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Identification of genetic determinants of antibiotic resistance in *Helicobacter pylori* isolates in Vietnam by high-throughput sequencing

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Abstract

The aim of this study was to identify genetic factors responsible for antibiotic resistance in *Helicobacter pylori,* a bacterium that can cause long-term gastroduodenal disease. The primary resistance of *H. pylori* to commonly used antibiotics was studied, and high-throughput next-generation sequencing (NGS) was employed to discover genetic determinants of resistance using a reference-based approach. A total of 123 *H. pylori* strains were cultured and tested for antibiotic susceptibility using an E test. Genotypic analysis was performed using NGS data with ARIBA v2.14.7 and PlasmidSeeker v1.3 for plasmid detection. Statistical correlations between resistant genotypes and phenotypes were evaluated. In addition, a genome-wide association study (GWAS) and linear mixed model were used to identify genetic variants associated with antimicrobial resistance phenotypes while adjusting for covariates such as population structure. Our results showed that 78.2% of the strains were resistant to metronidazole (MTZ), 22.5% to levofloxacin (LVX), 43.5% to clarithromycin (CLR) and 13.7% to amoxicillin (AMX). Resistance to tetracycline was not detected. Multi-drug resistance was detected in 48.8% of the strains. While plasmids were not detected, chromosomal genetic determinants of resistance to CLR, LVX, and AMX were identified, including mutations in 23S *rRNA* (A2142G and A2143G), *gyrA* (N87K/Y and D91Y/N/G), and *pbp1 A* (F366L, S414R, F473V, G595_V596insE, as well as the mutations T558S and T593A/G/P/S). Additionally, missense, frameshift, and nonsense mutations in *rdxA* were identified as genetic determinants of resistance to MTZ. No genetic determinants associated with tetracycline resistance were detected. A strong correlation was observed between resistance genotypes and phenotypes for CLR, LVX, AMX, and MTZ. In addition, we found that missense, frameshift and nonsense mutations in *rdxA* were genetic determinants of resistance to MTZ. We did not detect any genetic determinants associated with tetracycline resistance. There was a strong correlation between resistance genotypes and phenotypes for CLR, LVX, AMX, and MTZ. Furthermore, unitig-based GWAS revealed that AMX, LVX, and CLR resistance in *H. pylori* was mainly caused by chromosomal mutations that affected the targets of these antibiotics (*pbp1 A*, *gyrA*, and 23S *rRNA*, respectively). Our results highlight the need for regular evaluation and alternative therapies in Vietnam, given the high rates of *H. pylori* resistance to CLR,

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MTZ, and LVX. Our study also demonstrated the high capacity of NGS to detect genetic resistance determinants and its potential for implementation in local treatment policies.

Keywords *Helicobacter pylori*, Pangenome-wide association study, Antibiotic resistance, High-throughput sequencing

Introduction

Long-term *Helicobacter pylori* infection in the human stomach is associated with gastroduodenal disease and an increased risk of developing gastric cancer [1]. *H. pylori* can cause dysbiosis in the gastric microbiota and activate internal signaling in the carcinogenesis pathway [2]. Early treatment of *H. pylori* infection can restore homeostasis, which is crucial for preventing severe clinical outcomes [2]. *H. pylori* antimicrobial resistance is increasing globally, particularly in the Asia–Pacific region, leading to treatment failure and burdening the healthcare system and patients [3]. The most recently reported primary resistance prevalences in the Asia–Pacific region were 30% for clarithromycin (CLR), 61% for metronidazole (MTZ), 35% for levofloxacin (LVX), 4% for tetracycline (TET), and 6% for amoxicillin (AMX) [3]. Routinely performing antibiotic susceptibility testing (AST) on each isolate in clinical laboratories, is recommended in certain situations to guide treatment, especially when patients have failed initial treatment, have allergies to first-line antibiotics, or live in areas with high resistance rates.

Traditional culture-based methods for detecting *H. pylori* infection are limited by long wait times and the need for well-equipped laboratories. Furthermore, interpreting the results is subjective and difficult to replicate. However, PCR-based techniques offer faster results and can be used directly on various samples. Additionally, molecular-based test kits that detect *H. pylori* and mutations that confer resistance to CLR and LVX are available, providing results within hours. However, detecting resistance to MTZ and AMX remains challenging because of the diverse molecular mechanisms involved [4]. High-throughput sequencing technologies, including whole-genome sequencing (WGS), offer promising alternatives for monitoring *H. pylori* antibiotic resistance, as well as infection with antimicrobial resistant strains, in clinical microbiology laboratories [5].

The mechanism by which *H. pylori* eliminates antibiotics has been extensively studied. Three resistance profiles have been identified, namely: single drug resistance, multidrug resistance, and heteroresistance [6]. These resistance profiles have overlapping fundamental mechanisms and clinical implications. The most common molecular mechanisms that have been studied involve chromosomal mutations that alter the structure of antibiotic targets [4]. Advances in understanding the fundamental molecular aspects of drug resistance in *H. pylori* have facilitated the development of molecular techniques for the rapid detection of antibiotic resistance in clinical specimens [7]. Specific mutations in domain V of 23S rRNA, which is a component of the bacterial protein translation machinery, cause CLR resistance [8]. The two most common mutations in 23S rRNA, A2142G and A2143G, are the main causes of CLR resistance in *H. pylori* [4]. Additionally, resistance to LVX is mainly caused by the specific mutations N87 K/I/Y/H and D91G/N/Y/A in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene, which encodes DNA gyrase [9]. PCR-based techniques are used to detect these mutations in *H. pylori* clinical specimens [7]. Mutations in the gene encoding *pbp1 A*, which possesses both transglycosylase and transpeptidase activity, confer resistance to AMX [4]. The mechanism of MTZ resistance primarily involves the inactivation of both nitroreductase enzymes, *frxA* and *rdxA*. However, the mechanisms underlying resistance to MTZ and AMX are complex and still not well understood [4].

Bacterial genome-wide association studies (GWASs) are a new and exciting research technique in which human GWAS methods are used to understand how variations in microbial genomes affect host or pathogen phenotypes, such as drug resistance, virulence, host specificity, and prognosis [10]. However, this approach is prone to false positives due to correlations among genetic variations. *H. pylori*, which has a high rate of homologous recombination, does not exhibit this correlation, thus eliminating the problem of false positives [11, 12]. Although chromosomal mutations are widely accepted genetic determinants of antibiotic resistance in *H. pylori*, the *H. pylori* genome contains many genes that help the bacterium adapt to environmental changes such as antibiotics, host interactions, and the gastric environment [13].

