THE NATURE OF GENOTYPIC RESISTANCE TO FLUOROQUINOLONES IN *MYCOBACTERIUM TUBERCULOSIS* CIRCULATING IN RUSSIAN FEDERATION

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Fluoroquinolones are the main group of drugs used for treatment of multidrug resistant tuberculosis (MDR-TB). The study was aimed to assess the diversity of mutation in the *gyrA* gene and to evaluate the association of *gyrA* mutations with the phenotypic resistance to levofloxacin and the general drug resistance profile of the pathogen. The study involved assessment of diagnostic materials obtained from 2836 patients with pulmonary tuberculosis. TB-BIOCHIP-2 and Amplitube-FQ-RV kits were used for identification of the *gyrA* mutations. Phenotypic drug susceptibility of *M. tuberculosis* (MTB) was defined using the BACTEC MGIT 960 test system. It was shown that mutations D94G (41.63%; 95% CI: 38.03–45.32%) and A90V (21.32%; 95% CI: 18.44–24.50%) prevailed in MTB, although some isolates carrying these mutations were obtained from the newly diagnosed patients with pulmonary tuberculosis. It was found that mutation D94A was not strongly associated with the phenotypic resistance to fluoroquinolones. Fluoroquinolone resistance was usually associated with multiple drug resistance (93.52%; 95% CI 91.43–95.12%). In 2.31% (95% CI 1.78–3.00%) of cases, genotypic heteroresistance to fluoroquinolones was detected: mixed populations included 2–4 MTB pools with various structure of the *gyrA* QRDR. The results obtained lead to the conclusion that resistance to fluoroquinolones that is usually associated with the existing MDR arises in the modern MTB population. MTB carrying *gyrA* mutations D94G and A90V seems to be the most promising in evolutionary terms.

Keywords: *M. tuberculosis*, fluoroquinolones, resistance, *gyrA*, mutations, preXDR tuberculosis

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ОСОБЕННОСТИ ГЕНОТИПИЧЕСКОЙ РЕЗИСТЕНТНОСТИ К ФТОРХИНОЛОНАМ У *MYCOBACTERIUM TUBERCULOSIS*, ЦИРКУЛИРУЮЩИХ В РОССИЙСКОЙ ФЕДЕРАЦИИ

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Фторхинолоны — основная группа препаратов, применяемых для лечения туберкулеза с множественной лекарственной устойчивостью (МЛУ-ТБ). Целью исследования было оценить разнообразие мутаций в гене *gyrA*, а также установить ассоциацию мутаций в *gyrA* с фенотипической устойчивостью к левофлоксацину и общим профилем лекарственной устойчивости возбудителя. Исследование проведено на диагностическом материале от 2836 больных туберкулезом легких. Для определения мутаций в *gyrA* использовали наборы «ТБ-БИОЧИП-2» или «Амплитуб-FQ-РВ». Фенотипическую лекарственную чувствительность *M. tuberculosis* (МБТ) определяли в системе BACTEC MGIT 960. Показано, что у МБТ доминировали мутации D94G (41,63%; 95%ДИ: 38,03–45,32%) и A90V (21,32%; 95%ДИ: 18,44–24,50%), причем изоляты с этими мутациями были получены в том числе и от впервые выявленных больных туберкулезом легких. Установлено, что мутация D94A не являлась строго ассоциированной с фенотипической устойчивостью к фторхинолонам. Устойчивость к фторхинолонам, как правило, была ассоциирована с множественной лекарственной устойчивостью (93,52%; 95%ДИ 91,43–95,12%). В 2,31% (95%ДИ 1,78–3,00%) случаев выявлена генотипическая гетерорезистентность к фторхинолонам: смешанные популяции включали 2–4 пула МБТ с разной структурой QRDR *gyrA*. На основании полученных результатов можно заключить, что в современной популяции МБТ происходит формирование устойчивости к фторхинолонам, как правило, на фоне уже имеющейся МЛУ. Наиболее перспективными в эволюционном плане представляются МБТ с мутациями в *gyrA* D94G и A90V.

