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Resistance Patterns to Metronidazole and Levofloxacin in Russian *Helicobacter pylori* Clinical Isolates Based on Whole-Genome Sequencing

Starkova D.[1], Gladyshev N.[2], Polev D. [1], Saitova A. [1], Svarval A. [1]

[1] St. Petersburg Pasteur institute, St. Petersburg, Russia

[2] Avtsyn Research Institute of Human Morphology of Federal state budgetary scientific institution "Petrovsky National Research Centre of Surgery", Moscow, Russia

INTRODUCTION & AIM

The resistance of *H. pylori* to antimicrobials is currently considered one of the most serious issues. Moreover, a steady decline in the effectiveness of eradication therapy has been observed in recent years alongside the development of resistance to antibacterial drugs.

Metronidazole (MTZ) is frequently used in eradication therapy as a prodrug that is activated by intracellular reduction of the nitro group, resulting in the production of cytotoxic radicals that damage DNA and disrupt the functioning of bacterial cells. Primary resistance to MTZ in *H. pylori* is due to inactivation of the genes encoding nitroreductase RdxA and flavin oxidoreductase FrxA. Other potential mechanisms of metronidazole resistance include mutations in the ferredoxin-like protein FdxB and the ferric uptake regulator Fur.

Another antibiotic is used in rescue treatment, levofloxacin (LVX), exerts its antibacterial effect by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV,- key enzymes involved in DNA replication and recombination. The most common mechanism of *H. pylori* resistance to fluoroquinolones is caused by point mutations at codons 87 and 91 in the *gyrA* gene. Mutations in the *gyrB* gene may also may be involved in fluoroquinolones resistance development [1, 2].

Due to the lack of data on antimicrobial resistance patterns in Russian *H. pylori* isolates, we aimed to comprehensively investigate resistance determinants in *H. pylori* clinical isolates to metronidazole and levofloxacin and to evaluate the correlation between genotypic and phenotypic antimicrobial susceptibility testing (AST).

METHODS

A retrospective analysis of 43 *H. pylori* clinical isolates from adult patients with gastroduodenal diseases (2014-2022) was performed. The susceptibility of *H. pylori* isolates to MTZ and LVX was determined by conventional disc diffusion method. The results of the disk diffusion method were interpreted based on the threshold values presented by Zhong et al. (2021): *H. pylori* strains were considered resistant to metronidazole (MTZ-R) with an inhibition zone diameter ≤ 16 mm, susceptible (MTZ-S)- with a diameter ≥ 17 mm; resistant to levofloxacin (LVX-R)- with an inhibition zone diameter ≤ 17 mm, susceptible (LVX-S) - with a diameter ≥ 18 mm [3].

The total DNA of *H. pylori* isolates was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH, Germany) according to the manufacturer's guidelines. Whole-genome shot-gun DNA libraries were prepared using MGIEasy FS DNA Library Prep Set and then sequenced on a DNBSEQ-G50 sequencer (MGI Tech Co. Ltd, Beijing, China).

The raw paired-end reads were initially analyzed using FastQC software (v.0.12.1; Babraham Institute, Cambridge, UK), trimmed and filtered by Trim Galore! (version 0.6.7). Bacterial genomes were assembled de novo using SPAdes assembler software (version 3.13.1), and the results were evaluated with QUAST (version 5.2.0). Next, high-quality reads were aligned to the *H. pylori* 26695 reference genome (GenBAnk acc.no. AE000511.1). To evaluate the genetic variations between *H. pylori* isolates and reveal potential genotype-to-phenotype correlations, the insertions/deletions (indels) and SNVs were called from alignments using Snippy pipeline v.4.6.0 (https://github.com/tseemann/snippy) and were visually analyzed using UGENE v. 38.1. All genome assemblies were deposited to the NCBI and available under BioProject "Whole-genome sequence variations in Russian *Helicobacter pylori* isolates" (Accession: PRJNA1011037 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1011037).

All statistics was performed in R programming language, Environment version 4.3.1. The association between genotypic and phenotypic groups was screened using Chi-square and Fisher's exact tests. The significance level of the differences was set at α=0.05.

RESULTS & DISCUSSION

- Phenotypic AST results revealed that 31 isolates were susceptible to LVX (LVX-S) and 11- to MTZ (MTZ-S), while 12 were resistant to LVX (LVX-R) and 32- to MTZ (MTZ-R) (Fig. 1).

