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Determinants of resistance to levofloxacin and metronidazole in Russian clinical isolates of *Helicobacter pylori* based on whole-genome sequencing data

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Abstract

Introduction. Helicobacter pylori infection, however, data on the mechanisms of metronidazole (MTZ) and levofloxacin (LVX) resistance in Russia remain scarce. and levofloxacin (LVX) in Russia.

The aim of the study is to identify the determinants of resistance in clinical isolates of *H. pylori* to MTZ and LVX using whole-genome sequencing data.

Materials and methods. A retrospective analysis of 43 *H. pylori* isolates obtained from adult patients (2014–2022) was conducted. Susceptibility to antibiotics was determined using the bacteriological disk diffusion method. Whole-genome sequencing of 43 *H. pylori* strains was performed using a DNBSEQ-G50 sequencer.

Results. The evaluation of the phenotypic drug susceptibility test results showed that 11 isolates were susceptible to MTZ (MTZ-S), 31 were susceptible to LVX (LVX-S), while 32 isolates were resistant to MTZ (MTZ-R), and 12 were resistant to LVX (LVX-R). To identify the association between phenotypic and genotypic resistance, an analysis of nucleotide substitutions in the *gyrA, gyrB, rdxA, frxA, fdxB* and *fur* genes was conducted. Of all the mutations identified in the *gyrA* and *gyrB* genes, only *D91/GNY* in the *gyrA* gene was associated with phenotypic resistance to LVX and was found in 4/12 (33.3%) of the isolates ($p < 0.05$). The combined mutation *D91G/N/Y+N87K* in the *gyrA* gene was detected in 6/12 (50.0%) of LVX-R isolates ($p < 0.001$). Point mutations in the *rdxA* gene were detected in 21.9% (7/32) of MTZ-R isolates, leading to a frameshift or premature termination of protein synthesis. None of the mutations in the *frxA*, *fur* and *fdxB* genes were associated with *H. pylori* resistance to MTZ.

Conclusion. Based on the results of whole-genome sequencing of Russian clinical isolates of *H. pylori*, the detection of the combined mutation *D91G/N/Y+N87K* in the *gyrA* gene can serve as a predictor of the phenotypic resistance of *H. pylori* to levofloxacin.

Keywords: *Helicobacter pylori, whole-genome sequencing, antibiotic resistance, resistance determinants, metronidazole, levofloxacin*

Ethics approval. The study was conducted in strict compliance with confidentiality standards: all patient data was made anonymous and encrypted. Informed consent was obtained from each study participant. The study protocol was approved by the Independent Local Ethics Committee of the Pasteur Research Institute of Epidemiology and Microbiology (protocol No. 50/04–2019 dated June 22, 2020).

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Оригинальное исследование
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Детерминанты резистентности к левофлоксацину и метронидазолу российских клинических изолятов *Helicobacter pylori* по результатам полногеномного секвенирования

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Аннотация

Введение. Устойчивость к антибактериальным препаратам является одной из ключевых проблем в лечении *Helicobacter pylori*-инфекции, однако в России практически отсутствуют данные о механизмах резистентности к метронидазолу (MTZ) и левофлоксацину (LVX).

Цель работы — выявление детерминант резистентности у клинических изолятов *H. pylori* к MTZ и LVX с использованием данных полногеномного секвенирования.

Материалы и методы. Проведён ретроспективный анализ 43 изолятов *H. pylori*, выделенных от взрослых пациентов (2014–2022 гг.). Чувствительность к антибактериальным препаратам определяли бактериологическим диско-диффузионным методом. Полногеномное секвенирование 43 штаммов *H. pylori* проводили с использованием секвенатора «DNBSEQ-G50».