Ключевые слова: *M. tuberculosis*, фторхинолоны, устойчивость, *gyrA*, мутации, преШЛУ туберкулез

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The spread of tuberculosis caused by drug resistant pathogen is a major public health concern. Particularly worrisome is the wide spread of multidrug-resistant tuberculosis (MDR-TB), i.e. resistant to both of two most effective antituberculosis drugs, rifampicin and isoniazid. According to the WHO, the effectiveness of MDR-TB therapy is only 59% [1]. Russia is among the countries with a high burden of MDR-TB. Despite the fact that the prevalence of MDR-TB in the country has started to decline in recent years (from 20.6 per 100,000 population in 2020 to 18.1 per 100,000 population in 2021), the rate is still high [1, 2].

Regimens for treatment of MDR-TB necessarily include fluoroquinolones (group A drugs according to the WHO classification reflecting priorities of the drug inclusion in the treatment regimens) [3]. DNA gyrase, the enzyme essential for replication and transcription in the *M. tuberculosis* (MTB)

cell, is a target of fluoroquinolones [4, 5]. In 60–90% of cases, fluoroquinolone resistance is associated with mutations in the quinolone resistance-determining region (QRDR) of the gyrA gene encoding the DNA gyrase α-subunit [6, 7]. The TBDreamDB database (http://www.tbdreamdb.com) contains information about 17 variants of QRDR mutations associated with fluoroquinolone resistance, among which 10 variants show high reliability in terms of resistance to this group of medications [8].

The development of additional resistance to fluoroquinolones by the MDR MTB results in pre-extensively drug resistant tuberculosis (pre-XDR TB), the treatment of which requires expensive long-term chemotherapy. The WHO estimates that up to 20% of tuberculosis cases in 105 countries fall in this category. To improve the effectiveness of pre-XDR TB treatment it is necessary to adjust the course of chemotherapy based on the fluoroquinolone susceptibility defined by molecular genetic methods as early as possible. However, according to the WHO, the global coverage of the fluoroquinolone susceptibility testing is still low: it accounts for 50% of the identified worldwide cases of tuberculosis [1]. Two domestic test systems for rapid detection of MTB susceptibility to fluoroquinolones are used in Russia. The first one, TB-BIOCHIP-2 (Biochip-IMB; Russia), includes biochips that detect 10 variants of the QRDR spot mutation. The second one, Amplitube-FQ-RV (Syntol; Russia) based on the allele-specific PCR, detects six mutations in the *gyrA* QRDR.

The study was aimed to assess the diversity of mutation in the *gyrA* gene and to evaluate the association of *gyrA* mutations with phenotypic resistance to levofloxacin and the general drug resistance profile of the pathogen.

METHODS

Research object

Diagnostic materials obtained from patients of all age groups admitted to the diagnostic and clinical departments of the Central Tuberculosis Research Institute in 2011–2019 were assessed.

Study design

Retrospective analysis of the data on mutations in the *gyrA* QRDR and MTB phenotypic drug resistance obtained within 9 years (2011–2019) from patients with pulmonary tuberculosis, who were treated in the Central Tuberculosis Research Institute, was conducted. Diagnostic materials were assessed in accordance with the standard algorithm accepted by Microbiology Department of the Central Tuberculosis Research Institute: each sample of diagnostic material was simultaneously assessed by the culture-based and molecular genetic methods. Diagnostic materials were decontaminated using the standard procedure, then the MGIT tubes were inoculated for further growth in the BACTEC MGIT 960 system [9]. DNA was isolated from the amount of diagnostic material remaining after inoculation, and PCR was conducted to detect the MTB DNA. Upon receipt of a positive PCR result, mutations in the genes associated with resistance to rifampicin, isoniazid, and fluoroquinolones were identified using biochips or allele-specific PCR. The MTB culture obtained was tested for susceptibility to eight antituberculosis drugs.

DNA extraction

DNA was extracted from diagnostic materials using the Amplitube-RV reagent kit for isolation, detection and quantification of the *Mycobacterium tuberculosis* complex DNA by real-time PCR, kit № 1 (Syntol; Russia) according to the instructions.

Identification of MTB DNA

To detect the MTB DNA, PCR was conducted using the Amplitube-RV reagent kit for isolation, detection and quantification of the *Mycobacterium tuberculosis* complex DNA by real-time PCR, kit № 2 (Syntol; Russia) according to the instructions. Amplification was performed in the CFX96 thermal cycler with optical module (Bio-Rad; USA).