The aim of this study was to determine how *H. pylori* develops antibiotic resistance by analyzing WGS data. Both reference-based and panGWAS approaches were used to identify genetic variations linked to antimicrobial resistance in *H. pylori*.

Methods

Bacterial isolation, whole-genome sequencing, and assembly

Using conventional culture techniques, *H. pylori* strains were isolated from patients who underwent upper endoscopy at Cho Ray Hospital in Ho Chi Minh City, Vietnam and 108 Military Hospital in Hanoi, Vietnam from November 2012 to May 2014 as described in our previous studies [14]. Exclusion criteria included a history of gastrectomy; previous *H. pylori* eradication therapy; treatment with bismuth-containing compounds, H2-receptor blockers, or proton pump inhibitors within 2 weeks of the start of the study; and patients with suspected recurrent Billroth anastomosis cancer, entire stomach tumor, or cardiac tumor. Briefly, antral biopsy specimens were homogenized, inoculated onto *H. pylori*–selective plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and incubated for 3 to 10 days at 37 °C under microaerophilic conditions. The resulting purple colonies were subcultured in Brucella broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 7% horse blood. Bacterial DNA was extracted in 2014 using the DNeasy Blood and Tissue Kit (Qiagen Inc., Hilden, Germany) for whole-genome sequencing. DNA concentrations were quantified using a Quantus Fluorometer (Promega Corporation, Madison, WI, USA) to confirm a minimum of 10 ng double-stranded DNA (dsDNA) per sample required for library preparation. Library preparation was performed using the TruSeq Nano DNA Library Prep Kit according to the manufacturer's protocol. Bacterial genomes were sequenced on either the HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) with 2× 100 or 2× 150 bp paired-end reads, or the MiSeq platform with 2× 300 bp paired-end reads, in accordance with the manufacturer's instructions.

The complete genome sequence of *H. pylori* was obtained using a long-read sequencing platform (Pacific Biosciences of California, Inc., Menlo Park, CA, USA) and used as a reference. The SMRTbell Template Prep Kit 1.0 (Pacific Biosciences of California, Inc., Menlo Park, CA, USA) was used to prepare a SMRTbell library. DNA fragments greater than 17 kbp were selected using the BluePippin system (Sage Science, Inc., Beverly, MA, USA). Each *H. pylori* strain was sequenced on one SMRT cell using the PacBio RS II System with P6/C4 or P6/C4v2 chemistry and 360-min movies. The SMRT sequencing data were analyzed using SMRT Analysis v2.3.0 through the SMRT Portal. RS_HGAP_Assembly.2 was used to assemble the reads. The overlapping ends were trimmed, and the chromosomal contig was started from the ori-sequence. The contig was then resequenced with RS_Resequencing.1 to generate consensus sequences.

Determination of antibiotic resistance phenotypes and genotypes

A total of 123 clinical *H. pylori* isolates, previously obtained in a study conducted in Vietnam [14], were analyzed. From 2012–2014, the antimicrobial susceptibility testing (AST) was assessed using Epsilometer-test (Etest®, bioMerieux) for five antibiotics (amoxicillin, clarithromycin, levofloxacin, tetracycline, and metronidazole) following European Committee on Antimicrobial Susceptibility Testing (EUCAST) v.21.01.01 protocols on Müller–Hinton agar plates supplemented with 5% horse blood. The minimum inhibitory concentrations (MICs) of the antibiotics were monitored daily after incubation for 3–6 days. *H. pylori* strain 26,695 was used as a control strain. The clinical breakpoints distinguishing between resistant and susceptible strains were determined in accordance with the EUCAST guidelines, accessible at http://www.eucast.org/.

Bioinformatics analysis

The integrity of the raw sequence data obtained from high-throughput sequencing was assessed using FastQC v0.12.1. Reads of substandard quality (Q < 15), along with adapter sequences and sequences shorter than 36 residues, were eliminated using Trimmomatic v0.32 [15]. Three bases were truncated from both the beginning and end of a read if the sequence fell below the quality threshold, using the following function: TruSeq3-PE.fa:2:30:10:2:True LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. The raw sequence reads from each strain were assembled into contigs using Unicycler v0.5.0 [16]. Contigs shorter than 200 base pairs were excluded. To identify genome features within a collection of genomic DNA sequences, the assembled *H. pylori* genomes were annotated using Prokka v1.14.5, a rapid prokaryotic genome annotation pipeline [17]. The core genome alignment was extracted from the pangenome analysis of 123 strains using Panaroo v1.3.4, based on the GFF files generated by Prokka [18]. The maximum-likelihood (ML) phylogenetic tree based on core genome alignment was generated by IQTREE v2.2.2.7 using the generalized time-reversible model, 1000 bootstrap replicates, and ancestral state [19]. The ML phylogenetic tree, the distribution of susceptible/resistant phenotypes, and the distribution of genetic determinants of antibiotic resistance were visualized using Phandango [20].

Analysis of drug susceptibility genotypes encoded by plasmids in H. pylori isolates

Short-read sequences were used to identify plasmids that encode genes related to antibiotic resistance. This was

achieved using PlasmidSeeker v1.3, with *H. pylori* 26,695 (NC_000915.1) serving as the reference genome [21]. The read coverage of short DNA fragments of length k (k-mers) was analyzed against a publicly available plasmid reference database (http://bioinfo.ut.ee/plasmidseeker/). The abundance of k-mers was used to differentiate between plasmid and bacterial sequences. A plasmid was deemed to be present if at least 80% of the k-mers were detected.

Analysis of drug susceptibility genotypes encoded by the genomic DNA of H. pylori isolates

H. pylori genomic sequences associated with resistance phenotypes to AMX, LVX, CLR, TET, and MTZ were retrieved and used to construct a variant-calling database. Reference sequences were obtained from *H. pylori* strain 26,695 (CP003904.1), including *pbp1 A* (AMX), 23S *rRNA* (CLR), *gyrA* (LVX), *gyrB* (LVX), 16S *rRNA* (TET), and *rdxA* (MTZ). ARIBA v2.14.7, a local-assembly tool for identifying antibiotic resistance genes [22], was used to detect relevant genetic variants. For variant calling, ARIBA used two inputs: a FASTA file of reference sequences, and paired-sequencing reads. Detailed information, including variants and amino acid changes in both coding and noncoding sequences between the sequencing reads and the reference sequences, is reported.