Результаты. Оценка результатов теста фенотипической лекарственной чувствительности показала, что 11 изолятов являлись чувствительными к MTZ (MTZ-S), 31 — чувствительными к LVX (LVX-S), тогда как 32 изолята проявляли устойчивость к MTZ (MTZ-R), 12 — к LVX (LVX-R). Для выявления ассоциации между фенотипической и генотипической устойчивостью проведён анализ нуклеотидных замен в генах *gyrA*, *gyrB*, *rdxA*, *frxA*, *fdxB*, *fur*. Из всех мутаций, выявленных в генах *gyrA* и *gyrB*, только *D91/GNY* в гене *gyrA* была ассоциирована с фенотипической устойчивостью к LVX и обнаружена у 4/12 (33,3%) изолятов ($p < 0,05$). Комбинированная мутация *D91G/N/Y+N87K* в гене *gyrA* выявлена у 6/12 (50,0%) LVX-R-изолятов ($p < 0,001$). У 21,9% (7/32) MTZ-R-изолятов в гене *rdxA* выявлены точечные мутации, приводящие к сдвигу рамки считывания или преждевременной терминации синтеза белка. Ни одна из мутаций в генах *frxA*, *fur* и *fdxB* не была ассоциирована с устойчивостью *H. pylori* к MTZ.

Выводы. По результатам полногеномного секвенирования российских клинических изолятов *H. pylori* детекция комбинированной мутации *D91G/N/Y+N87K* в гене *gyrA* может служить предиктором фенотипической устойчивости *H. pylori* к LVX.

Ключевые слова: *Helicobacter pylori, полногеномное секвенирование, устойчивость к антибактериальным препаратам, детерминанты резистентности, метронидазол, левофлоксацин*

Этическое утверждение. Исследование проведено с соблюдением строгих норм конфиденциальности: все данные пациентов деперсонифицированы и зашифрованы. От каждого участника исследования получено информированное согласие. Протокол исследования одобрен независимым локальным этическим комитетом НИИ эпидемиологии и микробиологии им. Пастера (протокол № 50/04–2019 от 22.06.2020).

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Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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Introduction

Helicobacter pylori infection remains one of the most common chronic bacterial infections worldwide and is considered a major risk factor for the development of gastric cancer (approximately 90% of cases) [1]. According to the key principles of the Maastricht VI/Florence Consensus, which underpin national clinical guidelines, *H. pylori* infection (regardless of symptoms or complications) invariably causes gastritis, for which the only treatment is eradication therapy. Furthermore, the first principle of the consensus recommends conducting drug susceptibility testing prior to prescribing first-line therapy to ensure the rational use of antibiotics [2, 3].

H. pylori resistance to antibiotics is currently recognized as one of the most serious issues. Eradication therapy for *H. pylori* typically uses a combination of 2–3 antibiotics (such as amoxicillin, clarithromycin, metronidazole (MTZ), tetracycline, levofloxacin (LVX), or rifabutin), a proton pump inhibitor, and, in some regimens, a bismuth [2, 3]. However, the efficacy of *H. pylori* treatment has been steadily declining in recent years, in parallel with the growth of antibiotic resistance [4, 5]. Furthermore, the widespread use of antibiotics and low patient compliance not only foster resistance in *H. pylori* but also exert selective pressure on the broader gastrointestinal microbiome. This promotes the selection of resistance genes, enriches the resistome, and facilitates the spread of these genes among bacterial communities [6, 7].

The genetic mechanisms underlying the development of antibiotic resistance (AR) in *H. pylori* are not fully understood. It is generally believed that *H. pylori* resistance to antibiotics is due to *de novo* mutations in chromosomal DNA, which either alter the target of the antibiotic or prevent its activation within cells [6]. Nevertheless, a significant proportion of *H. pylori* strains resistant to antibiotics lack known genetic determinants of resistance. This indicates the complex and multifactorial nature of resistance mechanisms, which go beyond mutational activity and/or the activation of individual genes in response to antibiotic use. Potential mechanisms of *H. pylori* resistance include:

- increased expression of efflux system genes;
- synergistic interactions involving mutations, horizontal gene transfer, and activation of protective systems;
- cellular adaptation associated with the formation of biofilms and antibiotic-resistant coccoid forms;

Compensatory mutations that mitigate the fitness cost of resistance through epistatic interactions with resistance determinants;heteroresistance — a phenomenon where subpopulations of cells with different susceptibility to antibiotics are present simultaneously within a bacterial population.

