


## ISONIAZID-RESISTANT *MYCOBACTERIUM TUBERCULOSIS*: PREVALENCE, RESISTANCE SPECTRUM AND GENETIC DETERMINANTS OF RESISTANCE

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The lack of simple, rapid diagnostic tests for isoniazid-resistant rifampicin-susceptible tuberculosis infection (Hr-TB) can result in low treatment efficacy and further amplification of drug resistance. Based on the clinical data, this study sought to estimate the prevalence of Hr-TB in the general population and characterize the phenotypic susceptibility and genetic determinants of isoniazid resistance in *M. tuberculosis* strains. Molecular-genetic and culture-based drug susceptibility tests were performed on *M. tuberculosis* isolates and *M. tuberculosis* DNA obtained from the patients with pulmonary TB undergoing treatment at the Central Tuberculosis Research Institute between 2011 and 2018. The tests revealed that Hr-TB accounted for 12% of all TB cases in the studied sample. Hr-TB strains were either resistant to isoniazid only (45%) or had multiple resistance to 2–6 anti-TB agents. Resistance to isoniazid was caused by mutations in the *katG* gene. Based on the literature analysis and our own observations, we emphasize the importance of developing simple molecular drug susceptibility tests capable of detecting simultaneous resistance to rifampicin and isoniazid and the necessity of their translation into clinical practice.

**Keywords:** *M. tuberculosis*, isoniazid resistance, drug susceptibility, molecular diagnostics, single nucleotide polymorphism, tuberculosis

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**Compliance with ethical standards:** we retrospectively analyzed the results of routine laboratory tests performed on the patients undergoing treatment for tuberculosis at the Central Tuberculosis Research Institute. All patients gave informed consent.

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## ИЗОНАЗИД-РЕЗИСТЕНТНЫЕ *MYCOBACTERIUM TUBERCULOSIS*: ЧАСТОТА ВЫЯВЛЕНИЯ, СПЕКТРЫ РЕЗИСТЕНТНОСТИ И ГЕНЕТИЧЕСКИЕ ДЕТЕРМИНАНТЫ УСТОЙЧИВОСТИ

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Отсутствие ускоренной диагностики туберкулеза с устойчивостью возбудителя к изониазиду с сохраненной чувствительностью к рифампицину (ИР-ТБ) может быть причиной низкой эффективности терапии и приводить к амплификации лекарственной резистентности, в том числе к формированию множественной лекарственной устойчивости. Целью работы было определить частоту встречаемости ИР-ТБ в современной популяции, охарактеризовать фенотипическую чувствительность и генетические детерминанты устойчивости к изониазиду представителей этой группы *M. tuberculosis* на репрезентативном материале. Анализировали результаты определения лекарственной чувствительности, полученные при исследовании молекулярно-генетическими и/или культуральными методами изолятов *M. tuberculosis* / ДНК *M. tuberculosis*, выделенных от больных туберкулезом легких из клинических отделений Центрального научно-исследовательского института туберкулеза за период 2011–2018 гг. Частота ИР-ТБ составила 12% от всех выявленных случаев туберкулеза. *M. tuberculosis* с ИР были как монорезистентными к изониазиду (45%), так и полирезистентными (устойчивыми к 2–6 противотуберкулезным препаратам), а устойчивость к изониазиду была обусловлена мутациями в гене *katG*, приводящими к высокому уровню резистентности. На основании анализа литературных данных и собственных наблюдений подчеркивается важность разработки и внедрения новых простых молекулярных тестов для определения устойчивости одновременно к рифампицину и изониазиду.

**Ключевые слова:** *M. tuberculosis*, изониазид-резистентность, лекарственная чувствительность, молекулярная диагностика, однонуклеотидный полиморфизм, туберкулез

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**Соблюдение этических стандартов:** был проведен ретроспективный анализ результатов, полученных при выполнении рутинных лабораторных исследований для пациентов, проходящих лечение в Центральном НИИ туберкулеза; все пациенты подписали добровольное информированное согласие на проведение исследования.