PanGWAS approach to identifying genetic determinants of antibiotic resistance

The PanGWAS analysis was performed on a total of 255 *H. pylori* strains, comprising 159 WGS strains from the present study and 96 WGS strains obtained from two previous studies (Table S8) [5, 23]. To test associations between genetic variants and antibiotic resistance phenotypes, a single pan-genome-wide association study (panGWAS) was conducted using the Fast-LMM (factored spectrally transformed linear mixed model) algorithm implemented in *pyseer* v.1.3.11 [24]. PySeer estimates the effect of genetic variation in a bacterial population on a phenotype of interest while accounting for potentially very strong confounding population structure. The binary phenotype file for each antibiotic is supplied, with the sample names in the first column and the phenotype (1: resistant, 0: susceptible) in the last column. To reduce both computational and statistical complexity, *unitigs* identical to k-mers were used as input via the *–kmers* option. Unitigs are nodes in a compressed de Bruijn graph, and using them eliminates some of the redundancy present in k-mer counting, as well as requiring fewer tests [25]. The unitigs, which accounted for all sequence elements in the bacterial sequence data, were counted using the unitig-caller v.1.3.0 package (https://github.com/bacpop/unitig-caller). To reduce the false discovery rate, unitigs with a minor allele frequency greater than 5% were extracted. A unitig-based GWAS analysis was performed following the *pyseer* manual, utilizing a linear mixed regression model to control for population structure based on an $n \times n$ relatedness matrix calculated froma core-genome phylogenetic tree of *H. pylori* strains (https://pyseer.readthedocs.io/en/master/). A Q–Q plot was created using the qq_plot.py script provided by PySeer to assess the number and magnitude of observed associations between the number of unitigs and antibiotic resistance compared with the association statistics expected under the null hypothesis of no association.

Results

Primary *H. pylori* antibiotic susceptibility in Vietnam

Table 1 shows the antibiograms of 123 isolates from Vietnam. MTZ resistance was the most common—it was observed in 78.2% (95% CI, 69.7; 84.9) of the isolates. This was followed by CLR resistance in 43.5% (95% CI, 34.7; 52.7), LVX resistance in 22.5% (95% CI, 15.7; 31.1), and AMX resistance in 13.7% (95% CI, 8.4; 21.3). No isolates were found to be resistant to TET. Only 14 isolates (11.3%, 95% CI: 6.5; 18.5) were found to be susceptible to all antibiotics tested (Table S7).

Table 1 Antibiotic resistance of 123 *H. pylori* isolates from Vietnam

Susceptibility Phenotype	*n*	% (95% CI)
Susceptibility to all antimicrobials	**14**	**11.3 (6.53; 18.53)**
Resistance to at least one antimicrobial	**110**	**88.7 (81.4; 93.4)**
MTZ	97	78.2 (69.7; 84.9)
LVX	28	22.5 (15.7; 31.1)
AMX	17	13.7 (8.4; 21.3)
CLA	54	43.5 (34.7; 52.7)
Single drug resistance	**49**	**39.5 (30.9; 48.7)**
MTZ only	40	32.2 (24.3; 41.3)
CLR only	7	5.6 (2.5; 11.7)
LVX only	1	0.8 (0.0; 5.0)
AMX only	1	0.8 (0.0; 5.0)
Multidrug resistance	**61**	**49.2 (40.1; 58.2)**
LVX + MTZ	11	8.8 (4.7; 15.6)
AMX + LVX + MTZ	1	0.8 (0.0; 5.0)
CLR + LVX + MTZ	11	8.8 (4.7; 15.6)
AMX + CLR + LVX + MTZ	4	3.2 (1.0; 8.5)
AMX + MTZ	2	1.6 (0.2; 6.2)
CLA + MTZ	23	18.5 (12.3; 26.7)
AMX + CLA	4	3.2 (1.0; 8.5)
AMX + CLA + MTZ	5	4.0 (1.4; 9.6)

Notably, 39.5% (95% CI, 30.9; 48.7) of the isolates exhibited single drug resistance, 32.2% (95% CI, 24.3; 41.3) were MTZ-resistant, and 5.6% (95% CI, 2.5; 11.7) were CLR-resistant. Only one isolate each exhibited single drug resistance to AMX and to LVX. The rate of multidrug resistance was high, at 49.2% (95% CI, 40.1; 58.2). Dual drug resistance to CLR +MTZ and LVX +MTZ was found in 18.5% (95% CI, 12.3; 26.7) and 8.8% (95% CI, 4.7; 15.6) of the isolates, respectively and dual drug resistance to AMX +MTZ and AMX +CLR groups was found in 1.6% (95% CI, 0.2; 6.2) and 3.2% (95% CI, 1.0; 8.5) of the isolates, respectively. Triple drug resistance to CLR +LVX +MTZ, AMX +CLA +MTZ, and AMX +LVX +MTZ accounted was observed in 8.8% (95% CI, 4.7; 15.6), 4.0% (95% CI, 1.4; 4.6), and 0.8% (95% CI, 0.0; 5.0) of isolates, respectively. Finally, 3.2% (95% CI, 1.0; 8.5) of the isolates were found to be resistant to AMX +CLR +LVX +MTZ (Table S7).

Relationship between antibiotic resistance patterns and strain relationships in Vietnamese H. pylori isolates

Reconstruction of a maximum likelihood tree, which pertains to core single nucleotide polymorphisms concerning varying resistance patterns (Fig. 1) showed no association between strain relatedness and resistance to any individual antibiotic or combination of drugs. In contrast, the isolates exhibited unique susceptibility profiles, irrespective of their genetic relatedness.

Plasmid detection in *H. pylori* isolates from Vietnam

A plasmid reference database including a total of 19,782 known plasmids from published species, 69 of which are specific to *H. pylori*, was used to determine whether any of the isolates carried one or more plasmids. No plasmids encoding antibiotic resistance genes were found among the 123 analyzed *H. pylori* genomes.

Genetic determinants of antibiotic resistance in Vietnamese *H. pylori* isolates

CLR resistance

Our investigation of CLR resistance focused on genetic variations within the full-length 23S *rRNA* gene. A single point mutation in the peptidyl transferase region of domain V of this gene is known to be responsible for CLR resistance. Given the presence of two copies of the 23S *rRNA* gene in the *H. pylori* genome, the depth of reads mapped to each nucleotide variation position was analyzed to detect allelic variation. A total of 186 variants were identified among 53 CLR-resistant and 70 CLR-susceptible strains. Notably, only two mutations, A2142G ($p = 4 \times 10^{-11}$) and A2143G ($p = 5 \times 10^{-23}$), were significantly associated with CLR resistance (Table 2).

