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Genome Note

Genomic insights into virulence, biofilm formation, and antimicrobial resistance of multidrug-resistant *Helicobacter pylori* strains of novel sequence types isolated from Vietnamese patients with gastric diseases

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ABSTRACT

Helicobacter pylori (*H. pylori*) is a clinically important pathogen associated with gastric diseases. Here, we characterized the genome of multidrug-resistant *H. pylori* strains of novel sequence types, which were recovered from Vietnamese patients with gastritis or a stomach ulcer. Phenotypic-antibiotic susceptibility testing was performed against amoxicillin, clarithromycin, metronidazol, tetracycline, and levofloxacin using an E-test. The whole genome sequence of three *H. pylori* strains was de novo assembled, followed by in silico analysis of multilocus sequence typing (MLST), core-genome based phylogeny, genetic determinants associated with virulence, biofilm formation, and antibiotic-resistance. The genome sequence of *H. pylori* strains exhibited a high similarity with the average nucleotide identity (ANI) values of 98.5% to 99.2%, carried 5 to 7 pathogenicity islands, and 3 to 6 mobilomes. The MLST profile of strains revealed novel sequence types ST4511, ST4512, and ST4513. Core-genome based phylogeny exhibited that the three *H. pylori* strains belong to the Asian genotype. These strains possessed 128 to 131 virulence genes, including toxin-encoding genes *cag*A and *vac*A. Double amoxicillin-resistant mutations on *pbp*1 and *pbp*2, or a mutation on *pbp*3, triple clarithromycin-resistant mutations on 23S rRNA gene and a levofloxacin-resistant mutation on *gyr*A were detected in antibiotic-resistant strains. Mutations on *rdx*A were detected in both metronidazole-resistant and -sensitive strains, whereas *frx*A mutations were detected in only one metronidazole-sensitive strain. Finally, a rifamycin-resistant mutation in *rpo*B was also detected. This study provides insights into the genome of multidrug-resistant *H. pylori* strains of a novel sequence type circulating in Vietnam for future investigations of its pathobiology and spread through human populations.

1. Introduction

Helicobacter pylori (*H. pylori*) is the sole bacterial pathogen identified as a class I carcinogen in humans, because of its association with severe gastric diseases. It was estimated that among ap-

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proximately 4.4 billion individuals with positive *H. pylori*, 15% of infected cases could develop gastric diseases [1]. Treatment regimens frequently consist of a proton pump inhibitor along with tetracycline and metronidazole or amoxicillin, clarithromycin, and metronidazole combined with or without bismuth salts [2].

H. pylori exhibits genomic plasticity and diversity that facilitates host adaptation. The pathogen possesses various mechanisms of innate and acquired antibiotic resistance, and important virulence factors which are major concerns in tackling *H. pylori* infections. Furthermore, the ability of biofilm formation in *H. pylori* is strongly associated with colonization, persistent infection, multiple drug resistance, and eradication therapy failure. Genomes of *H. pylori* are highly heterogeneous according to their geographical locations and sequence variations are directly associated with resistance phenotypes and virulence.

Vietnam, among countries with the highest *H. pylori* infection, faces a rapid increase of antibiotic-resistant strains [3,4]. Unfortunately, the genomic characteristics of Vietnamese *H. pylori* strains are poorly comprehended. Here, we explored genetic determinants associated with resistance, virulence, biofilm formation, and biosynthesis of carcinogens in multidrug-resistant *H. pylori* isolates of novel sequence types recovered from Vietnamese patients with gastritis and peptic ulcers.

2. Materials and methods

H. pylori strains were recovered from biopsy pieces of patients undergoing endoscopy at 108 Military Central Hospital, Hanoi, Vietnam, between 2020 and 2022. These patients were diagnosed with gastro-duodenal ulcers and suspected gastric cancer using an endoscope system (Olympus CV-170) with 5 mm opening biopsy forceps.