All the mechanisms listed above not only contribute to the formation of *H. pylori* antibiotic resistance but also accelerate the development of multidrug resistance, which significantly complicates the eradication process and highlights the necessity for a thorough evaluation and improvement of therapeutic approaches [6–8].

The mechanism of action of MTZ, a first-line drug for *H. pylori* eradication therapy, involves the reduction of the nitro group under anaerobic conditions, leading to the formation of cytotoxic nitroanions and free radicals that damage DNA and disrupt the functioning of bacterial cells. *H. pylori* resistance to MTZ is due to a complex interplay of genetic and biochemical processes that are not yet fully understood. The main mechanism of resistance to MTZ is mediated by the inactivation of the reductase enzyme genes *rdxA* (which encodes an oxygen-insensitive nitroreductase) and *frxA* (which encodes flavin oxidoreductase), which reduces the ability of MTZ to be reduced to its active forms (NO_2^- and NO_2^{2-}) and, consequently, diminishes the drug's antimicrobial effect [9, 10]. Mutations in the *fur* gene, which regulates iron uptake, and *fdxB*, which encodes ferredoxin, are also thought to contribute to the formation of AR [7, 8, 10]. Increased levels of antioxidant enzymes and the efflux system, as well as mutational activity in genes responsible for repairing damaged DNA, act as additional factors contributing to the development of *H. pylori* resistance to MTZ [6].

Another antibiotic used as a reserve treatment for *Helicobacter pylori* infection, LVX, exerts its antibacterial effect by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV — key enzymes involved in DNA replication and recombination processes. The most common mechanism of *H. pylori* resistance to fluoroquinolones is due to point mutations in the quinolone resistance-determining regions (QRDR), particularly in codons 86, 87, 88, 91, 97 of the *gyrA* gene and codons 481, 484 and 463 of the *gyrB* gene [6, 7, 10]. However, the role of some of these mutations in the development of *H. pylori* resistance to LVX has not been proven.

The systematic and reliable data on the resistance rates of *H. pylori* to LVX and MTZ in Russia are scarce. Additionally, the genetic determinants of resistance to these antibiotics remain largely uncharacterized, impeding the development of effective molecular diagnostic tools for monitoring resistance in clinical practice. **The aim** of our study was to identify the determinants of resistance in *H. pylori* clinical isolates to MTZ and LVX using whole-genome sequencing data.

Materials and methods

A retrospective analysis was conducted on 43 clinical isolates of *H. pylori* obtained from adult patients with gastrointestinal diseases (2014–2022) at the Pasteur Research Institute of Epidemiology and Microbio-

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logy in St. Petersburg. The average age of the patients was 44.0 ± 4.5 years (range 22–70 years).

Biopsies from the gastric antrum and body, were placed in thioglycolate medium, homogenized and subsequently cultured on selective medium based on Columbia agar supplemented with 7% defibrinated horse blood and 1% IsoVitalex solution at 37°C under microaerophilic conditions (oxygen content ~5%) using GasPak 100 gas-generating pouches (BBL CampyPak Plus Microaerophilic System envelopes with Palladium Catalyst, BD Biosciences). *H. pylori* was identified using a set of biochemical tests (urease, catalase and oxidase) and a reagent kit for detecting *H. pylori* DNA by polymerase chain reaction (DNA-Technology).

To perform the antibiotic susceptibility test, the *H. pylori* bacterial culture was suspended to a density of 2 on the McFarland scale (~ 6×10^8 CFU/mL), 0.1 mL was applied to the surface of a Petri dish containing Müller–Hinton agar supplemented with 5% defibrinated horse blood, and evenly distributed across the surface with a spatula. The susceptibility of *H. pylori* isolates to MTZ and LVX was determined using the disk diffusion method: immediately after inoculation onto the agar surface, disks containing MTZ (5 µg/disk) and LVX (5 mcg/disk) were aseptically applied and incubated under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) at 37°C for 72 hours. After incubation was complete, the diameter of the zones of complete growth inhibition around the antibiotic disk was measured in millimeters. The interpretation of the disk diffusion method results was based on the threshold values presented in the publication by Z. Zhong et al.: *H. pylori* strains were considered resistant to MTZ (MTZ-R) when the inhibition zone diameter was ≤ 16 mm, and susceptible (MTZ-S) when the diameter was ≥ 17 mm; resistant to LVX (LVX-R) when the inhibition zone diameter was ≤ 17 mm, and susceptible (LVX-S) when the diameter was ≥ 18 mm [11].