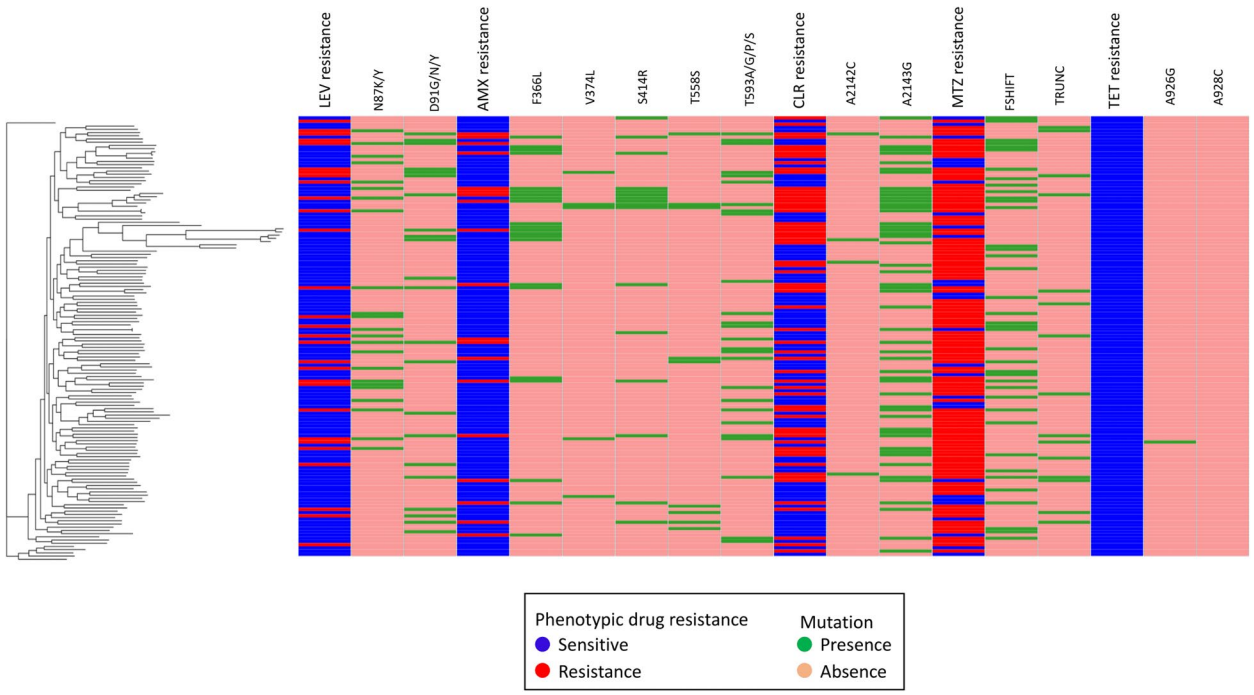

Fig. 1 The maximum likelihood phylogenetic tree based on whole-genome sequences, resistance patterns, and their corresponding genetic determinants for each antibiotic in the 123 *H. pylori* genomes sequenced in this study. The antibiotic sensitivity and resistance patterns are represented by dark blue and red rectangles, respectively. The presence and absence of mutations are indicated by green and pink rectangles, respectively

Table 2 Summary of the known and novel mutations in the 23S *rRNA* gene in *H. pylori* isolates

Variant		Variants		No variants		P value
		Resistant	Sensitive	Resistant	Sensitive	
Known variants	A2142G	4	0	49	70	4E-11
	A2143G	46	1	8	68	5E-23
	A2143 AG	2	0	52	69	2E-07

Among the CLR-resistant strains, 85% (51/60) carried the A2143G mutation, 6.7% (4/60) carried the A2142G mutation, and 8.3% (5/60) did not have either mutation. Conversely, all but one of the CLR-susceptible strains (98.6%, or 76/77) carried neither of the mutations.

Furthermore, strains harboring either the A2142G or A2143G mutations exhibited significantly greater CLR minimum inhibitory concentrations (MICs) than those without mutations (all $p < 0.05$) (Fig. 2A). However, there was no significant difference in the average MICs among strains with the A2142G mutation and those with the A2143G mutation. Interestingly, all reads (100%) from the 54 CLR-resistant strains mapped to either A2142G or A2143G, indicating that the mutations occurred in both copies of the 23S rRNA gene. Furthermore, two of the CLR-resistant strains carried the A2143G mutations in only one copy of the 23S *rRNA* gene (Table S1). This finding underscores the uniformity of the mutations in the CLR-resistant strains.

LVX resistance

Point mutations in the QRDR region of *gyrA* confer resistance to LVX in *H. pylori*. Genetic variations in *gyrA* were examined in 95 LVX-susceptible and 28 LVX-resistant strains. Among 213 coding mutations identified, only N87 K/Y and D91 N/G/Y ($p < 0.001$) were significantly associated with LVX resistance (Table 3 and Table S2). Notably, 21.4% (6/28), 14.28% (4/28), 3.5% (1/28), and 51.7% (15/28) of the LVX-resistant strains exhibited Gly-91, Asn-91, Tyr-91, and Lys-87/Tyr-87 mutations, respectively. Among the 28 LVX-resistant strains, 26 (92.8%) carried mutations at codons 87 and 91. Conversely, four strains did not exhibit any mutations at these codons, yet were LVX-resistant (Table S2).