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Drug-resistant tuberculosis (TB) is a serious public health concern. At present, the major focus is on fighting multidrug-resistant TB (MDR-TB), i.e. caused by strains resistant to at least 2 most effective anti-TB drugs: isoniazid and rifampicin

[1]. Russia has the third-highest burden of MDR-TB [2]. In 2018, the incidence and prevalence of MDR-TB in Russia stabilized at 5.6 and 23.6 cases per 100, 000 population, respectively. However, the share of patients with MDR-TB among individuals

with active TB disease increased both in terms of incident cases (from 27.4% in 2017 to 29.3% in 2018) and the total respiratory TB burden (from 54.0% in 2017 to 55.3% in 2018) [3].

By contrast, other forms of drug-resistant TB are receiving less attention, including isoniazid-resistant TB (Hr-TB) assigned to a separate category by WHO. Its causative agent is resistant to isoniazid but sensitive to rifampicin [4]. Isoniazid is a first-line drug that exerts a bactericidal effect on *M. tuberculosis* and is highly effective in treating active TB forms. Phenotypic resistance to isoniazid is associated with mutations in *katG*, *inhA*, *ahpC* and some other genes that encode proteins involved in the pharmacokinetics and pharmacodynamics of isoniazid in the bacterial cell [5, 6].

Inadequate therapy for Hr-TB promotes a high risk of acquiring resistance to other anti-TB drugs, including rifampicin, and results in MDR [7]. According to WHO, Hr-TB prevalence varies from 5 to 11% across WHO regions [8]. The data on Hr-TB prevalence in Russia is scarce.

This study aimed to estimate the prevalence of isoniazid-resistant *M. tuberculosis* in patients presenting with pulmonary TB at the clinical departments of Central Tuberculosis Research Institute between 2011 and 2018, as well as to provide an extensive phenotypic drug sensitivity profile and describe genetic determinants of resistance to isoniazid in this group of *M. tuberculosis* isolates.

## METHODS

### Object of research

In this study, we looked at *M. tuberculosis* isolates and/or DNA isolated from the clinical specimens collected from the patients with pulmonary TB who had presented at the counselling and clinical departments of Central Tuberculosis Research Institute in 2011–2018. All microbiological tests were performed on the same sample.

### Culture tests

The cultures were grown and analyzed for the presence of *M. tuberculosis* in a Middlebrook 7H9 broth base in a BACTEC MGIT 960 system (BD; USA) following the manufacturer's protocol [9]. For drug susceptibility testing, we used BACTEC MGIT 960 instrumentation (BD; USA) and a modified proportion method. The isolates were tested for sensitivity to 8 drugs taken at critical concentrations, including isoniazid (H, 0.1 µg/ml), rifampicin (R, 1.0 µg/ml), ethambutol (E, 5.0 µg/ml), pyrazinamide (Z, 100.0 µg/ml), ethionamide (Eto, 5.0 µg/ml), amikacin (Am, 1.0 µg/ml), capreomycin (Cm 2.5 µg/ml), and levofloxacin (Lfx, 1.0 µg/ml). Standard protocols were applied [9, 10].

### DNA isolation

DNA was isolated from the clinical specimens using an AmpliTub-RV reagent kit 1 for the isolation, detection and quantification of mycobacterial DNA by real-time PCR (Syntol; Russia) following the manufacturer's protocol.

Detection of *M. tuberculosis* DNA was performed using an AmpliTub-RV reagent kit 2 for the isolation, detection and quantification of mycobacterial DNA by real-time PCR (Syntol; Russia) following the manufacturer's protocol. DNA fragments were amplified in a thermocycler equipped with a CFX96 optical reaction module (Bio-Rad; USA).

Genotypic resistance to rifampicin, isoniazid and fluoroquinolones was tested using either TB-Biochip-1 and

TB-Biochip-2 kits that utilize a microarray technology (Biochip-IMB; Russia) or AmpliTub-MDR-RV and AmpliTub-FQ-RV kits (Syntol; Russia). All procedures were carried out in compliance with the manufacturers' guidelines.

### Statistical analysis

Descriptive statistics were used to analyze the results of the study, including the number of observations, frequencies, percentages, and 95% CI. The analysis was conducted in MS Excel (Microsoft; USA).