Bacterial strains were isolated on Pylori Agar, examined by Gram stain and identified as *H. pylori* by the VITEK-MS system (BioMérieux, USA), followed by E-test-based antibiotic susceptibility testing against amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin according to the guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2022. Genomic DNA of *H. pylori* strains was extracted using the DNeasy PowerLyzer Microbial Kit (Qiagen, Hilden, Germany). Whole-genome DNA sequencing was performed on the Illumina NovaSeq PE150 sequencing platform, 2 Gb/sample (Azenta Life Science, South Plainfield, NJ). The draft genomes were de novo assembled with SPAdes version 3.15.5, and checked for completeness by CheckM, then annotated using Prokka version 1.14.6. Multilocus sequence typing (MLST) was performed according to the Institute Pasteur scheme. The allele profile of novel sequence types was registered in the PubMLST database. COGclassifier (version 1.0.5) was used with the basic command to analyse the Cluster of Orthologous Genes (COGs) and amino acid sequences in FASTA format were uploaded to BlastKOALA (version 3.0) to assign KO (K number). To investigate genomic relatedness, average nucleotide identity (ANI) was calculated between the Vietnamese *H. pylori* strains and 53 globally representative strains using FastANI version 1.33. The resulting pairwise similarity matrix was visualized in R software version 4.2.2. For pangenome-based phylogenetic reconstruction, core genome alignments were generated using Roary version 3.13.0, and the phylogeny was visualized using Proksee. Reference strains were selected from the GenBank database (NCBI) to represent a geographically and genetically diverse set of clinical *H. pylori* strains, encompassing both established and novel sequence types. This selection strategy aimed to reflect the extensive genetic heterogeneity of *H. pylori* and to provide a comprehensive overview of its global phylogenetic landscape.

MobileElementFinder version 1.1.2 was used to detect mobile genetic elements (MGEs). In addition, a keyword search for "transposon" and "integron" was done based on the annotation from Prokka to detect MGEs. Genomic islands and pathogenicity islands were predicted using IslandPick, IslandPath-DIMOB, and SIGI-HMM in IslandViewer version 4. Virulence factors were predicted using VFanalyzer and the Virulence Factor Database. Genes encoding for biofilm formation were detected by ARIBA version 2.14.7. Genetic mutations and acquired antibiotic-resistance genes in *H. pylori* strains were identified in silico using a combination of tools, including ResFinder version 4.2.3, AMRfinderPlus version 3.11.14, Resistance Gene Identifier version 6.0.2, and the Comprehensive Antibiotic Resistance Database version 3.2.7.

3. Results and discussion

The genomes of *H. pylori* strains BT020, BT042, and BT063 comprises 1.65 Mb to 1.70 Mb in length with the GC content of 38.5% to 38.6%, and the completeness of 97.6% to 99.4% (Table 1). Among the CDSs identified, 1063 to 1097 genes are predicted to be functional proteins by the COGs, whereas 83 to 39 genes are poorly characterized or functionally unknown. In addition, each strain carried five to seven pathogenicity islands (PAIs), and three to six mobilomes (transposons, integrase, or recombinase) in the genome, in which two to four MGEs are located in PAIs. The genomes exhibit ANI values of 98.5% to 99.2%, similar to the genome of global *H. pylori* strains (Fig. 1A). The core genome-based phylogeny showed that these strains were clustered in a clade of the East Asia genotype (Fig. 1B). The MLST analysis of *H. pylori* strains BT063, BT042, and BT020 revealed novel sequence types, and therefore they are assigned as ST4511, ST4512, and ST4513, respectively, following the Institute Pasteur MLST scheme on PubMLST.org.

