

# Emergence of Amoxicillin Resistance and Identification of Novel Mutations of the *pbp1A* Gene in *Helicobacter pylori* in Vietnam

**Trung Thien Tran**

Department of Surgery, University of Medicine and Pharmacy at Ho Chi Minh City

**Anh Tuan Nguyen** (✉ [anh.nt@umc.edu.vn](mailto:anh.nt@umc.edu.vn))

Molecular Biomedical Center, University Medical Center, Ho Chi Minh City

**Duc Trong Quach**

Department of Internal Medicine, University of Medicine and Pharmacy at Ho Chi Minh City

**Dao Thi-Hong Pham**

Department of Genetics, University of Science, Vietnam National University Ho Chi Minh

**Nga Minh Cao**

Department of Microbiology-Parasitology, University of Medicine and Pharmacy at Ho Chi Minh City

**Uyen Thi-Hong Nguyen**

Department of Genetics, University of Science, Vietnam National University Ho Chi Minh

**An Nguyen-Thanh Dang**

Department of Genetics, University of Science, Vietnam National University Ho Chi Minh

**Minh Anh Tran**

Department of Surgery, University of Medicine and Pharmacy at Ho Chi Minh City

**Loc Huu Quach**

University Medical Center – Campus 2, Ho Chi Minh City

**Khiem Thien Tran**

University Medical Center – Campus 2, Ho Chi Minh City

**Nhan Quang Le**

Department of Endoscopy, University Medical Center, Ho Chi Minh City

**Viet Van Ung**

Department of Endoscopy, University Medical Center, Ho Chi Minh City

**Minh Ngoc-Quoc Vo**

Department of Endoscopy, University Medical Center, Ho Chi Minh City

**Danh Thanh Nguyen**

Molecular Biomedical Center, University Medical Center, Ho Chi Minh City

**Kha Dong Kha**

Molecular Biomedical Center, University Medical Center, Ho Chi Minh City

**Trung Le Tran**

Department of Oral Biology, Yonsei University College of Dentistry, Seoul

**Vy Thuy Nguyen**

Department of Genetics, University of Science, Vietnam National University Ho Chi Minh

---

## Research Article

**Keywords:** Amoxicillin resistance, Helicobacter pylori, mutation, pbp1A gene, Vietnam

**Posted Date:** December 2nd, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1099024/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published at BMC Microbiology on February 3rd, 2022. See the published version at <https://doi.org/10.1186/s12866-022-02463-8>.

# Abstract

## Background

Amoxicillin resistant *Helicobacter pylori* (*H. pylori*) strains seem to have increased over time in Vietnam. This threatens the effectiveness of *H. pylori* eradication therapies with this antibiotic. This study aimed to investigate the prevalence of primary resistance of *H. pylori* to amoxicillin and to assess its association with *pbp1A* point mutations in Vietnamese patients.

## Materials and Methods

Naive patients who presented with dyspepsia undergoing upper gastrointestinal endoscopy were recruited. Rapid urease tests and PCR assays were used to diagnose *H. pylori* infection. Amoxicillin susceptibility was examined by E-tests. Molecular detection of the mutant *pbp1A* gene conferring amoxicillin resistance was carried out by real-time PCR followed by direct sequencing of the PCR products. Phylogenetic analyses were performed using the Tamura-Nei genetic distance model and the neighbour-joining tree building method.