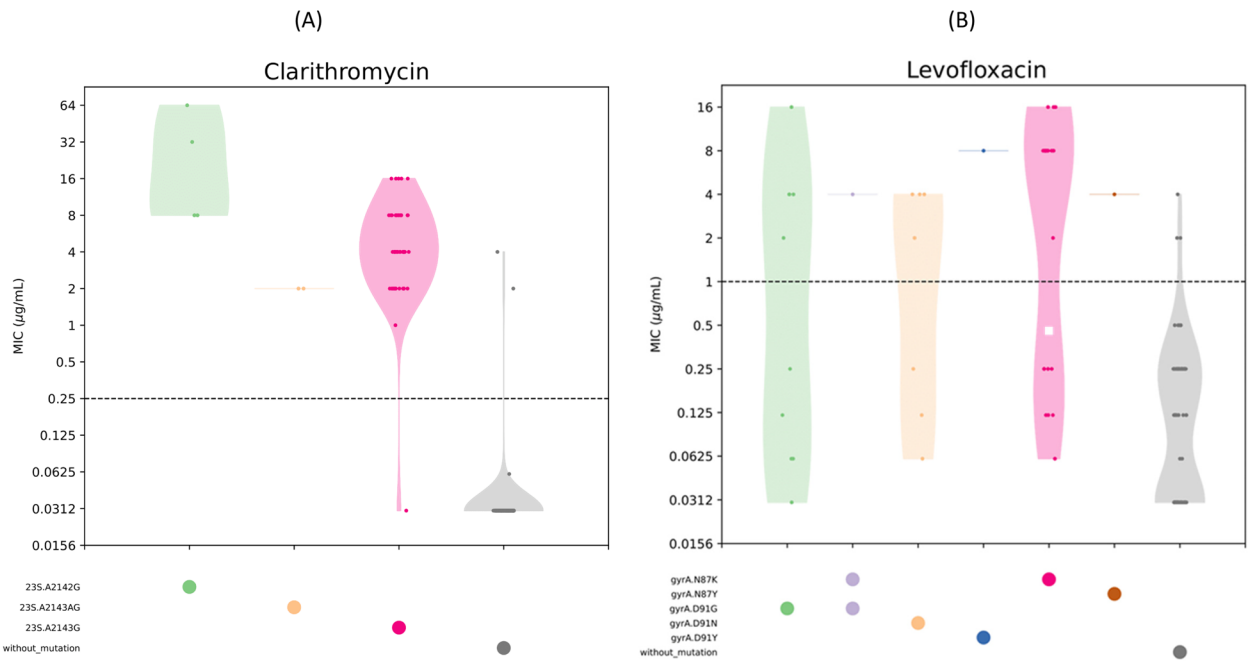

Fig. 2 **A** Distribution of clarithromycin minimum inhibitory concentrations (MICs) and corresponding mutations for all observed combinations of relevant antibiotic resistance determinants. **B** Distribution of levofloxacin MICs for all observed combinations of relevant antibiotic resistance determinants. Dotted horizontal lines mark clinical breakpoints. *** indicates $p < 0.001$ compared with isolates without mutations, ** indicates $p < 0.01$, * indicates $p < 0.05$

Table 3 Summary of known and novel *gyrA* mutations in *H. pylori* isolates

Variants		Variants		No variants		P value
		Resistant	Sensitive	Resistant	Sensitive	
	H57Y	**0**	**1**	**28**	**94**	**1**
Known variants	N87 K	14	8	14	87	2.70E-07
	N87Y	1	0	27	95	0.22
	A88 T	0	1	27	96	1
	D91 N	4	3	24	92	0.005
	D91G	6	5	22	90	0.088
	D91Y	1	0	27	95	0.22
	D99 A	1	0	27	95	0.22
	R130 K	2	13	26	82	0.517
Novel variants	A231 V	7	2	21	93	0.00037

In general, strains with mutations at either codon 87 or 91 had significantly greater LVX MICs than those without mutations (all $p < 0.05$) (Fig. 2B). Interestingly, only one strain was mutated at both codon 87 and codon 91, and it had a MIC of 4 μg/mL (Fig. 2B). Furthermore, it has been observed that two known variants D481E and R484 K of *gyrB* were detected in two LEV-R strains, while they were absent in LEV-S strains (Fig. S1 and Table S5).

AMX resistance

A comprehensive examination of *pbp1 A* mutations was conducted in 17 AMX-resistant and 106 AMX-susceptible strains, given the known role of this gene in AMX resistance. A total of 176 distinct coding mutations in *pbp1 A* were identified (Table S3). Notably, isolates with four different mutations—F366L ($p < 0.001$), S414R ($p < 0.001$), F473 V ($p < 0.001$), and G595_V596 insE ($p < 0.001$)—also carried a T558S mutation. T593 A/G/P/S ($p < 0.001$) mutations were significantly associated with AMX resistance (Table 4). In conclusion, strains harboring mutations at codons 366, 414, 473, 558, 593, and 595 demonstrated significantly higher AMX MICs than those without mutations (all $p < 0.05$) (Fig. 3A).

Metronidazole resistance

Loss-of-function (e.g., missense, nonsense, and frameshift) mutations in *rdxA* play a crucial role in MTZ resistance. All the MTZ-resistant strains had at least one missense/nonsense/frameshift mutation in the *rdxA* gene (Fig. 3B and Table S4 and S6). The MICs of most strains were greater than 8, indicating that mutation of this gene abolishes its function, leading to an increase in MTZ resistance. Several missense mutations were identified, including C19Y, K20 T, S43L, V55G, M56 V/L, N73 K, G145R, G162R, and G163D/S (Table S6). These missense mutations have been shown to encode functional codons in a previous study [26]. In addition to the nonsense mutations, frameshift and truncation mutations in *rdxA* were also identified (Table S6). As shown in Fig. 3B, the isolates carrying *rdxA* frameshift and truncation mutations were generally resistant to MTZ, indicating that these two types of mutations have a significant impact on *rdxA* function.

Correlations among antibiotic susceptibility genotypes and phenotypes

The effectiveness of WGS as a tool for predicting bacterial susceptibility to various antibiotics was assessed. To do this, the antibiotic resistance phenotypes were compared with the presence and absence of mutations. The results, as presented in Table 5, demonstrated a significant degree of concordance between genotypic and phenotypic drug resistance. This finding implies that the genetic makeup of bacteria (genotype) is a reliable predictor of their resistance or susceptibility to antibiotics (phenotype). More specifically, the concordance was found to be in perfect agreement for CLR (0.95), strong for AMX (0.90), moderate for MTZ (0.81), and in substantial agreement for LVX (0.60).

Pangenome analysis of antibiotic resistance in *H. pylori*

A De Bruijn graph, also known as a pangenome graph, was constructed from a total of 255 *H. pylori* genomes. Accession numbers and assembly levels of all genomes used were provided in Supplementary Table S8. The pangenome analysis included a total of 3,928,855 unitigs. Filtering based on a minor allele frequency of at least 5%excluded 2,346,859 unitigs, and the remaining 1,581,996 unitigs were tested for their associations with antibiotic resistance. Q–Q plots were created using the P values of the associations between unitigs and antibiotic resistance (Fig. 4) to assess the number and magnitude of

Table 4 Summary of *pbp1 A* mutations in *H. pylori* isolates

Variants		Variants		No variants		P value
		Resistant	Sensitive	Resistant	Sensitive	
Known variants	V45I	0	12	17	94	0.369
	V250I	0	10	17	96	0.354
	F366L	11	10	6	95	2.6E-11
	V374L	0	5	17	101	1
	S402G	0	1	17	105	1
	S414R	9	5	8	101	2.47E-06
	S455 N	0	1	17	105	1
	V469M	2	1	15	105	0.049
	F473 V	11	11	6	95	2.91E-06
	A474 T	0	7	17	99	0.59
	D535 N	1	50	16	56	0.51
	S543R	4	5	13	101	0.021
	T556S	0	5	17	101	1
	T558S	3	6	14	100	0.073
	N562H/Y	3	2	14	104	0.018
	T593 A/G/P/S	4	19	13	87	0.51
	T593 A/G/P/S.T558S	2	1	14	106	0.044
	G595S	2	20	15	86	0.73
	G595_V596 insE	12	17	5	89	1.49E-05
	D465_L466 insK	5	12	12	94	0.057
Novel variants	K315E	10	21	7	85	0.001
	K464_D465 insK	4	1	13	105	0.001
	D504 N	5	11	12	95	0.046