Genotypic resistance to rifampicin and isoniazid

The test was performed either by the microchip technique using the TB-BIOCHIP-2 kit (Biochip-IMB; Russia), or using the Amplitube-MDR-RV kit (Syntol; Russia). Both procedures were conducted in accordance with the manufacturers' instructions.

Genotypic resistance to fluoroquinolones

The samples obtained in 2011–2015 were tested by the microchip technique using the TB-BIOCHIP-2 kit (Biochip-IMB; Russia), while the samples obtained in 2015–2019 were tested using the Amplitube-FQ-RV kit (Syntol; Russia). Both procedures were conducted in accordance with the manufacturers' instructions. In case the tests conducted during various stages of therapy were available for one patient or different diagnostic materials (for example, sputum and excision material) obtained from one patient were tested, the results of testing each of the samples for *gyrA* mutations were compared.

Culture-based diagnosis

MTB was detected in the Middlebrook 7H9 liquid growth medium with the BACTEC MGIT 960 system (BD; USA) in accordance with the standard protocol developed by the manufacturer [9].

Phenotypic drug resistance

The test for susceptibility to eight antituberculosis drugs (rifampicin, isoniazid, ethambutol, pyrazinamide, ethionamide, amikacin, capreomycin, and levofloxacin) was performed by the modified proportion testing method in the BACTEC MGIT 960 system (BD; USA) in accordance with the manufacturer's guidelines [9, 10].

Methods of statistical analysis

Descriptive statistics was used to assess the results: number of observations, frequency, share (percentage), and 95% confidence interval (95% CI) were taken into account. Chi-squared test (χ^2) was used for intergroup comparison. The differences were considered significant at $p < 0.05$. Analysis was performed using Microsoft Excel (Microsoft; USA).

RESULTS

In 2011–2019, microbiological diagnosis of tuberculosis was performed in 4451 patients. The study involved materials obtained from 2836 patients with positive MTB DNA PCR test results. Of them in 2082 cases (73.41%, 95% CI: 71.76–75.01%) no *gyrA* mutations were detected by molecular genetic methods (hereinafter, the wild type *gyrA*).

Table 1. Frequency of single *gyrA* mutations in the total number of strains with mutations in *gyrA* (*n* = 699)

Codon of the *gyrA* QRDR	Amino acid substitution	Frequency, No. (%)	95% CI
88	G → C	1 (0.14)	0.03–0.81
90	A → V	149 (21.32)	18.44–24.50
91	S → P	53 (7.58)	5.84–9.78
94	D → A	102 (14.59)	12.17–17.40
	D → N	54 (7.73)	5.97–9.94
	D → G	291 (41.63)	38.03–45.32
	D → H	11 (1.57)	0.88–2.80
	D → Y	38 (5.44)	3.99–7.37

MTB with single *gyrA* mutations were isolated from 699 patients (24.65%, 95% CI: 23.10–26.27%). In MTB isolated from 55 patients (2.31%, 95% CI: 1.78–3.00%), the results of determining genotypic resistance to fluoroquinolones changed over time or according to the diagnostic material type. These cases designated as heteroresistance will be described in detail below.

Frequency of single mutations in the *gyrA* QRDR

Single mutations detected in the *gyrA* QRDR were located in codons 88, 90, 91 or 94 (Table 1). Mutations were most often found in codon 94 of the gene (496/699, 70.96%; 95% CI: 67.49–74.20%). These were represented by five single-nucleotide variants, among which a D94G substitution was the most common (291/496, 58.67%; 95% CI: 54.29–62.92% among mutations in codon 94 and 291/699, 41.63%; 95% CI: 38.03–45.32% among MTB with single *gyrA* mutations). The A90V substitution was the second most frequent mutation (149/699, 21.32%; 95% CI: 18.44–24.50%). In total, MTB carrying mutations D94G and A90V accounted for more than half of all cases of MTB carrying single *gyrA* mutations (440/699, 62.95%; 95% CI: 59.31–66.45%).

Phenotypic susceptibility to levofloxacin

Phenotypic susceptibility to levofloxacin was defined for MTB isolated from diagnostic materials obtained from 1326 patients by the culture-based method. Testing of these MTB isolates for *gyrA* mutations revealed MTB with the wild type *gyrA* in 846 cases and MTB carrying single *gyrA* mutations in 480 cases. MTB with the wild type *gyrA* were largely susceptible to levofloxacin (814/846, 96.22%, 95% CI: 94.71–97.31%), while MTB carrying mutations usually were levofloxacin-resistant (448/480, 93.33%, 95% CI: 90.74–95.24%) (Table 2).