## Results

There were 308 patients (46.1% men and 53.9% women,  $p = 0.190$ ) with *H. pylori* infection. The mean age of the patients was  $40.5 \pm 11.4$  years, ranging from 18 to 74 years old. The E-test was used to determine the susceptibility to amoxicillin (minimum inhibitory concentration (MIC)  $\leq 0.125$   $\mu\text{g/ml}$ ) in 101 isolates, among which the rate of primarily resistant strains to amoxicillin was 25.7%. Then, 270 sequences of *pbp1A* gene fragments were analysed. There were 77 amino acid substitution positions investigated, spanning amino acids 310–596, with the proportion varying from 0.4–100%. Seven amino acid changes were significantly different between amoxicillin-sensitive (Amox<sup>S</sup>) and amoxicillin-resistant (Amox<sup>R</sup>) samples, including Phe<sub>366</sub> to Leu ( $p < 0.001$ ), Ser<sub>414</sub> to Arg ( $p < 0.001$ ), Glu/Asn<sub>464–465</sub> ( $p = 0.009$ ), Val<sub>469</sub> to Met ( $p = 0.021$ ), Phe<sub>473</sub> to Val ( $p < 0.001$ ), Asp<sub>479</sub> to Glu ( $p = 0.044$ ), and Ser/Ala/Gly<sub>595–596</sub> ( $p = 0.001$ ). Phylogenetic analyses suggested that other molecular mechanisms might contribute to amoxicillin resistance in *H. pylori* in addition to the alterations in PBP1A.

## Conclusions

We reported the emergence of amoxicillin-resistant *Helicobacter pylori* strains in Vietnam and new mutations statistically associated with this antimicrobial resistance. Additional studies are necessary to identify the mechanisms contributing to this resistance in Vietnam.

## Introduction

*Helicobacter pylori* (*H. pylori*) is a Gram-negative microaerophilic pathogenic bacterium that colonises the human gastric mucosa. It has been categorised as a Group I carcinogen for gastric cancer [1]. It infects approximately 50% of individuals worldwide. In Vietnam, the prevalence of *H. pylori* is very high (75%) [2]. The rates of *H. pylori* infection in Vietnamese patients with chronic gastritis, gastric ulcers, duodenal ulcers and gastroduodenal ulcers range from 59.9-69.9%, 77.8%, 85%-95%, and 85.3%-93.6%, respectively [3]. The incidence of gastric cancer in Vietnam has been reported to be the highest compared to other southeast Asian countries [4]. Successful *H. pylori* eradication has been demonstrated to not only heal peptic ulcers but also prevent their recurrence and reduce the incidence of gastric cancer [3, 5].

Amoxicillin is one of the key antibiotics often used to eradicate *H. pylori* in standard triple therapy (amoxicillin, clarithromycin and proton pump inhibitor) as the first-line treatment [6]. However, the spread of amoxicillin- and/or clarithromycin-resistant *H. pylori* has become an important cause of eradication failure. A high rate of clarithromycin resistance of *H. pylori* (72.6%) has been well documented in Vietnam [7]. Additionally, recent data in Vietnam have shown that the amoxicillin resistance rate of *H. pylori* differs across regions and it seems to have increased over time, from 1.1% in 2015 to 10.4% in 2016 and 15% in 2018 [3, 8, 9]. This threatens the effectiveness of *H. pylori* eradication therapies with these antibiotics.

Point mutations in the genes coding for penicillin-binding proteins (PBPs) leads to a decreased affinity for the drug and results in amoxicillin resistance [10, 11]. Among these genes, the *pbp1A* gene seems to be one of the keys [12–15]. There have been several studies on the molecular antibiotic resistance of *H. pylori* in Vietnam, but most of them focused on clarithromycin and levofloxacin resistance [7, 9, 16]. This is the first study conducted to investigate the molecular mechanism of amoxicillin resistance in *H. pylori* in Vietnam.

The present study aimed to evaluate the prevalence of amoxicillin-resistant *H. pylori*-positive gastric biopsy specimens and to investigate point mutations in the *pbp1A* gene in *H. pylori*-sensitive and primary-resistant samples. This study contributes to uncovering novel point mutations in the *pbp1A* gene underlying this resistance effect.

## Materials And Methods

### Patient population and clinical specimens

This study was conducted between January 2019 and April 2021 at the University Medical Center, Ho Chi Minh City, Vietnam. Vietnamese patients presenting with naive dyspepsia and aged  $\geq 18$  years were recruited. The exclusion criteria included patients with gastric cancer and/or those who took any antibiotics within the last 4 weeks.