Hundred virulence genes were detected in *H. pylori* strains: n = 128 for BT020, n = 131 for BT042, and n = 129 for BT306. These genes encode for different virulence factors including adherence, effector delivery type IV secretion system, exotoxin, immune modulation, motility, stress survival, biofilm formation, and others. The high number of virulence factors associated with motility compared to adherence can facilitate rapid movement in the high-stress gastric environment rather than anchor them to the epithelial layer. All strains are positive for both toxin-encoding genes cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA), which induce ferroptosis, necrosis, and apoptosis in gastric epithelial cells [5,6]. This explains a long-standing infection with CagA and/or VacA-positive *H. pylori* as a promoter of the development of gastric cancer. In addition, these strains possessed 36 genes associated with biofilm formation, including *alp*B, *alp*A, *fla*A, *che*P, *mur*F, *fut*B, *ami*A, *csd*4, *tol*B, *cag*E, *rpo*N, *glu*P, *csd*3, *cgt, flg*R, *csd*6, *pse*B, *flg*E, *tlp*B, *csd*1, *csd*2, *che*Y, *che*A, *mot*A, *mot*B, *fli*A, *ars*R, *cag*D, *che*W, *lux*S, *sp*OT, *fur, nik*R, *ccm*A, *hom*D, and *flh*A, suggesting that they are high-biofilm producers. The genetic feature of these genotypes can increase the antibiotic resistance ability and decrease the eradication rate during the treatment course.

All *H. pylori* strains tested exhibited multidrug-resistant phenotypes (Table 2). Specifically, they were resistant to amoxicillin (minimum inhibitory concentrations [MICs] = 0.75 to 6 μg/mL) and clarithromycin (MIC = 256 μg/mL). Strain BT020 was resistant to additional metronidazole (MIC = 32 μg/mL), whereas strains BT042 and BT063 were resistant to additional levofloxacin (MIC = 32 μg/mL). All strains were sensitive to tetracycline (MICs < 0.23 μg/mL). Genomic analysis revealed concordance between phenotypic and genotypic resistance profiles for amoxicillin, clarithromycin, levofloxacin, and tetracycline, but it was discordant for metronidazole (Table 2). All HP strains harboured well-known amoxicillin-resistant mutations on *pbp*1 (S414R and F366L) and *pbp*2 (S494H and E572G), but particularly strain BT063 carried an additional mutation on *pbp*3 (A541T). Similarly, these strains carried clarithromycin-resistant mutations in 23S rRNA genes

Table 1
Genomic features of *H. pylori* strains.

Number	Feature	*H. pylori* strain BT20	*H. pylori* strain BT042	*H. pylori* strain BT063
1	Genome size (bp)	1,701,376	1,650,873	1,745,035
2	GC content (%)	38.55	38.64	38.55
3	Completeness	99.4%	99.4%	97.6%
4	rRNA genes	2	2	2
5	tRNA genes	37	37	36
6	COGs	1159	1152	1180
7	PAIs	7	5	5
8	MGEs	3	4	6
9	Positive CagA-PAI and VacA-PAI	+	+	+
10	MLST profile	*atp*A (2663), *efp* (2487), *mut*Y (2684), *ppa* (1965), *trp*C (1536), *ure*I (2786), *yph*C (2775)	*atp*A (2663), *efp* (2487), *mut*Y (2684), *ppa* (2592), *trp*C (1536), *ure*I (2754), *yph*C (2770)	*atp*A (2663), *efp* (2487), *mut*Y (2684), *ppa* (1965), *trp*C (1536), *ure*I (2754), *yph*C (2791)
11	Novel sequence type	ST4513	ST4512	ST4511

CagA, cytotoxin-associated gene A; COGs, Clusters of Orthologous Genes; *H. pylori, Helicobacter pylori*; MGE, mobile genetic element; MLST, multilocus sequence typing; PAI, pathogenicity islands; VacA, vacuolating cytotoxin A.

Table 2
Phenotypic and genotypic antibiotic susceptibility profile of *H. pylori* strains.