Polymorphism of MTB variants with mutant *gyrA* isolated in the new cases and previously treated patients with tuberculosis

Among 2836 patients with pulmonary tuberculosis, whose diagnostic materials were included in the study, there were 1253 new cases and 767 previously treated ones. No information about the status of another 816 patients was available.

MTB with the wild type *gyrA* were most often isolated in the new cases than in the previously treated ones: among 1475 isolates of MTB with the wild type *gyrA* obtained from patients with known status, 1012 (68.61%) were obtained in the new cases and 463 (31.39%) were isolated in the previously treated ones (p-value ≤ 0.001). The *gyrA* mutant variant A90V was significantly more common in the group of MTB isolated from the new cases; no significant differences were revealed for other mutant variants (Table 3).

gyrA mutations in MTB showing resistance of different types

MTB isolates with known genotypic susceptibility to fluoroquinolones were divided into five categories based on the resistance type (cases of heteroresistance were not included in the analysis): MDR MTB fell into the first category, polyresistant MTB (resistant to all combinations of antituberculosis drugs except the combination of rifampicin and isoniazid) fell into the

Table 2. MTB isolates with various *gyrA* QRDR structure showing phenotypic resistance to levofloxacin (*n* = 1326)

Mutation in QRDR *gyrA*	Strains with phenotypic resistance to levofloxacine	
	No. (%)	95% CI
G88C (*n* = 1)	1 (100)	20.65–100.00
A90V (*n* = 97)	95 (97.94)	92.79–99.43
S91P (*n* = 33)	32 (96.97)	84.68–99.46
D94A (*n* = 73)	49 (67.12)	55.73–76.81
D94N (*n* = 39)	39 (100)	91.03–100.00
D94G (*n* = 204)	200 (98.04)	95.07–99.23
D94H (*n* = 6)	6 (100)	60.97–100.00
D94Y (*n* = 27)	26 (96.30)	81.72–99.34
Total number of mutants (*n* = 480)*	448 (93.33)	90.74–95.24
WT (*n* = 846)	32 (3.78)	2.69–5.29

Note: WT — wild type *gyrA*; * — only MTB isolates with known phenotypic resistance to fluoroquinolones are taken into account.

Table 3. Abundance of the *gyrA* mutant MTB variants in the groups of the new cases and retreatment cases with tuberculosis

Patient category	Number of isolates carrying mutations, No. (%)							
	G88C	A90V	S91P	D94A	D94N	D94G	D94H	D94Y
NC (*n* = 239)	0 (0.00)	60 (25.10)	12 (5.02)	30 (12.55)	21 (8.79)	110 (46.03)	0 (0.00)	6 (2.51)
RC (*n* = 264)	1 (0.38)	44 (16.67)	25 (9.47)	41 (15.53)	16 (6.06)	117 (44.32)	6 (2.27)	14 (5.30)
P-value	0.341	0.038	0.066	0.375	0.26	0.776	0.02	0.117

Note: NC — new cases; RC — retreatment cases.

second, fluoroquinolone-monoresistant MTB fell into the third, MTB monoresistant to other antituberculosis drugs fell into the fourth, and MTB susceptible to all antituberculosis drugs fell into the fifth one (Table 4). MTB carrying *gyrA* mutations usually showed MDR or polyresistance (in total, 689/694, 99.28%, 95% CI: 98.32–99.69%). Fluoroquinolone-monoresistance was extremely rare (5/694, 0.72%, 95% CI: 0.31–1.68); in four cases, such MTB carried *gyrA* mutation D94A, and in one case mutation D94G was found. MTB with the wild type *gyrA* were almost equally divided between the categories of MDR MTB and MTB susceptible to all antituberculosis drugs.

Heteroresistance and multiple mutations

The listed above MTB isolates showed the same structure of the *gyrA* QRDR (wild type or single mutation) in all samples obtained from the same patient over time. However, the data on the *gyrA* QRDR structure obtained by dynamic monitoring of 55 patients differed (Table 5).