The sample size was calculated by RaoSoft® software (<http://www.raosoft.com/samplesize.html>) at a 95% confidence level and alpha set at 0.05. During upper gastrointestinal endoscopy, four biopsies were obtained from each patient. A total of 308 gastric biopsy specimens were collected through endoscopy. The first pair of specimens (one from the antrum and one from the corpus) was tested using both the

rapid urease test (NK-Pylori test, Nam Khoa Biotek Co., Ltd.) and a polymerase chain reaction-based test (PCR) (*AccuPid H. pylori* Genotyping Kit, ref. No. Q01HPY03.1A, Khoa Thuong Biotech Co., Ltd.). The second pair was stored in transport medium [17] and kept at 2-8°C until processed for culture within 3 hours after sampling. A total of 101 samples were collected for culture. The samples can also be stored at -80°C in BHI (BD) supplemented with 25% glycerol until culture. The *Pbp1A* gene fragment from all samples was subjected to direct sequencing. The patient characteristics are presented in Table 1.

## Culture conditions and bacteria identification

Gastric biopsy specimens with a positive rapid urease test were collected and maintained for a maximum of 3 hours in transport medium [17] until further processing. The samples were then ground in 100 µL brain heart infusion (BHI, BD) broth supplemented with 10% foetal bovine serum (FBS, Sigma–Aldrich) and cultured on Columbia agar (BD) plates supplemented with 10% lysed sheep blood (Nam Khoa Biotek Co., Ltd.), 1% isovitexin (BD), a skin antibiotic mixture (Sigma–Aldrich), and 2.5 µg/mL amphotericin B (Sigma–Aldrich). Plates were incubated for 4–5 days at 37°C in a microaerobic atmosphere. A single colony of a 4–5-day-old culture was identified through morphological observation, urease tests and PCR specific to the *cagA* and *vacA* genes. A colony with specific morphology, a positive urease test and identified *cagA* and *vacA* was subcultured on Columbia agar plates as described above for 4 days. Amplification and detection of the *cagA* and *vacA* genes was performed based on a previously published protocol [18].

## Determination of the Minimal Inhibitory Concentration

The minimal inhibitory concentration (MIC) of amoxicillin was determined by the E-test method (BioMerieux, ref# 412243). *H. pylori* strains stored at -80°C were thawed on Columbia agar plates supplemented with 10% lysed sheep blood and 1% Isovitalex and incubated at 37°C for 3–4 days in a microaerobic atmosphere. Colonies were subcultured on another plate for 3 more days. Each plate was swabbed with a sterile cotton-tipped applicator, and the cells were suspended in BHI to obtain a turbidity equivalent to a 3.0 McFarland standard. A 100 µl bacterial suspension was inoculated on a Columbia plate containing 10% lysed sheep blood and 1% isovitalex, and another sterile cotton-tipped swab was used to streak in three directions across the plate. The plates were left to dry for 2-3 min, and then the E-test strips containing a continuous exponential gradient of amoxicillin were placed on the agar surface. The plates were incubated at 37°C for 3-4 days in a microaerobic atmosphere. At the end of the incubation period, the MICs were determined by the intercept of the zones of growth inhibition with the graded E-test strip. The reference *H. pylori* strain ATCC® 43504™ susceptible to amoxicillin (Amx<sup>S</sup>) was always used as a quality control strain, and MIC determination was accepted only if the MIC of amoxicillin for this strain was less than or equal to 0.125 µg/ml. The susceptibility of the *H. pylori* isolates to amoxicillin was classified as resistant if the MIC was greater than 0.125 µg/ml, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints ([https://eucast.org/clinical\\_breakpoints/](https://eucast.org/clinical_breakpoints/)).