Total DNA from pure cultures of *H. pylori* was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH) according to the manufacturer's instructions. The DNA concentration of each sample was quantified using a Qubit 4.0 fluorometer. Whole-genome sequencing was performed on a DNBSEQ-G50 sequencer (MGI Tech Co. Ltd.).

The quality assessment of paired-end libraries, adapter removal, and low-quality sequence trimming (Q-score < 20) were performed using the FastQC v. 0.12.1 and Trim Galore! v. 0.6.7 programs. Bacterial genomes were assembled *de novo* using the genomic assembler SPAdes v. 3.13.1, and the results were evaluated using QUAST v. 5.2.0 [12, 13]. The obtained genomic sequences were aligned to the *H. pylori* 26695 reference strain (GenBank acc. no. AE000511.1). The Snippy v.4.6.0 program was used to assess genetic variations between isolates and identify nucleotide substitutions[1]. Aligned nucleotide sequences were visually analyzed using UGENE v. 38.1 [14]. All genome assemblies of clinical isolates of *H. pylori* were deposited in the NCBI GenBank database under registration number PRJNA1011037[2].

Statistical analysis was performed using the R programming language v. 4.3.2. The agreement between phenotypic and genotypic resistance profiles was evaluated using the χ^2 test and Fisher's exact test. Differences between groups were considered significant at $p < 0.05$.

Results

Phenotypic drug susceptibility testing of 43 clinical *H. pylori* isolates showed that 12 isolates were resistant to LVX (LVX-R), 32 to MTZ (MTZ-R); at the same time, 31 isolates were susceptible to LVX (LVX-S), and 11 to MTZ (MTZ-S). Among the resistant isolates, 9 were resistant to both antibiotics simultaneously (Group ***A***), while among the susceptible isolates, 8 were susceptible to both LVX and MTZ (Group ***B***: Fig. 1).

To identify the determinants of resistance to LVX and MTZ and their association with phenotypic resistance, all 43 isolates underwent whole-genome sequencing, followed by an analysis of nucleotide substitutions in the *gyrA, gyrB, rdxA, frxA, fdxB* and *fur* genes.

Of all the mutations in the *gyrA* gene, only D91N/Y/G was associated with phenotypic drug resistance and was detected in 33.3% (4/12) of LVX-R isolates ($p < 0.05$) (**Table**). The missense mutation *N87K* in the *gyrA* gene was detected in 16.7% (2/12) of LVX-R isolates and was not found in combination with *D91N/Y/G* and *A88P* mutations, while the *A88P* mutation was identified in only 1 (8.3%) resistant strain in combination with the *D91N* mutation. Given the absence of these mutations in LVX-S isolates, the combined *D91N/Y/G+N87K* mutation is significantly associated with resistance in clinical isolates and was detected in 50.0%

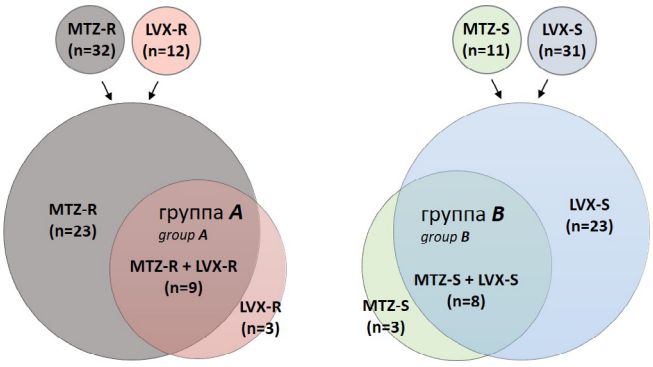

Fig. 1. Venn diagram showing the combinations of phenotypic drug susceptibility statuses of clinical isolates of *H. pylori* to MTZ and LVX

[1] URL: https://github.com/tseemann/snippy
[2] URL: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1011037

(6/12) of LVX-R isolates ($p < 0.001$). It should be noted that the *D91N/Y/G*, *N87K* and *A88P* mutations in the *gyrA* gene were found exclusively in group A isolates and were absent in monoresistant isolates. All mutations in the *gyrB* gene were present in *H. pylori* isolates regardless of their phenotypic resistance ($p > 0.05$). Mutations at positions 86 and 97 of the *gyrA* gene and 463 of the *gyrB* gene, which are presumably associated with *H. pylori* resistance to LVX, were not found in our sample.