Thus, when assessing diagnostic materials during chemotherapy, MTB with varying structure of the *gyrA* QRDR were isolated from 35 patients. Of them in 22 cases both MTB with the wild type *gyrA* and MTB carrying mutations were revealed in the samples obtained from one patient. The wild type *gyrA* and *gyrA* with single mutations (mostly D94G) were found in MTB isolated from 15 patients (Table 5, item 1.1.1). MTB with the wild type *gyrA* and *gyrA* carrying multiple mutations were isolated from seven patients (Table 5, item 1.1.2). In three out of these seven cases, co-existence of two pools of MTB carrying single mutations instead of the existence of one pool with the double *gyrA* mutation was proven, since the samples with single mutations were also isolated over time. In four out of seven cases, the existence of two pools with single mutations or one pool with the double *gyrA* mutation was not proven.

On different dates samples were obtained from 13 patients, from which MTB carrying various single mutations were isolated (eight out of 13), or among which samples carrying double or single mutations alternated (five out of 13). This could indicate that there were several MTB pools with mutant *gyrA* in the patient's body (Table 5, item 1.2).

In five cases, heteroresistance was revealed when checking exceptions of the tests for phenotypic and genotypic resistance: MTB DNA carrying *gyrA* mutations was obtained from the diagnostic sample, while MTB culture obtained from the sample showed phenotypic susceptibility to levofloxacin, or vice versa. In such a case the available DNA samples were repeatedly (up to eight times) tested for *gyrA* mutations, and fresh DNA was isolated from the diagnostic sample and tested for *gyrA* mutations. The data both consistent with the initial results and different from these results were obtained in each of these five cases in a series of tests for *gyrA* mutations. This could somehow prove the presence of the MTB mixed population in one diagnostic sample (Table 5, item 2).

In another 15 cases, we detected double mutations in one sample (only one sample per patient was available for testing) (Table 5, item 3). It was usually one of the most common mutations (D94G or A90V) combined with one rare mutation (nine cases out of 15). However, in five cases out of 15, two rare mutations were simultaneously detected: it was the S91P + D94A combination only. MTB carrying two most common mutations (D94G and A90V) were isolated from only one patient. In all 15 cases, another PCR test of DNA isolated from the diagnostic sample revealed two mutations again. This indicated that either one MTB pool with the genomes containing double *gyrA* mutations, or two MTB pools carrying single *gyrA* mutations and represented in equal proportions were isolated from one patient. In these cases, diagnostic materials were collected from patients only once, that is why

Table 4. MTB isolates with various resistance profiles and various structure of *gyrA**

Mutation	Resistance profile, No. (%; 95% CI)				
	MDR	Poly	Mono to FQ	Mono to other ATBD	sens
G88C (*n* = 1)	1 (100; 20.65–100)	–	–	–	–
A90V (*n* = 149)	140 (93.96; 88.92–96.79)	9 (6.04; 3.21–11.08)	–	–	–
S91P (*n* = 53)	52 (98.11; 90.06–99.67)	1 (1.89; 0.33–9.94)	–	–	–
D94A (*n* = 102)	92 (90.20; 82.89–94.59)	6 (5.88; 2.72–12.24)	4 (3.92; 1.54–9.65)	–	–
D94N (*n* = 54)	49 (90.74; 80.09–95.98)	5 (9.26; 4.02–19.91)	–	–	–
D94G (*n* = 287)	270 (94.08; 90.72–96.27)	16 (5.57; 3.46–8.86)	1 (0.35; 0.06–1.95)	–	–
D94H (*n* = 10)	10 (100.00; 72.25–100)	–	–	–	–
D94Y (*n* = 38)	35 (92.11; 79.20–97.28)	3 (7.89; 2.72–20.80)	–	–	–
Total, with single mutations (*n* = 694)	649 (93.52; 91.43–95.12)	40 (5.76; 4.26–7.75)	5 (0.72; 0.31–1.68)	–	–
WT (*n* = 1412)	779 (55.17; 52.57–57.75)	–	–	54 (3.82; 2.94–4.96)	579 (41.01; 38.47–43.59)
Total (*n* = 2106)	1428 (67.81; 65.78–69.77)	40 (1.90; 1.40–2.58)	5 (0.24; 0.10–0.55)	54 (2.56; 1.97–3.33)	579 (27.49; 25.63–29.44)

Note: MDR — multiple drug resistance; Poly — polyresistance; Mono — monoresistance; FQ — fluoroquinolones; ATBD — antituberculosis drugs; sens — sensitive to ATBD; WT — wild type *gyrA*; * — only samples with known resistance type were included in the analysis.