## Direct sequencing of the *pbp1A* gene fragment

Total genomic DNA was extracted from the gastric biopsy specimens using a Qiacube automated purification system and kit (QIAGEN) according to the manufacturer's standard instructions. Optimisation of the real-time PCR components and conditions was performed to determine the optimal conditions for the amplification of a specific fragment of the *pbp1A* gene of the bacteria in an automated thermal cycler (Roto-Gene Q, Qiagen). Sequences of the *pbp1A* primers were designed in this study as follows: *pbp1A*-F: 5'-CGATAGATTTGGATTACCAACGC-3'; *pbp1A*-R: 5'-ACGATTTCTTTACGCAAGCC-3'. The size of the expected amplicon was 1035 bp. The *pbp1A*-F/R primers (300 nM), MgCl<sub>2</sub> (1 mM), h-Taq DNA polymerase (2 units), dNTP mix (0.2 mM), 1X PCR buffer, 1X EvaGreen and 5 µl of the extracted DNA were used in a total reaction volume of 25 µl. The optimal amplification of the target DNA was set at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. After amplification, the samples were denatured at 95°C for 30 sec and cooled to 65°C, where they were held at that temperature for 30 sec. Then, the samples were slowly heated to 95°C at a ramping rate of 0.5°C/s with continuous acquisition of the decline in fluorescent value. Melting curves were plotted automatically and analysed with Roto-Gene Q software. The specific melting temperature (T<sub>m</sub>) of the *pbp1A* PCR products was 87.2 ± 0.2°C.

The PCR products were purified with ethanol. The purified products were sequenced using a BigDye™ Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). The sequencing PCR products were purified with the BigDye XTerminator™ and read in an ABI 3130 Genetic Analyser. Nucleotide sequences of both chains obtained were aligned and transformed into amino acid sequences using Geneious Prime software version 2021.1.1 (Auckland, New Zealand) and compared to the sequences of strains 26695 (AE000511) and Hargenberg (AF479617) deposited in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) for detection of mutations in the *pbp1A* gene.

## Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 20.0. Descriptive statistical analysis was used to describe the characteristics of the patients' gender, age, residency, gastric disease status and susceptibility to amoxicillin of the strains isolated from the clinical samples. A one-sample binomial test was used to determine whether the proportion of cases (sex, amoxicillin resistance, coinfection) was equal to the previously documented corresponding proportion. The chi-square test was used to correlate the presence of mutations in the *pbp1A* gene and the susceptibility to amoxicillin. Fisher's exact test was used alternatively when more than 20% of the expected counts were less than 5. Monte Carlo estimates of the exact significance were used when the data did not meet the assumption of the asymptotic method. A p-value less than 0.05 was considered significant.

## Results

### *Demographic characteristics*

There were 308 qualified patients recruited in this study. Details on the demographic characteristics of these patients are presented in Table 1.

There were 46.1% (142/308; 95% CI: 40.3–51.6) men and 53.9% (166/308; 95% CI: 48.4–59.7%) women. The gender ratio occurred with probabilities of 0.5 and 0.5 ( $p = 0.190$ ; binomial test). The mean age of the patients was  $40.5 \pm 11.4$  (95% CI: 39.2–41.8) years, ranging from 18–74. Age followed a normal distribution ( $p = 0.247$ ; skewness test). The highest percentage of patients was from Ho Chi Minh City (31.5%). The proportions of patients from nearby regions, including the southeastern area, Mekong River Delta, and central region, were similar, with proportions of 19.5%, 22.1% and 25.3%, respectively; a very small percentage of patients (1.6%) were from the Red River Delta. Most patients were diagnosed with gastritis (93.5%; 288/308), and only a small percentage of them were diagnosed with gastric ulcers (6.5%; 20/308).

Table 1  
Demographic characteristics and diagnosis of the recruited patients

Characteristics	Number of cases (n)	Rate (%)
Gender		
Male	142	46.1
Female	166	53.9
Mean age $\pm$ SD (range) (year)	40.5 $\pm$ 11.4 (18–74)	
Residency		
Ho Chi Minh	97	31.5
Southeast	60	19.5
Mekong River Delta	68	22.1
Central	78	25.3
Red River Delta	5	1.6
Diagnosis		
Gastritis	288	93.5
Gastric ulcer	20	6.5
E-test for amoxicillin		
Sensitive	75	74.3
Resistant	26	25.7
Amoxicillin MIC was determined randomly in 101 samples (32.8% of the entire study group). Among these samples, 74.3% (75/101) of strains were sensitive, and 25.7% (26/101) of strains were primarily resistant to amoxicillin. The isolates with phenotypic resistance to amoxicillin exhibited a MIC range of 0.190–1.5 mg/l (Figure 1).		