In total, 56 point mutations in the *rdxA* gene were identified. Among these, 4 (*H97fs, S43fs, I182fs, M120fs*) were frameshift mutations, 2 (*Q50*stop*, *W52*stop*) were nonsense mutations leading to premature termination of the reading frame, and *Ter211R_ext** mutation resulted in the loss of stop codon, and fusion of *rdxA* gene with the adjacent *HP_0953* gene. Two mutations (*S108A/P, L62V*) identified for the first time in MTZ-R isolates, showed no association with phenotypic resistance ($p > 0.05$). The *R131K*, *T31E* and *D59N* mutations were found in *H. pylori* isolates from both phenotypic groups and were not associated with phenotypic resistance to MTZ.

We identified 7 frameshift mutations in the *frxA* gene, 4 of which (*Y19fs, R23fs, I44fs, A70fs*) were detected only in MTZ-R isolates, while 3 (*K18fs, R106fs,*

Mutations in LVX and MTZ resistance genes in *H. pylori* clinical isolates of compared to the *H. pylori* 26695 reference genome, *n* (%)

Gene (locus)	Amino acid substitution	LVX-R (*n* = 12)	LVX-S (*n* = 31)	MTZ-R (*n* = 32)	MTZ-S (*n* = 11)	*p*
gyrA (HP_0701)	*D91N/Y/G*	4 (33.3)	0			0.0040
	N87K	2 (16.7)	0			0.0730
	A88P	1 (8.3)	0			0.2790
gyrB (HP_0501)	*D481E*	3 (25.0)	8 (25.8)			~ 1.0000
	R484K	3 (25.0)	8 (25.8)			~ 1.0000
rdxA (HP_0954)	*S108A/P*			7 (21.9)	0	0.1628
	R16C			7 (21.9)	0	0.1628
	L62V			6 (18.7)	0	0.3122
	Ter211R_ext stop lost & splice region*			1 (3.1)	0	~ 1.0000
	*Q50*stop*			1 (3.1)	0	~ 1.0000
	*W52*stop*			1 (3.1)	0	~ 1.0000
	H97fs			1 (3.1)	0	~ 1.0000
	S43fs			1 (3.1)	0	~ 1.0000
	I182fs			1 (3.1)	0	~ 1.0000
	M120fs			1 (3.1)	0	~ 1.0000
	R131K			12 (37.5)	1 (9.1)	0.1290
	T31E			13 (40.6)	5 (45.4)	0.7794
	D59N			30 (93.7)	9 (81.8)	0.2665
frxA (HP_0642)	*K18fs*			17 (53.1)	5 (45.4)	0.6606
	Y19fs			1 (3.1)	0	~ 1.0000
	*Q27*stop*			1 (3.1)	0	~ 1.0000
	R23fs			1 (3.1)	0	~ 1.0000
	I44fs			1 (3.1)	0	~ 1.0000
	A70fs			1 (3.1)	0	~ 1.0000
	R106fs			1 (3.1)	1 (9.1)	0.4507
	*W137*stop*			1 (3.1)	0	~ 1.0000
	*Q141*stop*			1 (3.1)	0	~ 1.0000
	V215fs			10 (31.2)	6 (54.5)	0.1679
fdxB (HP_1508)	*N424fs*			1 (3.1)	1 (9.1)	0.4507
	K426fs			1 (3.1)	1 (9.1)	0.4507
fur (HP_1027)	*C150Y*			3 (9.4)	2 (18.2)	0.5890
	N118Q			7 (21.9)	3 (27.3)	0.6982

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V215fs) were found in isolates from both phenotypic groups. At the same time, 3 nonsense mutations leading to premature protein termination were found exclusively in MTZ-R strains (Table). In the *fdxB* gene, 2 mutations (*N424fs, K426fs*) were harbored by both MTZ-R and MTZ-S isolates. None of the missense mutations in the *frxA* and *fdxB* genes were associated with resistant phenotype.