Table 5. Heteroresistance to fluoroquinolones

Description	Number (abs)	Patient category			Nature of resistance to ATBD			
		NC	RC	Undefined	MDR	Poly	Mono FQ	N/D
1. Different structure of the *gyrA* QRDR in various samples obtained from one patient, such as:	35	1	27	7	31	1	2	1
1.1 Sequential isolation of MTB with the WT *gyrA* and *gyrA* mutations from various samples, including	22	1	18	3	18	1	2	1
1.1.1 WT + single mutations (2 pools):	15	1	11	3	12	1	1	1
WT + D94G	9	–	8	1	7	1	–	1
WT + A90V	4	1	2	1	3	–	1	–
WT + D94N	1	–	–	1	1	–	–	–
WT + D94Y	1	–	1	–	1	–	–	–
1.1.2 WT + multiple mutations	7	–	7		6	–	1	–
1.1.2.1 (3 pools)	5	–	5		4	–	1	–
WT + D94G + S91P	1	–	1			–	1	–
WT + D94G + A90V	1	–	1		1	–	–	–
WT+D94G+D94N, then D94N only	1	–	1		1	–	–	–
WT+D94G+D94N, then D94N only	1	–	1		1	–	–	–
WT+D94G+A90V, then D94N only	1	–	1		1	–	–	–
1.1.2.2 (4 pools)	2	–	2		2	–	–	—
WT + A90V + S91P + D94N	1	–	1		1	–	–	–
WT + A90V + D94G + D94N	1	–	1		1	–	–	–
1.2 Sequential isolation of MTB with various *gyrA* mutations from various samples	13	–	9	4	13	–	–	–
1.2.1 Various single mutations (2 pools)	8	–	6	2	8	–	–	–
A90V or D94G	4	–	2	2	4	–	–	–
A90V or D94A	1	–	1		1	–	–	–
D94H or D94Y	1	–	1		1	–	–	–
D94G or D94N	1	–	1		1	–	–	–
D94G or D94H	1	–	1		1	–	–	–
1.2.2 Alternating double and single mutations	5	–	3	2	5		–	–
1.2.2.1 (2 pools)	4	–	2	2	4	–	–	–
D94G + A90V or A90V	1	–	1	–	1	–	–	–
D94G + A90V or D94G	1	–	1	–	1	–	–	–
A90V + S91P or A90V	1	–	–	1	1	–	–	–
D94N + D94G or D94G	1	–	–	1	1	–	–	–
1.2.2.2 (3 pools)	1	–	1	–	1	–	–	–
A90V + D94N + D94Y or A90V	1	–	1	–	1	–	–	–
2 Different variants of QRDR in one sample	5	1	4	–	3	1	1	–
2.1 (2 pools)	4	1	3	–	2	1	1	–
WT + D94G	1	–	1	–	1	–	–	–
WT + A90V	1	–	1	–	–	1	–	–
WT + S91P	1	–	1	–	1	–	–	–
WT + D94N	1	1	–	–	–	–	1	–
2.2 (3 pools)	1	–	1	–	1	–	–	–
WT + D94Y + A90V + (A90V и D94Y)	1	–	1	–	1	–	–	–
3. Double mutation	15	–	9	6	11	4	–	–
A90V + D94N	3	–	–	2	2	–	–	–
S91P + D94A	5	–	4	1	3	2	–	–
S91P + D94G	1	–	–	1	1	–	–	–
A90V + D94A	2	–	1	1	2	–	–	–
A90V + D94H	3	–	3	–	1	2	–	–
A90V + D94G	1	–	–	1	1	–	–	–
S91P + D94N	–	–	1	–	1	–	–	–
Total	55	2	40	13	45	6	3	1
Of those:	–	–	–	–	–	–	–	–
2 pools*	46	2	31	13	37	6	2	1
3 pools	7	–	7	–	6	–	1	–
4 pools	2	–	2	–	2	–	–	–

Note: NC — new cases; RC — retreatment cases; ATBD — antituberculosis drugs; FQ — fluoroquinolones; MDR — multiple drug resistance; Poly — polyresistance; Mono — monoresistance; N/d — no data available; WT — wild type *gyrA*; * — cases of the detected double *gyrA* mutation are included.

no ongoing monitoring allowing us to clarify the data obtained was performed.