## Virulence factors of *H. pylori*

The presence of the *cagA* or *vacA* gene was investigated in all 308 clinical specimens positive for the rapid urease test. Based on the *vacA* genotypes, coinfection was discovered: 38 (12.3%) specimens were colonised by at least two *H. pylori* strains (*vacA* m1 and *vacA* m2). All of the coinfection specimens were excluded from the analysis to assess the association between bacterial genotypes and clinical outcome as well as antibiotic resistance.

The *cagA* gene was present in 225 (83.3%) of the 270 *H. pylori* specimens. There was no association between gastritis and peptic ulcers with the presence or absence of the *cagA* gene ( $p = 1.000$ ).

Regarding the *vacA* genotypes, most of the specimens had the *vacA* s1 allele (98.4%). No association was detected between the *vacA* signal sequences and the clinical outcome ( $p = 0.265$ ). However, there was a relationship between the *vacA* middle sequences and the clinical outcome ( $p = 0.021$ ). The *vacA* m1 allele was found to be significantly higher in 12 (75.0%) specimens from gastric ulcer patients, and the *vacA* m2 allele was significantly higher in 141 (55.5%) of the specimens from gastritis patients. The combination of the *vacA* signal and middle sequences with the clinical outcome also presented a similar significant relationship ( $p = 0.008$ ). *VacA* s1m1 was higher in the ulcer group (75.0%), while *vacA* s1m2 was higher in the gastritis group (53.9%). The combination of *cagA* status and *vacA* genotypes was also investigated. There was an association between the combined genotypes and the clinical outcome ( $p = 0.032$ ). In particular, no association was observed between the genotypes and the resistance to amoxicillin ( $p > 0.05$ ) (Table 2).

Table 2  
The genotypes of the *H. pylori* strains and the clinical outcome

Genotype	Clinical outcome			<i>Amoxicillin susceptibility</i>		
	Gastritis % (n=254)	Gastric ulcer % (n=16)	p value	Resistant % (n=19)	Sensitive % (n=66)	p value
<i>cagA</i>						
<i>cagA</i> <sup>+</sup>	83.1 (211)	87.5 (14)	1.000	78.9 (15)	80.3 (53)	1.000
<i>cagA</i> <sup>-</sup>	16.9 (43)	12.5 (2)		21.1 (4)	19.7 (13)	
<i>vacA</i>						
s1	98.4 (250)	93.8 (15)	0.265	94.7 (18)	97.0 (64)	0.537
s2	1.6 (4)	6.2 (1)		5.3 (1)	3.0 (2)	
m1	44.5 (113)	75.0 (12)	0.021	68.4 (13)	43.9 (29)	0.060
m2	55.5 (141)	25.0 (4)		31.6 (6)	56.1 (37)	
s1m1	44.5 (113)	75.0 (12)	0.008	68.4 (13)	43.9 (29)	0.086
s1m2	53.9 (137)	18.8 (3)		26.3 (5)	53.0 (35)	
s2m2	1.6 (4)	6.2 (1)		5.3 (1)	3.0 (2)	
<i>cagA</i> and <i>vacA</i>						
<i>cagA</i> <sup>+</sup> <i>vacA</i> s1m1	42.1 (107)	68.8 (11)	0.032	63.2 (12)	40.9 (27)	0.235
<i>cagA</i> <sup>+</sup> <i>vacA</i> s1m2	40.2 (102)	18.8 (3)		15.8 (3)	37.9 (25)	
<i>cagA</i> <sup>+</sup> <i>vacA</i> s2m2	0.8 (2)	0.0 (0)		0.0 (0)	1.5 (1)	
<i>cagA</i> <sup>-</sup> <i>vacA</i> s1m1	2.4 (6)	6.2 (1)		5.3 (1)	3.0 (2)	
<i>cagA</i> <sup>-</sup> <i>vacA</i> s1m2	13.8 (35)	0.0 (0)		10.5 (2)	15.2 (10)	
<i>cagA</i> <sup>-</sup> <i>vacA</i> s2m2	0.8 (2)	6.2 (1)		5.3 (1)	1.5 (1)	