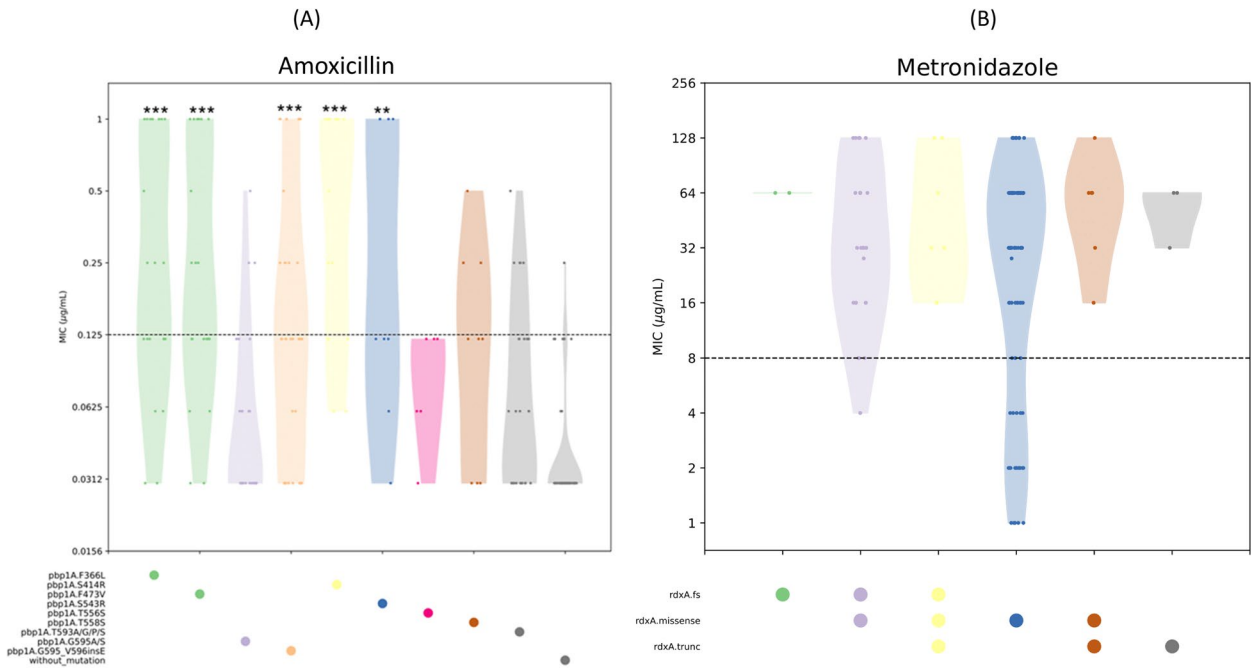

Fig. 3 **A** Distribution of amoxicillin minimum inhibitory concentrations (MICs) and corresponding mutations. **B** Distribution of metronidazole MICs for all observed combinations of relevant antibiotic resistance determinants. Dotted horizontal lines mark clinical breakpoints. *** indicates $p < 0.001$ compared with groups without mutations, ** indicates $p < 0.01$, * indicates $p < 0.05$

Table 5 Agreement between antibiotic resistance genotypes and phenotypes of in *H. pylori* strains isolated in Vietnam

Antibiotic	Mutations	Phenotype		Cohen's kappa (95%CI)
		Resistant	Susceptible	
		n (%)	n (%)	
AMX (*pbp1 A*)	Resistant (mutation)	17 (100)	3 (29.2)	0.90 (0.796;1)
	Susceptible (no mutation)	0	93 (70.7)	
CLR (23S *rRNA*)	Resistant (mutation)	52 (96.3)	1 (1.4)	0.95 (0.89;1)
	Susceptible (no mutation)	2 (3.7)	68 (95.5)	
LVX (*gyrA*)	Resistant (mutation)	24 (85.7)	16 (16.8)	0.6 (0.44; 0.75)
	Susceptible (no mutation)	4 (14.2)	79 (83.1)	
MTZ (*rdxA*)	Resistant (mutation)	91 (100)	8 (100)	0.81 (0.69; 0.93)
	Susceptible (no mutation)	0	24 (0)	

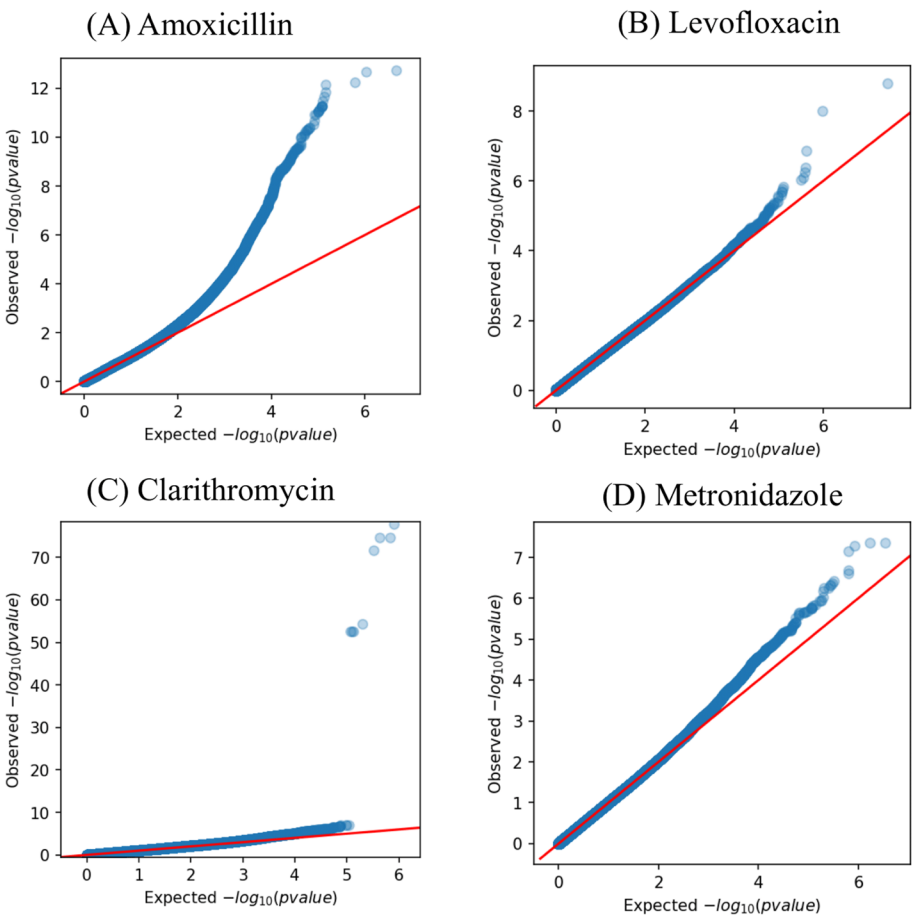

Fig. 4 Q–Q plot constructed to assess the genome-wide association study results. Each dot in (**A**), (**B**), (**C**), and (**D**) indicates a unitig. Y-axis: observed − log10(P) of each unitig, where P is its P value. X-axis: expected − log10(P) under the null hypothesis of no association

the observed associations compared with the statistical values expected for the null hypothesis of no association. The pangenome GWAS showed that the P values of most unitigs were as expected under the null hypothesis for AMX, LVX, CLR, and MTZ resistance (Fig. 4A, 4B, 4C, 4D). In addition, there was a strong association between LVX resistance, CLA resistance, and AMX resistance and the unitigs.