A total of 12 missense mutations were identified in the *fur* gene, of which *C150Y* and *N118Q* predominated, however, none of mutations were associated with MTZ-R phenotype . Furthermore, no frameshift or nonsense mutations were detected in the *fur* gene among the studied isolates.

Discussion

The steady increase in *H. pylori* antibiotic resistance worldwide significantly impacts the effectiveness of eradication therapy regimens. Meta-analysis data from Russia (2011–2020) indicate that the most significant rise in *H. pylori* resistance was observed for MTZ (33.95%) and LVX (20.0%) [15]. However, subsequent studies from 2015–2019 and 2020–2024 revealed a slight decrease in LVX resistance from 18.3% to 17.1% [15, 16]. Nevertheless, it must be acknowledged that data on the levels and prevalence of antibiotic resistance are still lacking for most Russian regions [2].

Our study, which included 43 *H. pylori* clinical isolates from St. Petersburg (2014–2022), demonstrated a high level of MTZ resistance — 74.4%. This finding underscores the necessity for a full-scale survey of *H. pylori* resistance in this region and calls into question the efficacy of MTZ in local eradication regimens. In contrast, resistance to LVX (a second- and third-line drug) was lower in our sample, at 27.9%. Notably, among the LVX-R isolates, only 15.0% were monoresistant.

[17]. Summarizing the previously presented data on clarithromycin resistance, 20.9% of *H. pylori* isolates are multidrug-resistant, exhibiting simultaneous resistance to three antibiotics: LVX, MTZ, and clarithromycin [17]. It is well known, that multidrug-resistant *H. pylori* strains represent a major obstacle to successful eradication therapy and pose a significant challenge to global gastroenterological health. Treatment failure rates can reach 30% with single antibiotic resistance and exceed 70% with dual resistance [18, 19]. Since the choice of eradication regimen is empirical, the data obtained highlight the necessity for global, regional, and local monitoring of *H. pylori* antibiotic resistance in our country. They also emphasize the need to adapt treatment strategies in each region based on these data and to implement a rational antibiotic use program in eradication therapy regimens [20].

Given that whole-genome sequencing is the most accurate, reliable, rapid and efficient method for identifying known resistance patterns, as well as searching for new ones, this method was used in the study to identify LVX and MTZ resistance determinants and their association with the phenotypic drug resistance of the Russian *H. pylori* population.

Analysis of the obtained data showed that among all mutations in the *gyrA* and *gyrB* genes, only *D91Y/N/G* in the *gyrA* gene was significantly associated with phenotypic resistance of *H. pylori* to LVX ($p < 0.05$). Another mutation associated with the development of resistance to fluoroquinolones, *N87K*, was detected in only 16.7% of resistant isolates in our study ($p > 0.05$). Nevertheless, considering the low detection frequency of the *D91Y/N/G* mutation, as well as the absence of *D91* and *N87* mutations in LVX-S isolates, the combined detection of *D91Y/N/G* and *N87K* mutations should be considered a more reliable predictor of *H. pylori* resistance to LVX. On the other hand, given that 50.0% of LVX-resistant strains lack resistance markers in their genome, genotyping the *gyrA* gene alone may be insufficient to detect phenotypic resistance to LVX, which in turn casts doubt on the rationality of using the *D91+N87 gyrA* genotype as the sole targets when developing PCR tests for determining *H. pylori* antibiotic resistance in clinical practice. It should be noted that mutations *D91* and *N87* were present in the genome of only multidrug-resistant isolates, while these mutations were not detected among LVZ-monoresistant isolates. This could indicate both the existence of other, unstudied resistance mechanisms and the involvement of phenotypic resistance mechanisms, such as changes in the expression levels of efflux systems, biofilm formation and others.Moreover, multidrug-resistant strains may emerge under the selective pressure of combination antibiotic therapy, which promotes the accumulation of mutations, including in the *gyrA* gene, which is associated with resistance to fluoroquinolones. The obtained data require further investigation using an expanded sample of *H. pylori* isolates resistant and susceptible to LVX.