## RESULTS

Clinical specimens collected from 4056 patients with pulmonary TB were subjected to culture-based and molecular testing. In 71 cases, neither *M. tuberculosis* DNA nor tubercle bacilli themselves were detected; so those cases were excluded from the analysis. Phenotypic/genotypic drug susceptibility was determined for *M. tuberculosis* DNA/cultures isolated from the remaining 3985 samples. If the results of culture tests contradicted those of molecular tests, priority was given to culture-based data (Table 1). For example, 38 strains that demonstrated resistance to both isoniazid and rifampicin in culture tests but had no mutations in the *rpoB* gene implicated in rifampicin resistance were put into the MDR category because molecular rifampicin susceptibility tests used in our study could only detect a limited number of mutations, meaning that genetic determinants of rifampicin resistance might have been overlooked in the analysis. And, vice versa, 29 strains that tested positive for mutations in the *rpoB* gene and did not have the rifampicin-resistant phenotype were categorized as isoniazid-resistant.

The total sample of drug-resistant *M. tuberculosis* strains was dominated by MDR isolates (Table 1). However, isoniazid-resistant strains that were susceptible to rifampicin were also well represented in the sample (502/3985; 12.60%).

The analysis of clinical data over the period from 2011 to 2018 revealed that Hr-TB amounted to about 14% of all TB cases per year reported in 2011–2012 and 2017–2018. In 2013–2016, the rate of detection for this TB form was lower (10–11%). We were unable to describe this linear trend with a sufficient degree of reliability (Table 2).

Because culture-based tests are less sensitive than molecular methods, the growth of *M. tuberculosis* in culture media was not detected for some specimens. Therefore, phenotypic sensitivity to anti-TB drugs was only determined for 260 isoniazid-resistant isolates of *M. tuberculosis* (Table 3). The following definitions were applied to identify the type of drug resistance of *M. tuberculosis* isolates [1]: monoresistance, i.e. resistance of the mycobacterium to only one anti-TB drug, and polyresistance, i.e. resistance of the mycobacterium to 2 or more anti-TB drugs but not to the combination of isoniazid and rifampicin.

Mono-resistant isolates amounted to 117/260 (45%) cases. The rest 143 (55%) isolates were polyresistant (to 2–6 drugs). Polyresistant isolates were equally represented by *M. tuberculosis* strains resistant to both isoniazid and first-line drugs (42/143; 29.37%) and by the strains resistant to both isoniazid and second-line drugs (38/143; 26.57%); resistance to second-line drugs almost always included resistance to ethionamide (31/38; 81.58%). Co-resistance to first- and second-line drugs was the most common among the polyresistant isolates (63/143; 44.06%). Of them, co-resistance to isoniazid, ethambutol and ethionamide (HEEto), including

their combinations with other second-line medications, was detected in 20/63 (31.75%) cases; polyresistance to isoniazid, pyrazinamide and ethionamide (HZEto), including their combinations with other second-line drugs, was not so common (9/63 cases or 14.29%). Polyresistance to HEZEto was observed in 15/63 (23.81%) isolates. In 19/63 (30.16%) isolates, resistance spectra included other combinations of drugs (a total of 12 resistance spectra with 3 to 5 drugs).

Data on mutations in the genes associated with resistance to isoniazid were acquired for 451 *M. tuberculosis* isolates resistant to isoniazid (Table 4). In most cases (386/451 isolates or 85.59%), single nucleotide polymorphisms (SNPs) were detected in one of the genes associated with resistance to isoniazid. The presence of SNPs in 2 genes associated with isoniazid resistance was not so common (65/451 cases or 14.41%). The most prevalent were mutations at codon 315 of the *katG* gene (413/451 cases or 91.57%). In 348/413 (84.26%) cases, mutations were detected only in *katG*; in 62/413 (15.01%) isolates, mutations in *katG* co-occurred with SNPs in the *inhA* gene. In single cases, *katG* mutations co-occurred with SNPs in the *ahpC* gene.

The *inhA15\_C*->T substitution was quite common (94/451; 20.84%); in 33/94 (35.11%) cases it was the only mutation detected. In other samples, this mutation co-occurred with an SNP at codon 315 of the *katG* gene.