The hits column indicates the number of unitigs mapping to the pangenome. -log10(P value): The

-log10 of the Bonferroni P value of the unitigs. The average minor allele frequency is the second highest frequency of variants in the population. Average beta: effect size of variants on the antimicrobial resistance phenotype.

Mapping significant unitigs to our isolate dataset identified 42 and four genes related to AMX and LVX resistance, respectively (Fig. S2 and Table 6). In contrast, significant CLR-resistance unitigs only mapped to 23S rRNA, while no unitigs were found to be associated with MTZ-resistance—all P values were under the significance threshold. In brief, the *pbp1 A*, *gyrA*, and 23S rRNA genes exhibited the strongest correlations with AMX-resistance, LVX- resistance, and CLR-resistance, respectively (Table 6). Genetic variations in 23S rRNA were most strongly related to CLR resistance, with an effect size of 0.84, a -log10 (P value) of 77, and 77 hits. For AMX resistance and LVX resistance, *pbp1 A* and *gyrA* mutations were most common, with 15 and 21 hits, respectively, and -log10(P values) of 12.7 and 8.7, respectively (Table 6). Additionally, two genes associated with AMX resistance, encoding components of the type IV secretion system: *virB11* and *virD4*—were identified. Both *virB11* and *virD4* play crucial roles in DNA processing during horizontal gene transfer. Furthermore, *lpxD*, which encodes UDP-3-O-acylglucosamine N-acyltransferase, a bacterial outer membrane biogenesis protein, and RluB, which encodes RNA pseudouridine synthase, an enzyme-directed rRNA binding gene, correlated with AMX resistance [27, 28]. In addition to *gyrA*, we identified another candidate gene, *tlpC*, that was strongly associated with LVX resistance. *tlpC* gene encodes a methyl-accepting chemotaxis transducer that plays a vital role in lactate utilization in *H. pylori* [11].

Discussion

The aims of this study were to identify genetic factors associated with antibiotic resistance and to investigate the practicality of using genomic NGS techniques to monitor antibiotic resistance in *H. pylori* clinical samples originating from Vietnam. As a preliminary step, the susceptibility of *H. pylori* strains to AMX, CLR, LVX, and MTZ were evaluated. These critical antimicrobials are traditionally used in both the first- and second-line treatment of *H. pylori* infection, typically in combination with proton pump inhibitors.

A large dataset of Illumina-based WGS data were examined and detected no plasmids in our isolates, confirming that all resistance determinants were encoded within the bacterial genome. Next, WGS-based approach were employed to identify mutations in genes related to antimicrobial resistance in each isolate. Our findings showed that phenotypic resistance to AMX was primarily associated with mutations in the *pbp1 A* gene located within penicillin-binding protein (PBP) motifs, specifically SAIK367-371, KTG555-557, and SNN559-561, as well as within the C-terminus at codons T593 and G595. Intriguingly, novel mutations were also identifed near or outside the PBP motifs, namely: F366L, S414R, F473 V, S543R, T558S, and N562H/Y. These mutations may confer AMX resistance, and thus necessitate further investigation in vitro. Notably, previous studies have also reported the presence of mutations in *pbp1 A* outside of the PBP motif and C-terminus that are relevant to AMX resistance, suggesting that other regions of this gene are potentially significant to the development of AMX resistance [6, 29–31]. Thus, the AMX resistance observed in our isolates could be due to reduced affinity of the antibiotic for the protein encoded by the *pbp1 A* gene. The CLR resistance observed in our *H. pylori* isolates stemmed from two point mutations in the peptidyl

Table 6 Genes significantly related to antibiotic resistance and their functions

Antibiotic	Gene	Hits	-$\log_{10}$(p value)	Average minor allele frequency	Average beta (effect size)	Function
AMX	*pbp1 A*	15	12.7	0.09	0.47	Penicillin-binding protein 1 A
	VirD4	17	12.2	0.08	0.46	Type IV secretion system
	VirB11 (*ptlH*)	8	11.2	0.08	0.46	Type IV secretion system
	lpxD	3	10.9	0.07	0.456	UDP-3-O-acylglucosamine N-acyltransferase
	RluB	7	10.0	0.07	0.47	RNA pseudouridine synthase
LVX	*gyrA*	21	8.7	0.28	0.33	Type II topoisomerase that negatively supercoils closed circular double-stranded (ds) DNA in an ATP-dependent manner
	tlpC	3	6.2	0.125	0.43	Methyl-accepting chemotaxis transducer
CLR	23S *rRNA*	32	77	0.45	0.84	Protein synthesis

transferase–encoding region of the 23S rRNA that account for nearly 90% of primary CLR resistance in *H. pylori* isolates from Western countries. The predominance of A2143G in CLR resistance (92%) raises concerns because this is the main mutation thought to be responsible for failure of *H. pylori* eradication. Although mutations outside of domain V of the 23S *rRNA* gene (T2182 C, C2195 T) have been reported to be linked to CLR resistance, any such mutations were not detected in our isolates [32, 33]. The LVX resistance observed in our study was primarily attributed to mutations in the QRDR regions of the *gyrA* and *gyrB* genes. In the *gyrA* QRDR, mutations were detected at codons 87 (N to K, Y) and 91 (D to N, G, Y) in 87% of the resistant strains. Additionally, the A231 V mutation, which is located outside the *gyrA* QRDR, was seen more frequently in LVX-resistant isolates than in LVX-sensitive isolates ($p < 0.001$). The relevance of this mutation to LVX resistance requires further investigation.

