides for calibration. Each mass spectrum was collected at 30 laser pulses at constant laser power and constant threshold value, in order to enhance the resolution.

Registration and analysis of the obtained spectra were carried out using XMASS and XT of v.1.1 (Bruker Daltonics) software.

Conclusions about the nucleotide composition of extended primers were inferred on the basis of the appearance of peaks of calculated *m/z* ratio in mass spectra. If the unforeseen peaks arise, we could determine the nucleotide sequence by calculating the mass difference between neighbouring lines in spectra (Figure 1).

DNA sequencing of rpoB, katG and fabG1 gene PCR fragments

Cycle sequencing reactions were performed using an ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, USA; Hitachi, Japan) according to the manufacturers' instructions.

Results

Susceptibility testing

Among 412 *M. tuberculosis* isolates included in the study, 260 (63.1%) were identified as MDR, (i.e. resistant to both rifampicin and isoniazid), 84 (20.4%) were susceptible to rifampicin and isoniazid and 11 (2.7%) and 57 (13.8%) isolates were resistant, respectively, to either rifampicin or isoniazid.

Table 2. Distribution of mutations seen in 412 isolates of *M. tuberculosis* from three regions of the Russian Federation

Locus	Nucleotide change	Amino acid change	No. of isolates (%)
Mutation in RRDR of *rpoB* gene			
533	CTG→CCG	Leu→Pro	4 (1)
531	TCG→TTG	Ser→Leu	171 (41.5)
	TCG→TGG	Ser→Trp	5 (1.2)
526	CAC→TAC	His→Tyr	6 (1.5)
	CAC→AAC	His→Asn	3 (0.75)
	CAC→GAC	His→Asp	10 (2.4)
	CAC→CGC	His→Arg	8 (2)
	CAC→CTC	His→Leu	1 (0.25)
516	GAC→GTC	Asp→Val	18 (4.4)
	GAC→GTA	Asp→Val	1 (0.25)
	GAC→GGC	Asp→Gly	1 (0.25)
514	ins TTC	ins Phe	2 (0.5)
513	CAA→CCA	Gln→Pro	1 (0.25)
511	CTG→CCG	Leu→Pro	2 (0.5)
531 and 526	TCG→TTG and CAC→TAC	Ser→Leu and His→Tyr	4 (1)
	TCG→TTG and CAC→GAC	Ser→Leu and His→Asp	1 (0.25)
	TCG→TTG and CAC→CGC	Ser→Leu and His→Arg	1 (0.25)
	TCG→TGG and CAC→CTC	Ser→Trp and His→Leu	1 (0.25)
516 and 511	GAC→GGC and CTG→CCG	Asp→Gly and Leu→Pro	1 (0.25)
Wild-type			171 (41.5)
Total			412
Mutations in codon 315 of *katG* gene and *inhA* promoter region			
KatG315	AGC→ACC	Ser→Thr	243 (59)
	AGC→ACA	Ser→Thr	1 (0.25)
KatG315 and *inhA*	AGC→ACC and −15C→T	Ser→Thr	41 (9.9)
	AGC→ACC and −8 T→C	Ser→Thr	2 (0.5)
	AGC—ACC and −8T→A	Ser→Thr	1 (0.25)
inhA	−15 C→T		3 (0.75)
Wild-type			121 (29.35)
Total			412

Detection of mutations in the rpoB gene

Mutations in the RRDR of the *rpoB* gene were detected in 241 (88.9%) of 271 rifampicin-resistant *M. tuberculosis* isolates. Thirteen different types of mutations were identified in Leu-533, Ser-531, His-526, Asp-516, Gln-513 and Leu-511 codons and one variant insertion between codons 514 and 515.

No substitutions in Ser-522 were detected for *M. tuberculosis* isolates investigated. Mutation TCG→TTG (Ser→Leu) in codon 531 was the most prevalent; it was detected in 171 (41.5% of total number) isolates. Five different types of point nucleotide substitutions were found in the His-526 codon of 28 (6.8%) isolates. Twenty (4.85%) isolates revealed three variants of mismatches in Asp-516. Four (1%), one (0.25%) and two (0.5%) isolates carried mutations in Leu-533, Gln-513 and Leu-511 codons, respectively. Insertion Phe-514a was found for two *M. tuberculosis* isolates.

Eight isolates carried point mutations in two separated codons: Ser-531 and His-526 (seven isolates) and Asp-516 and Leu-511 (one isolate).

Detection of mutation in the Ser-315 codon of the katG gene and in the inhA promoter

Substitutions in the Ser-315 codon of *katG* and/or the *inhA* promoter region were found in 291 (91.7%) of 317 isoniazid-resistant *M. tuberculosis* isolates. Most of the *M. tuberculosis* isolates—243 (59%)—had mutation Ser-315→Thr (AGC→ACC). Mutation Ser-513→Thr, caused by double nucleotide alteration AGC→ACA, was revealed in one isolate only.