Antibiotic	*H. pylori* Strains					
	BT020		BT042		BT063	
	MIC[a]	Genetic Mutations	MIC	Genetic Mutations	MIC	Genetic Mutations
Amoxicillin	0.75 (R)	*pbp*1 (F366L, S414R) and *pbp*2 (S494H, E572G)	6 (R)	*pbp*1 (F366L, S414R) and *pbp*2 (S494H, E572G)	6 (R)	*pbp*1 (F366L, S414R), *pbp*2 (S494H, E572G) and *pbp*3 (A541T)
Clarithromycin	256 (R)	23S rRNA (C1707T, A2144G, A2147G)	256 (R)	23S rRNA (C1707T, A2144G, A2147G)	256 (R)	23S rRNA (C1707T, A2144G, A2147G)
Metronidazole	32 (R)	*rdx*A (A118T, C49T, D59N)	**0.016 (S)**[b]	***frx*A (A16T, I44F, V7I, Y62D); *rdx*A (A118T, C49T, D59N)**	**0.016 (S)**[b]	***frx*A (A16T, M126F, V7I, Y62D)**
Levofloxacin	0.19 (S)	ND	32 (R)	*gyr*A (N87K)	32 (R)	*gyr*A (N87K, V172I)
Tetracycline	0.016 (S)	ND	0.125 (S)	ND	0.23 (S)	ND
Rifabutin	ND	ND	ND	ND	ND	*rpo*B (K2068R)

[a] The MIC was determined using E-test.
[b] Discordance between phenotypic and genotypic resistant profile.*H. pylori, Helicobacter pylori*; MIC, minimum inhibitory concentration; ND, not determined.

(A2147G, C1707T, and A2144G). For levofloxacin resistance, strain BT042 harboured a mutation in *gyr*A (N87K), whereas strain BT063 carried double mutations on this gene (V172I and N87K). None of the 16S rRNA gene mutations were detected in these strains. The metronidazole-resistant strain BT020 possessed mutations on *rdx*A (A118T, C49T, and D59N), nonetheless, these mutations along with mutations on *frx*A (A16T, I44F, V7I, and Y62D) were also detected in the metronidazole-sensitive strain BT042. In addition, the same mutations on *frx*A were found in the metronidazole-sensitive strain BT063. This finding underlines the challenges in phenotypic susceptibility testing for *H. pylori* due to its slow and fastidious growth. Furthermore, Marques et al. 2019 found some metronidazole-resistant *H. pylori* without any mutations in *rdx*A and *frx*A, suggesting that other molecular mechanisms need to be investigated [7]. Finally, a rifamycin-resistant mutation in *rpo*B (K2068R) was detected in strain BT063, but its phenotypic susceptibility test was not available. Our findings suggest that multi-drug resistant *H. pylori* strains carrying multiple antibiotic-resistant mutations are circulating in Vietnam. It is crucial to determine the fitness effect of resistance and to investigate fitness compensatory mechanisms to estimate the risk of transmission of these genotypes in the population.

In summary, *H. pylori* strains with novel ST4511, ST4512, and ST4513 possess various distinct virulent factors which promote its phenotypic plasticity for quick adaptability, flexibility, and persistence under the highly stressful gastric environment. Because of a high mutation rate (10^{-5} to 10^{-6} mutations/site/year) [8], *H. pylori* can acquire and accumulate multiple resistance mutations during the treatment leading to multidrug-resistant hypermutable *H. pylori* strains. Insight into their genome could help develop new