Thus, we have shown that the patient could be infected with 2–4 MTB pools showing different structure of the *gyrA* QRDR. Mixed populations were most often represented by two MTB pools (46/55, 83.64%, when 15 cases of double mutations with unproven membership in two different pools were included in the analysis). Of those in 19 cases the population consisted of the MTB pool with the wild type *gyrA* and MTB pool carrying single *gyrA* mutations. In all other cases, (27, when 15 cases of double mutations for which no data of the ongoing monitoring were available were included in the analysis) MTB population was represented by two MTB pools with various *gyrA* mutations

In seven cases, MTB population found in one patient was represented with three pools showing different *gyrA* structure. In six cases, one MTB pool contained the wild type *gyrA* and two pools carried different *gyrA* mutations, while in one case all three MTB pools carried various *gyrA* mutations. Co-existence of three MTB pools showing different *gyrA* structure in one patient was proven by ongoing monitoring in five cases out of seven.

The presence of four MTB pools showing different *gyrA* QRDR structure could be suspected, since in one case susceptible MTB were isolated, and in another case tree *gyrA* mutations were identified in two samples of diagnostic material. It is hard to imagine that independent sequential processes of spontaneous mutagenesis could result in the emergence of three mutations at once within the same gene region, that is why it is reasonable to assume that there are three independent MTB pools carrying different mutations.

Mixed populations of MTB were usually isolated from the previously treated patients (40/55, 72.73%, 95% CI: 59.77–82.72). These populations were represented mostly by MDR MTB (45/55, 81.82%, 95% CI: 69.67–89.81). However, in two cases out of 55, mixed populations of MTB characterized by levofloxacin monoresistance were isolated from the new cases.

DISCUSSION

A retrospective study that covered a significant number of tuberculosis cases diagnosed in 2011–2019 was carried out to assess the diversity of mutation in the *gyrA* gene QRDR of MTB.

Of the eight identified nutant variants, seven were highly credible in terms of developing resistance to fluoroquinolones [8]. Mutations D94G and A90V were the most common, which was typical for the entire world's population [6, 7]. Our data on the frequency of these mutations (40.42% for D94G and 21.26% for A90V) showed that it was slightly higher compared to the global population (21–32% and 13–20%, respectively) [6].

The featured study shoes that mutations in the gyrA QRDR were most often associated with phenotypic resistance to fluoroquinolones, however, in rare cases, testing of MTB for *gyrA* mutations by the culture-based method revealed no fluoroquinolone resistance. This was explained by the MTB population heteroresistance after conducting additional studies. It is known that in case the share of one strain in the mixture is less than 5% when assessing phenotypic and genotypic resistance to fluoroquinolones, it is impossible to define its genotype and phenotype, and the results reflect the characteristics of the strain that dominates in the mixture [11]. This is important to consider when interpreting the mismatching results of testing for fluoroquinolone resistance by the culture-based and molecular genetic methods, since the initial low concentration of MTB with certain genotype in the cell mix and the likelihood of uneven distribution of MTB cells with various genotypes among samples collected for further molecular genetic and culture-based tests cannot be excluded.

When D94A mutation was detected in *gyrA* in 24 cases out of 73 (32.88%), MTB showed phenotypic resistance to levofloxacin. It is hard to explain such a high percentage by the undetected heteroresistance or errors in the culture-based or genotypic testing. Furthermore, other papers also report cases of the gyrA_D94A genotype corresponding to the susceptible phenotype: phenotypic resistance to fluoroquinolones was detected in one out of seven and four out of 12 MTB strains carrying this mutation, depending on the studied population [12, 13]. Therefore, it can be concluded that regardless of the fact that mutation D94A is highly credible in terms of developing resistance, mutation is not strongly associated with the fluoroquinolone resistance.