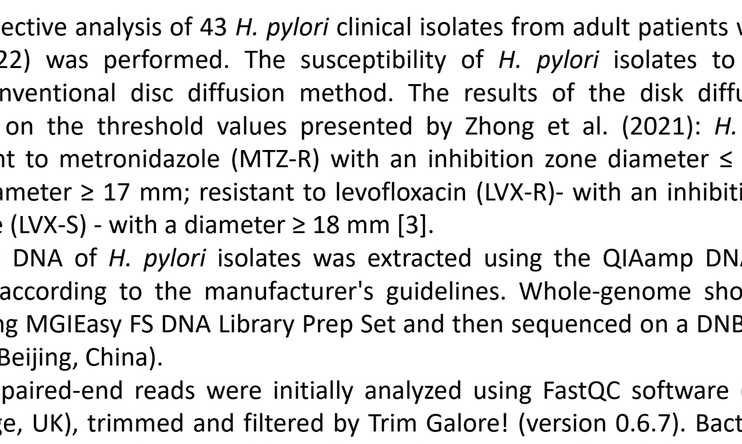

Figure 1. Venn diagram illustrating the combinations of phenotypic drug susceptibility statuses of clinical *H. pylori* isolates to metronidazole and levofloxacin. MTZ-R and LVX-R stands for mono-resistant to MTZ and LVX *H. pylori* isolates, respectively; MTZ-S and LVX-S stands for mono-susceptible to MTZ and LVX *H. pylori* isolates, respectively; MTZ-R+LVX-R (group *A*) represents isolates resistant to both MTZ and LVX; MTZ-S+LVX-S (group *B*) represents isolates susceptible to both MTZ and LVX.

- To identify associations between phenotypic and genotypic resistance, a comprehensive analysis of nucleotide substitutions was performed in the following genes: *gyrA*, *gyrB*, *rdxA*, *frxA*, *fdxB*, and *fur* (Table 1);
- Of all mutations identified in *gyrA* and *gyrB*, only **D91G/N/Y** in *gyrA* was associated with phenotypic resistance to LVX, being present in 4 of 12 (33.3%) LVX-R isolates ($p<0.05$);
- The combined mutation **D91G/N/Y+N87K** in the *gyrA* was detected in 6 of 12 (50.0%) LVX-R isolates ($p<0.001$);
- Mutations D91N/Y/G, N87K and A88P in the *gyrA* gene were found exclusively in isolates from ***group A*** (Fig.1) and were absent in mono-resistant isolates;
- No mutations in *rdxA* were associated with resistance to MTZ; however, 7 of 32 (21.9%) MTZ-R isolates had point mutations leading to a frameshift or premature termination of protein synthesis;
- Similarly, no mutations in *frxA*, *fur*, or *fdxB* were associated with resistance to MTZ.

Table 1. Mutations in levofloxacin and metronidazole resistance genes in *H. pylori* clinical isolates compared to the *H. pylori* 26695 reference genome.

Gene (Locus)	Amino acid change	LVX-R (n=12) N (%)	LVX-S (n=31) N (%)	MTZ-R (n=32) N (%)	MTZ-S (n=11) N (%)	p-value
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

CONCLUSION

- This study presents the first WGS insight into genetic diversity of *H. pylori* in Russia with a particular focus on the molecular basis of resistance to metronidazole and levofloxacin;
- It has been shown that among all genetic variants we obtained, only **D91G/N/Y** mutation in the *gyrA* was associated with phenotypic resistance to levofloxacin;
- Despite the N87K mutation in *gyrA* being found at a low frequency, the detection of combined mutation **D91G/N/Y+N87K** can serve as a predictor of phenotypic resistance to levofloxacin in Russian *H. pylori* clinical isolates;
- None of mutations in the *rdxA*, *frxA*, *fur*, or *fdxB* genes were associated with resistance to metronidazole.

FUTURE WORK / REFERENCES

Given that 50.0% of resistant isolates lacked known quinolone resistance determinants challenges the reliability of diagnostic molecular assays for predicting levofloxacin resistance in *H. pylori*. This underscores the necessity for more extensive studies into antibiotic resistance mechanisms, alongside ongoing surveillance of *H. pylori* antibiotic resistance in Russia.

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