Forty-seven (11.4%) isolates had point nucleotide mutations in the −15 and −8 positions of the *inhA* promoter region. In 44 of them, substitutions in Ser-315 of *katG* and the *inhA* promoter were detected.

In one isolate, a new substitution (T→C) in the −8 position of the *inhA* promoter region was found.

Sequencing of gene fragments

Direct DNA sequencing of gene fragments obtained as a result of PCR was carried out for one hundred isolates and confirmed perfectly the results of minisequencing analysis.

All genetic variants revealed in the present study are shown in Table 2.

Discussion

Comparative analysis of genotypes and phenotypes of *M. tuberculosis* isolates is shown in Table 3. Resistance to rifampicin was correctly identified by MALDI–TOF MS analysis in 90.4% of MDR isolates and in 54.5% of isolates demonstrating isolated phenotypical resistance to this antibiotic; no mutations were detected for phenotypically susceptible isolates.

No substitutions in the studied loci of RRDR were observed for 29 (11.1%) rifampicin-resistant *M. tuberculosis* isolates. It has been previously observed that ~10% of rifampicin-resistant strains do not have any mutations in the RRDR.[7]

For isoniazid, resistance was predicted correctly in 94.25% of MDR isolates and in 80.7% of isolates with isolated phenotypical resistance to isoniazid. Twenty-six (8.2%) isoniazid-resistant isolates had no detectable changes in the two targets analysed.

The sequencing procedure of the RRDR of the *rpoB* gene, fragments of the *katG* gene and the *inhA* promoter region from isolates that had a resistant phenotype, but had no mutations in the studied loci, confirmed the results of minisequencing analysis and confirmed the absence of mutations in the 81 bp RRDR of *rpoB*, fragments of the *katG* gene and the *inhA* promoter. Probably, in these cases, resistance might be concerned with changes in other loci of the *M. tuberculosis* genome.

The codons most frequently affected by point mutations were Ser-531, His-526 and Asp-516, with frequencies of 64.8%, 10.3% and 7.7%, respectively, in agreement with previous data.[12–14] Mutations in the Leu-533 codon were revealed for four rifampicin-resistant isolates. The wild-type Leu-511 was altered in two isolates, and the Gln-513 codon was altered in one isolate.

Contrary to our data, Toungoussova *et al.*[15] detected mutations in codon Gln-513 more frequently, for 10% of rifampicin-resistant isolates. The insertion Phe-514a (TTC) was identified for two isolates. Eight (3.3%) rifampicin-resistant isolates had point mutations in two separate codons.

Most isoniazid-resistant isolates—244 (76.97%)—contained nucleotide substitutions in the Ser-315 codon of the *katG* gene. This agrees with the fact that the frequency of mutations in this codon is 93% for isoniazid-resistant strains in the Russian Federation.[12,14] Only three isoniazid-resistant isolates had changes in the *inhA* promoter and an intact Ser-315 codon.

Mutations in two loci (*katG* and *inhA* promoter) were found simultaneously in 44 (13.88%) samples.

Table 3. Comparison of genotype analysis results for MDR isolates with equivalent phenotypic results

Phenotype[a]	Genotype[b] [no. (%)]							
	$rpoB_{mut}$	$rpoB_{mut}$ $katG_{mut}$	$rpoB_{mut}$ $katG_{mut}$ $inhA_{mut}$	$katG_{mut}$ $inhA_{mut}$	$katG_{mut}$	$rpoB_{mut}$ $inhA_{mut}$	$inhA_{mut}$	WT
RIFrINHr ($n = 260$)	5 (1.9)	195 (75)	34 (13.1)	4 (1.5)	10 (3.85)	1 (0.4)	1 (0.4)	10 (3.85)
RIFrINHs ($n = 11$)	6 (54.5)	0	0	0	0	0	0	5 (45.5)
RIFsINHr ($n = 57$)	0	0	0	6 (10.5)	39 (68.4)	0	1 (1.8)	11 (19.3)
RIFsINHs ($n = 84$)	0	0	0	0	0	0	0	84 (100)

WT, wild-type.

[a]RIFrINHr, resistant to rifampicin and resistant to isoniazid; RIFrINHs, resistant to rifampicin and susceptible to isoniazid; RIFsINHr, susceptible to rifampicin and resistant to isoniazid; RIFsINHs, susceptible to rifampicin and susceptible to isoniazid.

[b]$rpoB_{mut}$, mutations in the RRDR region of the *rpoB* gene; $katG_{mut}$, mutation in codon 315 of the *katG* gene; $inhA_{mut}$, mutations in the −15 or −8 positions of the *inhA* promoter region.