For 209 isolates of *M. tuberculosis* with phenotypically confirmed resistance to isoniazid, the following distribution of mutant variants was observed: 152/209 (72.73%) carried a mutation in the *katG* gene only (315\_Ser->Thr(1)); 32/209 (15.31%) carried a combination of *katG*315\_Ser->Thr(1) and *inhA15\_C*->T; 17/209 (8.13%) only *inhA15\_C*->T was detected. The remaining 8 (3.83%) isolates with phenotypically confirmed resistance to isoniazid had mutations in other regions of the genes associated with isoniazid resistance (*ahpC*10\_C->T in

the absence of other mutations, *katG*315\_Ser->Asn; co-occurring *katG*315\_Ser->Gly + *inhA15\_C*->T, *katG*315\_Ser->Thr(1) + *inhA8\_T*->G, *katG*315\_Ser->Thr(1) + *ahpC*10\_C->T).

Thus, our sample of isoniazid-resistant *M. tuberculosis* was dominated by the *katG*315\_Ser->Thr(1) mutation corresponding to the substitution AGC->ACC (333/451; 73.84%), followed by the co-occurring *katG*315\_Ser->Thr(1) + *inhA15\_C*->T (60/451; 13.30%), and single *inhA15\_C*->T (33/451; 7.32%). On the whole, these 3 mutant variants amounted to 426/451 (94.46%) isoniazid-resistant *M. tuberculosis* isolates.

## DISCUSSION

We attempted to estimate the prevalence of isoniazid-resistant, rifampicin-susceptible *M. tuberculosis* strains isolated from the patients with pulmonary TB, who had presented at clinical departments of Central Tuberculosis Research Institute in 2011–2018.

The prevalence of this TB form and the rate of its spread vary across the world's regions. For example, the analysis of data on drug susceptibility collected by WHO from 131 specialized healthcare institutions in 1994–2009 reveals that Eastern Europe had the highest burden of Hr-TB (15%), followed by Western and Central Europe (11%); in other WHO regions, Hr-TB prevalence did not exceed 8% [11]. In some regions, the prevalence of Hr-TB tended to decrease, whereas in others, it was increasing. No clear linear dynamics were established for the majority of WHO regions. In our study, the prevalence of isoniazid-resistant *M. tuberculosis* (12%) was similar to that in Eastern Europe and its dynamics were non-linear, just like in the majority of the world's regions.

The systematic review of the link between primary resistance to isoniazid and the acquisition of secondary resistance to other anti-TB drugs [12] concludes that monoresistant strains acquire

**Table 1.** *M. tuberculosis* isolates and drug susceptibility tests

Types of sensitivity to antituberculous drugs	Number of isolates for which data on drug sensitivity was obtained (abs.)			The total number of <i>M. tuberculosis</i> isolates assigned to each sensitivity type based on culture and molecular testing	
	Molecular and culture tests	Culture tests only	Molecular tests only	abs.	% (95% CI)
Sensitive	478	207	673 <sup>1)</sup>	1358	34.08 (32.62–35.56)
MDR	1179 <sup>2)</sup>	256	613	2048	51.39 (49.84–52.94)
Hr	209 <sup>3)</sup>	51	242	502	12.60 (11.60–13.66)
Other <sup>4)</sup>	42	23	12	77	1.93 (1.55–2.41)
Total	1908	537	1540	3985	100

**Note:** <sup>1</sup> — including cases for which no mutations were detected in the genes associated with resistance to isoniazid, rifampicin and fluoroquinolones; <sup>2</sup> — including 38 *M. tuberculosis* isolates for which no mutations in the *rpoB* gene (see the article) were detected; <sup>3</sup> — including 29 *M. tuberculosis* isolates carrying mutant *rpoB* but having no resistance phenotype; <sup>4</sup> — mono- or polyresistance to antituberculous drugs, excluding isoniazid.

**Table 2.** The frequency of detection of isoniazid-resistant *M. tuberculosis* in 2011–2018

Year	Number of <i>M. tuberculosis</i> isolates		
	Total isolates studied (abs.)	Hr isolates	
		abs.	% (95% CI)
2011	458	67	14.63 (11.69–18.16)
2012	355	52	14.65 (11.35–18.70)
2013	530	54	10.19 (7.89–13.06)
2014	554	65	11.73 (9.31–14.68)
2015	569	65	11.42 (9.06–14.30)
2016	502	56	11.16 (8.69–14.21)
2017	557	76	13.64 (11.04–16.75)
2018	460	67	14.57 (11.64–18.08)

additional resistance (no necessarily MDR) to other anti-TB drugs 5.1 times more often than drug-susceptible strains. High occurrence rates of polyresistant strains demonstrated in our study (55% of all Hr-TB isolates) insensitive to 1–5 drugs apart from isoniazid corroborate the possibility of drug resistance amplification in isoniazid-resistant *M. tuberculosis*.