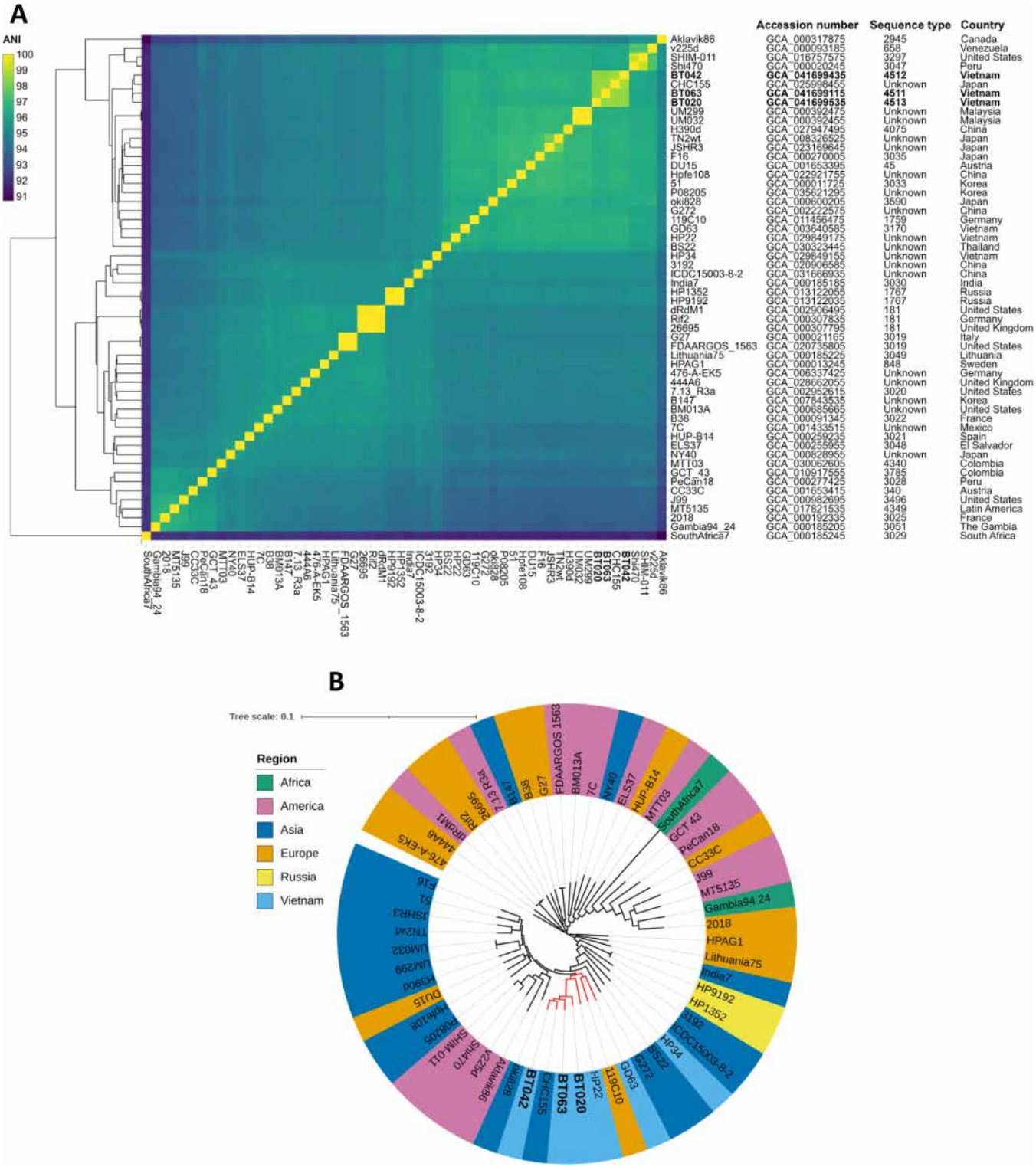

Fig. 1. Heatmap of ANI relationships (**A**) and a pangenome-based phylogenetic tree (**B**) of *H. pylori* strains from diverse genotypes and geographic origins. Strains are labelled by name; GenBank accession numbers, sequence types, and countries of origin are listed in the accompanying table (top left). The three Vietnamese *H. pylori* strains from this study, representing novel sequence types, are highlighted in bold in both panels and the table. ANI, average nucleotide identity; *H. pylori, Helicobacter pylori*.

treatment strategies in order to cure, stop the spread of *H. pylori*, and decrease treatment failure.

Competing interests

None declared.

Accession numbers

The genome sequence of *H. pylori* strains BT20, BT042, and BT063 was submitted to the NCBI GenBank database with BioProject PRJNA1145883, BioSample SAMN43304330, SAMN43304332, and SAMN43304333, respectively.

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Ethical approval

The bacterial isolates were collected in the framework of a project that was approved by the Ethical Committee of 108 Military Central Hospital according to Decision number 5054/GCN-BV dated on 19 October 2021.

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