Table 4. Frequency of mutation in rifampicin-resistant and isoniazid-resistant *M. tuberculosis* isolates from different regions of the Russian Federation

Region (total number of isolates)	No. (%) resistant isolates with mutations																				
	Mutations in RRDR of rpoB																Mutations in codon 315 of the *katG* gene and the *inhA* promoter					
	CTG-533→CCG (Leu→Pro)	TCG-531→TTG (Ser→Leu)	TCG-531→TGG (Ser→Trp)	CAC-526→TAC (His→Tyr)	CAC-526→AAC (His→Asn)	CAC-526→GAC (His→Asp)	CAC-526→CGC (His→Arg)	CAC-526→CTC (His→Leu)	GAC-516→GTC (Asp→Val)	GAC-516→GTA (Asp→Val)	GAC-516→GGC (Asp→Gly)	514 ins TTC (ins Phe)	CAA-513→CCA (Gln→Pro)	CTG-511→CCG (Leu→Pro)	Mutations in two Separated codons	No mutation in Study loci	AGC-*katG*315→AAC (Ser→Thr)	AGC-*katG*315→ACA (Ser→Asn)	AGC-*katG*315→ACC + *inhA* C(−15)T	*inhA* C(−15)T	Other	No mutation in Study loci
Central region (98)	3 (3.8)	45 (57)	3 (3.8)	2 (2.5)	2 (2.5)	0	4 (5)	1 (1.3)	7 (8.8)	0	0	0	1 (1.2)	0	3 (3.8)	8 (10.1)	54 (66.6)	0	20 (24.7)	2 (2.5)	0	5 (6.1)
Ural region (160)	0	77 (70)	0	2 (1.8)	1 (0.9)	4 (3.6)	2 (1.8)	0	7 (6.3)	0	0	2 (1.8)	0	1 (0.9)	5 (4.5)	9 (8.1)	113 (85.6)	1 (0.75)	12 (9.1)	0	2 (1.5)	4 (3)
West Siberian region (154)	1 (1.2)	49 (59.7)	2 (2.4)	2 (2.4)	0	6 (7.3)	2 (2.4)	0	4 (4.9)	1 (1.2)	1 (1.2)	0	0	1 (1.2)	1 (1.2)	12 (14.6)	76 (73)	0	9 (8.4)	1 (1)	1 (1)	17 (16.3)

Previously, it was noted that the structure and frequency of occurrence of mutant codons for *M. tuberculosis* varied for different regions of the world.[16,17] By comparison of the presented data with those in the literature, it was observed that high prevalence mutations in Ser-531 also occurred in strains from Germany (75.7%),[18] Peru (68.1%)[19] and Italy (63.3%).[20] His-526 mutations are predominantly observed in the USA (44%)[21] and China (40%)[22] and Asp-516 in Hungary (37.9%),[23] whereas in our samples, the frequency of these mutations was much lower.

Mutations in Ser-315 of *katG* also have geographical variations, for example, they were not detected in Equatorial Guinea[24] but were detected in a substantial 93.6% of isolates from Northwest Russia.[25] Moreover, in our samples, only three (~1%) isoniazid-resistant strains had isolated *inhA* promoter mutations, whereas in some world regions, prevalence of these mutations can reach up to 80%.[24]

In our work, we made an attempt to compare groups of drug-resistant isolates among themselves, using their mutation profiles. The spread of resistance to rifampicin and isoniazid was analysed for Central, West Siberian and Ural regions of the Russian Federation. The 79, 82 and 110 rifampicin-resistant isolates and 81, 104 and 132 isoniazid-resistant isolates were collected from these three regions, respectively. It is significant that previously[12,13,25,26] mutation profiles of resistant *M. tuberculosis* strains were studied mainly for the European part of the Russian Federation (Moscow and Moscow Oblast, Samara Oblast, Archangel Oblast, St Petersburg etc.); strains from the Eastern part (Ural, Siberian and War West) were poorly investigated.

Comparison analysis reveals that strains circulating in different geographical regions possess different mutation profiles (Table 4). However, future monitoring studies are needed to confirm the reliability of observed differences for evaluation of the epidemiological features of circulating drug-resistant strains. It is evident that the frequency and type of mutations in one country or geographical region are not applicable more generally.

In the present study, molecular analysis of the loci of *rpoB* and *katG* genes and the *inhA* promoter region of 412 *M. tuberculosis* clinical isolates from various parts of the Russian Federation was carried out. Unfortunately, mutations which lead to high-level resistance were prevalent for all regions from where isolates were collected.

For *M. tuberculosis* mutation identification, a new method by means of a minisequencing approach based on MALDI–TOF MS was used. We succeeded in realization of the so-called 'one-tube' technique, when all analysis steps are implemented in one reaction tube, without transferring material between tubes. It decreases the probability of error and chances of cross-contamination. Furthermore, the cost of analysis can be compared practically with the cost of two standard PCR reactions, but the accuracy is similar to direct sequencing of gene fragments. Execution time for the analysis is several hours (but is possible within a working day).

Thus, this method may be interesting and useful for rapid and accurate monitoring of the spread of drug resistance. At the same time, this approach may be suitable for timely correction of antituberculosis treatment in accordance with the mutation profile of *M. tuberculosis* strains. This should help avoid the generation and transmission of drug-resistant strains in the community.

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Transparency declarations

None to declare.

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