Because first-line antituberculous drugs ethambutol and pyrazinamide are included in the standard chemotherapy regimen often prescribed empirically to newly diagnosed patients, it would be reasonable to expect high prevalence rates of Hr *M. tuberculosis* strains additionally resistant to these 2 drugs. Indeed, resistance to ethambutol was detected in almost half of all polyresistant *M. tuberculosis* isolates analyzed in our study (70/143, 48.95%; 95% CI: 40.89–57.06%); pyrazinamide-resistant isolates were slightly rarer (57/143, 39.86%; 95% CI: 32.20–48.05%).

Polyresistant *M. tuberculosis* strains resistant to ethionamide (the second-line medication) were much more prevalent (80/143, 55.94%; 95% CI: 47.76–63.82%). This can be explained by the fact that ethionamide is a structural analogue of isoniazid; it inhibits synthesis of mycolic acids, thereby disrupting the structure of the bacterial cell wall. Therefore, these two drugs may have common targets and genetic determinants of resistance [5, 13].

In general, the use of first-line drugs in the therapy of Hr-TB leads to poor outcomes, including the lack of therapeutic effect, relapses, acquired MDR. Besides, standard empiric treatment of Hr-TB can promote MDR-TB epidemics, especially in the regions where such TB forms are not rare [7]. At the same time, timely adjustments to the regimen based on results of isoniazid susceptibility testing and the use of modified regimens reinforce therapeutic success and reduce the risk of relapses [14–16].

In this light, 2 clinical studies should be mentioned that aimed to establish an association between mutations in *M. tuberculosis* and the efficacy of treatment of Hr-TB with high doses of isoniazid [17, 18]. It is known that mutations in the *katG* gene, which dominated our sample, result in a high level of resistance to isoniazid whereas mutations in *inhA*, in a low level of resistance [5]. The studies revealed that therapy with isoniazid was effective when mycobacteria carried mutations in the *inhA* gene; *katG* mutations were associated with poor treatment outcomes [17, 18].

This emphasizes the need for effective regimens for the therapy of Hr-TB [8, 19]. Rapid drug susceptibility testing is critical. Molecular diagnostic methods are highly sensitive and rapid (1–2 days in comparison with weeks required for culture); they also provide valuable information about mutations carried by the strain and the level of isoniazid resistance [1]. Therefore, the demand for molecular methods in the diagnosis of TB and drug susceptibility testing is high. However, although tests based on allele specific PCR, bioarray technologies or DNA strips used in large TB healthcare centers expedite diagnosis, they impose strict requirements on staff qualifications and laboratory infrastructure.

Today, the only molecular test that can be deployed in any laboratory is Xpert MTB/RIF that utilizes the GeneXpert platform [20]. Unfortunately, this test can detect only genotypic resistance to rifampicin because these days all diagnostic procedures are largely focused on detecting MDR strains, which are resistant to rifampicin. Therefore, Xpert MTB/RIF cannot detect resistance to isoniazid in the strains that are sensitive to rifampicin (12% of our isolates). In the absence of additional diagnostic tools, isoniazid resistance of such strains will never be revealed, leading to inadequate chemotherapy regimens and amplification of MDR. This indicates the need for

a simple molecular test that is as convenient as Xpert MTB/RIF and can be used in any laboratory.

## CONCLUSIONS

Isoniazid-resistant tuberculosis can be regarded as a potential predecessor of MDR disease. It is important to control the spread of primary resistance to isoniazid and prevent acquisition of further resistance. Our analysis revealed high prevalence