Various mutations in *rdxA* and *frxA*, such as null mutations, frameshift mutations, and missense mutations, have been reported to confer resistance to MTZ. In our study 97 out of 123 samples (78.8%) exhibited MTZ resistance, and all of the resistant strains carried *rdxA* mutations, confirming that mutations in *rdxA* were responsible for the high rate of MTZ resistance. Some of the isolates with mutations exhibited an MTZ MIC ≤ 8 μg/ml, and all of these mutations were missense mutations. In contrast, the MTZ-resistant isolates predominantly carried frameshift and truncation mutations. In total, 24 out of 27 (88.8%) of the MTZ-resistant isolates analyzed in our study carried missense mutations. Our previous study, conducted in Cambodia, indicated a 96.4% rate of MTZ resistance, and loss of function due to nonsense or frameshift mutations in *rdxA* was shown to play an important role in MTZ resistance. Out of the 30 MTZ-resistant Cambodian isolates examined, 15 (50%) carried frameshift mutations, and 15 (50%) carried nonsense mutations. However, the kappa index results from the current study were similar to those from the study performed in Cambodia [5]. There are two factors contributing to the low Cohen's kappa values observed for LVX (0.6) and MTZ (0.81). First, the *gyrA/gyrB* and *rdxA* genes may have multiple mutations, some of which may not directly correlate with phenotypic resistance. This genetic variability can lead to discrepancies between genotype and phenotype [34]. The *rdxA* gene can have various mutations, including missense, nonsense, and frameshift mutations. Not all of these mutations result in a functional change that affects metronidazole activation [35]. Certain mutations in *rdxA* are related to the phylogenetic origin of the strain rather than directly causing resistance [36]. Second, bacteria may have compensatory mechanisms that mitigate the effects of certain mutations, leading to discrepancies between the presence of a mutation and the actual resistance phenotype [37].

In this study, genetic variations were explored in the *H. pylori* pangenome associated with resistance to first- and second-line antibiotics. Using a unitig-GWAS approach, mutations in *pbp1 A*, *gyrA*, and 23S *rRNA* were found to associate with resistance to AMX, LVX, and CLR, respectively, indicating that chromosomal mutations disrupt the interaction between antibiotics and their targets. *pbp1 A* gene had an effect size of 0.47 on AMX resistance. In *H. pylori*, *pbp1 A* encodes PBP1 A, which is involved in peptidoglycan biosynthesis, similar to *Streptococcus pneumoniae*, in which mutations in PBP1 A confer resistance to beta-lactams [38]. Previous studies have also detected antibiotic resistance–associated mutations in other genes participating in the peptidoglycan biosynthesis pathway, including *pbp2x*, *pbp1 A*, *pbp2B, mraW*, and *mraY* [38]. In addition to *pbp1 A*, our GWAS identified novel mutations in four other genes. The first two genes, *virD4* and *virB11,* are involved in the type IV secretion system, which helps translocate effector proteins directly into neighboring cells [39]. The *H. pylori* type IV secretion system may confer antibiotic resistance by promoting biofilm formation, rendering microbial populations impervious to antibiotics, environmental insults, and host defenses [40]. The type IV secretion system is not present in all *H. pylori* isolates [41]; and indeed, our previously study found that the severity of *H. pylori* infection in patients with gastroduodenal disease depends on the presence of the functional type IV secretion system [42]. Additionally, there is a statistically significant correlation between the presence of a functional type IV secretion system and resistance to penicillin in *Neisseria gonorrheae* [43].

The third and fourth genes with novel mutations identified in this study include *rluB* and *lpxD. lpxD* is involved in lipid A biosynthesis in gram-negative bacteria, and mutations in this gene may confer resistance to antimicrobial peptides that target the outer membrane [44, 45]. *RluB*, which encodes RNA pseudouridine synthase, confers resistance to streptomycin and is an important factor affecting the bacterial response to low doses of antibiotics from different families [28, 46]. The *tlpC* gene encodes a methyl-accepting chemotaxis receptor protein involved in promoting *H. pylori* growth in the stomach and mediating responses to lactate attractants; however, its potential role in LVX resistance remains unexplored [47].

Finally, although our results provide valuable insights, they are based on hypothetical scenarios and computational predictions. Future work, in particular in vitro

transformation analyse, is crucial to eliminate potential confounding biases, such as concomitant mutations, and to validate the hypotheses presented here [48].

Conclusions

Standard triple therapy for eradicating *H. pylori* in Vietnam may no longer be effective because of the high rates of resistance to CLR, MTZ, and LVX. Therefore, it is necessary to periodically evaluate antibiotic resistance in this area. Given the increasing resistance rates, clinicians should consider antimicrobial susceptibility testing before prescribing therapy to ensure effective treatment. Reliable genetic markers for the rapid and accurate prediction of *H. pylori* resistance to CLR, LVX, and AMX were identified: 23S *rRNA* (A2142G and A2143G), *gyrA* (N87 K/Y and D91Y/N/G), and *pbp1 A* (F366L, S414R, F473 V, G595_V596 insE, T558S, and T593 A/G/P/S). While mutations in the *rdxA* gene are associated with MTZ resistance, their significance varies, as not all mutations result in phenotypic resistance due to genetic variability, phylogenetic background, and potential compensatory mechanisms. We also showed that an NGS-based approach is a useful and reproducible method for detecting antibiotic resistance–related genetic determinants for rapid molecular diagnostics, enabling personalized treatment strategies that can improve eradication rates and reduce treatment failures. Our findings suggest that the mechanisms underlying AMX and MTZ resistance are polygenic and require further exploration. Through our bacterial genome-wide association study, we identified several novel antimicrobial resistance mutation candidates in *H. pylori*, and further research is necessary to elucidate the mechanism of drug resistance in *H. pylori.*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03990-w.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Authors' contributions

Conceptualization, B.H.P., and Y.Y.; methodology, B.H.P.; software, B.H.P.; conducting experiments, B.H.P, T.M., and J.A.; validation, B.H.P and Y.Y.; formal analysis, B.H.P. and T.T.T; investigation, B.H.P. and Y.Y.; resources, Y.Y.; data curation, B.H.P. and Y.Y.; writing—original draft preparation, B.H.P. and T.T.T.; writing—review and editing, B.H.P., C.N.M.T, J.A., T.M., and Y.Y.; visualization, B.H.P.; supervision, B.H.P. and Y.Y.; project administration, B.H.P.; funding acquisition, B.H.P. and Y.Y. All authors have read and agreed to the submitted version of the manuscript.

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Data availability

Briefly, the *H. pylori* genome assemblies were downloaded from ENA under BioProject accession numbers PRJDB10671 (*Helicobacter pylori* type 4 secretion system within the integrative and conjugative element and cag pathogenicity island are gastroduodenal disease markers), PRJNA566177 (*Helicobacter pylori* from the Bronx, New York), and PRJNA547954 (Draft genomes of *Helicobacter pylori* isolated from Cambodia).
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Declarations

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Oita University. Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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