Given the high heterogeneity of *H. pylori* strains, elucidating the mechanisms of MTZ resistance remains a complex challenge. It is generally accepted that *H. pylori* resistance to MTZ is primarily due to the inactivation of the *rdxA* and *frxA* genes, which encode the reduced form of nicotinamide adenine dinucleotide phosphate (NAD(P)H) nitroreductase and flavin nitroreductase, respectively, which catalyze the reduction of MTZ levels within the cell [21]. Numerous international studies have demonstrated that most MTZ-R *H. pylori* strains carry multiple nonsense and/or frameshift mutations, leading to the loss of nitroreductase functional binding sites.

In the current study, mutations causing stop codon loss, premature termination or frameshifts in the *rdxA* gene were found in 21.9% of MTZ-R isolates and were absent in the MTZ-S group. This finding suggests a potential role of this gene in the development of antibiotic

resistance in the Russian *H. pylori* population. According to E.G. Chua et al., the *R16H/C* mutation in the *rdxA* gene is associated with phenotypic resistance of *H. pylori* isolates to MTZ [22]. Our results showed that the *R16C* mutation, as well as the *S108A/P* and *L62V* mutations in the *rdxA* gene, were found only in MTZ-R isolates, with frequencies of 21.9%, 21.9% and 18.7%, respectively. However, due to the uneven distribution of MTZ-R and MTZ-S isolates in our sample (74.4% and 25.6%), the impact of these mutations on the development of phenotypic resistance to MTZ remains to be elucidated.

Similarly, loss-of-function mutations in the *frxA* and *fdxB* genes have been proposed as potential predictors of *H. pylori* phenotypic resistance to MTZ. However, in our study, frameshift mutations in these genes were found in isolates from both resistant and susceptible phenotypic groups. Furthermore, no mutations associated with phenotypic resistance to MTZ were detected in the *fur* gene. These results suggest that *frxA*, *fdxB*, and *fur* genes are unlikely to play a primary role in the development of *H. pylori* resistance to MTZ. Nevertheless, for a more precise understanding of the resistance mechanisms, further research is necessary, including a comprehensive analysis of other potential genetic factors in conjunction with possible synergistic or epistatic interactions.

Research limitations. This study has several limitations. The sample size was limited both numerically and geographically, preventing a comprehensive analysis of *H. pylori* resistance patterns across Russia. Furthermore, an uneven distribution of resistant and susceptible isolates was observed, which significantly complicates the interpretation of the data and reduces the statistical reliability of the results. However, despite these limitations, this study provides important information about the resistance patterns of Russian *H. pylori* clinical isolates and also highlights the necessity for a systematic, nationwide antibiotic resistance monitoring in our country.

Conclusion

Based on whole-genome sequencing data, this study presents the first comprehensive analysis of phenotypic and genotypic resistance to LVX and MTZ in Russian *H. pylori* clinical isolates. Our results have demonstrated a high prevalence of resistance to both MTZ and LVX, alongside a high frequency of polyresistant isolates resistant to three antibiotics (LVX + MTZ + clarithromycin). Despite the limited sample size, we were able to confirm the key role of *D91* and *N87* mutations in the *gyrA* gene in the development of *H. pylori* resistance to LVX. However, the high frequency of resistant isolates that do not carry known resistance determinants calls into question the effectiveness of current and emerging molecular diagnostic tests for determining *H. pylori* susceptibility and dictates the necessity for larger-scale studies to elucidate the full spectrum of antibiotic resistance mechanisms in *H. pylori* .

Our findings emphasize the exceptional importance of issues related to the continuous monitoring for *H. pylori* antibiotic resistance in our country, as well as tracking genome dynamics and resistance development mechanisms. Such efforts could become a prerequisite for optimizing eradication therapy regimens and improving treatment effectiveness for *H. pylori* infections across different regions of Russia. .

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