**Table 3.** Resistance spectra of isoniazid-resistant strains of *M. tuberculosis*

Resistance spectra	Number of <i>M. tuberculosis</i> strains	
	abs.	% (95% CI)
<b>Monoresistance (H)</b>	<b>117</b>	<b>45.00 (39.07–51.08)</b>
Polyresistance (H + other anti-TB drugs except R):	143	55.00 (48.95–61.05)
<b>To 2 anti-TB drugs:</b>	<b>71</b>	<b>27.31 (22.25–33.02)</b>
HE	17	
HZ	16	
H Eto	31	
H Am	3	
H Cp	1	
H Lfx	3	
<b>To 3 anti-TB drugs:</b>	<b>36</b>	<b>13.85 (10.17–18.57)</b>
HEZ	9	
HE Eto	13	
HE Lfx	1	
HZ Cp	1	
HZ Eto	7	
H AmCp	2	
H EtoAm	3	
<b>To 4 anti-TB drugs:</b>	<b>21</b>	<b>8.08 (5.34–12.03)</b>
HEZ Eto	6	
HEZ Am	1	
HEZ Lfx	3	
HE AmCp	2	
HE EtoAm	4	
HE EtoLfx	2	
HZ EtoCp	1	
H EtoAmCp	1	
H AmCpLfx	1	
<b>To 5 anti-TB drugs:</b>	<b>12</b>	<b>4.62 (2.66–7.89)</b>
HEZ EtoAm	2	
HEZ EtoLfx	4	
HEZ AmCp	2	
HE EtoAmLfx	1	
HZ AmCpLfx	1	
HZ EtoAmLfx	1	
H EtoAmCpLfx	1	
<b>To 6 anti-TB drugs:</b>	<b>3</b>	<b>1.15 (0.39–3.34)</b>
HEZ EtoAmCp	2	
HEZ EtoAmLfx	1	
<b>Total</b>	<b>260</b>	<b>100</b>

**Note:** in the lists of resistance spectra, first-line drugs are separated from second-line medications by a space character.



**Table 4.** Isoniazid-resistant *M. tuberculosis* isolates with different combinations of mutations in the genes associated with isoniazid resistance

Mutations in <i>katG</i> Mutations in <i>ahpC</i> and <i>inhA</i>			Not detected	At codon 351, substitution of Ser to					Total
				Arg	Asn	Gly	Thr(1)*	Thr(2)*	
No detected mutations in <i>ahpC</i> or <i>inhA</i>			–	4 (0.89; 0.35–2.26)	7 (1.55; 0.75–3.17)	3 (0.67; 0.23–1.94)	333 (73.84; 69.59–77.68)	1 (0.22; 0.04–	348 (77.16; 73.07–80.80)
SNP in <i>ahpC</i> at position	6	G→A	–	–	–	–	1 (0.22; 0.04–1.25)	–	1 (0.22; 0.04–1.25)
	10	C→A	1 (0.22; 0.04–1.25)	–	–	–	–	–	1 (0.22; 0.04–1.25)
		C→T	1 (0.22; 0.04–1.25)	–	–	–	1 (0.22; 0.04–1.25)	–	2 (0.44; 0.12–1.60)
	12	C→T	–	–	–	–	1 (0.22; 0.04–1.25)	–	1 (0.22; 0.04–1.25)
SNP in <i>inhA</i> at position	8	T→A	2 (0.44; 0.12–1.60)	–	–	–	–	–	2 (0.44; 0.12–1.60)
		T→G	–	–	–	–	1 (0.22; 0.04–1.25)	–	1 (0.22; 0.04–1.25)
	15	C→G	1 (0.22; 0.04–1.25)	–	–	–	–	–	1 (0.22; 0.04–1.25)
		C→T	33 (7.32; 5.26–10.10)	–	–	1 (0.22; 0.04–1.25)	60 (13.30; 10.48–16.75)	–	94 (20.84; 17.35–24.83)
Total			38 (8.43; 6.20–11.35)	4 (0.89; 0.35–2.26)	7 (1.55; 0.75–3.17)	4 (0.89; 0.35–2.26)	397 (88.03; 84.70–90.71)	1 (0.22; 0.04–1.25)	451 (100)

**Note:** SNPs occurring in only one gene associated with isoniazid resistance are highlighted in gray; other cells show combinations of >1 mutation. \* Thr(1) represents the AGC→ACC substitution, Ser→Thr(2) represents the AGC→ACA substitution.

of Hr-TB (over 12% of all analyzed cases) among isoniazid-resistant rifampicin-susceptible *M. tuberculosis* strains isolated from patients with pulmonary TB. The majority of such isolates carried mutations causing strong resistance to isoniazid. Our

findings indicate the importance of rapid testing for sensitivity to both rifampicin and isoniazid based on molecular-genetic methods. There is a need for simple point-of-care tests that do not impose high requirements on laboratory infrastructure.

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