There is a theory that the widespread use of fluoroquinolones for treatment of nontuberculous infections may result in fluoroquinolone resistance developed by patients with undiagnosed tuberculosis [14]. In this regard it was important to evaluate the nature of resistance in the fluoroquinolone-resistant MTB: association of this parameter with multiple drug resistance of the pathogen can indicate that resistance to fluoroquinolones develops during treatment of MDT-TB, while identification of the cases of fluoroquinolone monoresistance, especially in the new cases, may be an indicator of fluoroquinolone resistance developed by MTB during treatment of other infectious diseases. We showed that the MTB genotypic resistance to fluoroquinolones was most often associated with MDR: 649 isolates carrying single *gyrA* mutations out of 694 (93.52%) and 45 cases of mixed populations out of 55 (81.00%) fell into the MDR category.

The findings prove that fluoroquinolone resistance is developed by MTB during treatment of MDR-TB. However, we cannot rule out the fluoroquinolone resistance developing during treatment of nontuberculous infections, since MTB showing fluoroquinolone monoresistance have been also found. Unfortunately, we do not know, whether these patients were previously treated with fluoroquinolones.

The study of the MTB genome structure, that involved MTB circulating in Samara region, performed by whole genome sequencing, allowed the authors to conclude that MTB were more likely to acquire fluoroquinolone resistance during therapy, and the cases of human infections caused by the fluoroquinolone-resistant MTB clones were rare. Based on this observation, it was assumed that the development of fluoroquinolone resistance resulted in the reduced MTB fitness [13]. The population-based study of the MTB primary fluoroquinolone resistance distribution across the Novosibirsk region also showed that resistance to fluoroquinolones most often resulted from the use of drugs of this group in chemotherapy of MDR-TB [15].

The findings presented here also confirm that the development of fluoroquinolone resistance during treatment is more frequent: MTB carrying *gyrA* mutations were more often isolated from the previously treated patients than from the newly diagnosed ones. However, we have found that fluoroquinolone-resistant MTB could be also isolated from the newly diagnosed patients with tuberculosis. In these cases *gyrA* mutations D94G and A90V were the most common, although the frequency of A90V mutation in MTB isolated from the newly diagnosed patients was significantly higher than in that isolated from the previously treated ones. Therefore, it can be concluded that MTB carrying this mutation are transmitted rather actively between humans.

The described possibility of the co-existence of several MTB population varying in *gyrA* mutations in one patient refers to the development of fluoroquinolone resistance in the population. A number of studies also showed that there could be several MTB clones with different structure of *gyrA* in one diagnostic sample; such samples accounted for 1–3% of the total number, which was consistent with our results [16–18].

Thus, we have shown that fluoroquinolone resistance is currently developing in the population of MTB circulating in the RF, which is usually associated with the pre-existing MDR. MTB resistance to fluoroquinolones has good prospects in terms of evolution, since it is developed in favorable genetic conditions during treatment of tuberculosis, caused by pathogen showing MDR, that results from the combination of mutations, that do not reduce MTB fitness [19]. The fact, that MTB carrying *gyrA* mutations D94G and A90V were also rather frequent in the new cases, allows us to conclude that it is these mutants that would play a key role in the spread of pre-XDR tuberculosis across the RF.

CONCLUSIONS

Retrospective analysis of the range of mutations in the QRDR of the *gyrA* gene of MTB isolated in 2011–2019 showed that genotypic resistance to fluoroquinolones was detected in 26.96% of the MTB clinical isolates, including cases of heteroresistance. Mutations D94G and A90V were the most common, their frequency totaled 62.95% of the number of MTB carrying a single *gyrA* mutation. It was also shown that these two mutations were rather common in MTB isolated in the new cases (D94G was found in 46.03%, and A90V was found in 25.10% MTB in this group), thus confirming successful spread of these MTB mutant variants in the modern population. The presence of *gyrA* mutations was usually associated with phenotypic resistance to levofloxacin, except for mutation D94A that was associated with phenotypic resistance to levofloxacin in only 67% of cases. The *gyrA* mutations were found mostly in MDR MTB: 93.52% of the strains carrying *gyrA* mutation were also resistant to rifampicin and isoniazid. The findings proved that resistance to fluoroquinolones was developed during treatment of MDR-TB. Furthermore, in 2.31% of cases heteroresistant MTB populations were found that included 2–4 MTB pools with different structure of the *gyrA* QRDR. Fluoroquinolone heteroresistance indicates active development of resistance to this group of medications in the current conditions.

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