## Mutational changes in or adjacent to the acyl transpeptidase conserved sequence of the *pbp1A* gene

Amoxicillin resistance in Amx<sup>R</sup> *H. pylori* isolates is mediated by mutations in the *pbp1A* gene [13]. To investigate the genetic diversity of *pbp1A* genes in this study by gene sequencing, the *pbp1A* gene sequences of the *H. pylori* reference strain 26695 (O25319, Amx<sup>S</sup>) and Hargenberg (AF479617, Amx<sup>R</sup>) were used as controls to compare and number the isolates according to the corresponding deduced

amino acid sequences. A total of 270 sequences of *pbp1A* gene fragments spanning amino acids 310–596 were analysed. The results showed that the proportion of amino acid substitutions varied from 0.4–100% depending on the amino acid position (Figure 2). Several amino acid positions in the acyl transpeptidase domain of PBP1A had a high proportion of substitutions, such as Asp<sub>535</sub> to Asn (100%), Ser<sub>589</sub> to Gly (96.7%), Asp<sub>479</sub> to Glu (86.6%), and Asn<sub>504</sub> to Asp (74.1%). Amino acid positions with substitution proportions equal to or lower than 1% are not shown on the graph (Figure 2).

## ***Association between amino acid substitution and amoxicillin susceptibility***

To investigate which *pbp1A* gene mutations were involved in amoxicillin resistance in the Amox<sup>R</sup> isolates in this study, we evaluated all 77 amino acid substitutions in the acyl transpeptidase domain of PBP-1A, from amino acids 310 to 596. Seven amino acid changes were found to be significantly different between the Amox<sup>S</sup> and Amox<sup>R</sup> samples (Table 3), including Phe<sub>366</sub> to Leu ( $\chi^2 = 19.055$ ;  $p < 0.001$ ), Ser<sub>414</sub> to Arg ( $\chi^2 = 31.056$ ;  $p < 0.001$ ), Glu/Asn<sub>464-465</sub> ( $\chi^2 = 6.898$ ;  $p = 0.009$ ), Val<sub>469</sub> to Met ( $\chi^2 = 7.304$ ;  $p = 0.021$ ), Phe<sub>473</sub> to Val ( $\chi^2 = 21.223$ ;  $p < 0.001$ ), Asp<sub>479</sub> to Glu ( $\chi^2 = 4.060$ ;  $p = 0.044$ ), and Ser/Ala/Gly<sub>595-596</sub> ( $\chi^2 = 10.356$ ;  $p = 0.001$ ). In particular, two novel insertion mutations, Glu/Asn<sub>464-465</sub> and Ser/Ala/Gly<sub>595-596</sub>, were discovered for the first time in this study, together with changes in Val<sub>469</sub> to Met and Asp<sub>479</sub> to Glu. These four novel, never-reported mutations were in or adjacent to the second (SKN<sub>402-404</sub>) and third (KTG<sub>555-557</sub>) conserved PBP motifs. The other three mutations, including Phe<sub>366</sub> to Leu, Ser<sub>414</sub> to Arg, and Phe<sub>473</sub> to Val, have been documented [11, 13, 15].

We noticed that the previously described amino acid changes, such as Glu<sub>406</sub> to Ala ( $\chi^2 = 0.017$ ;  $p = 1.000$ ), Ser<sub>417</sub> to Thr ( $\chi^2 = 0.291$ ;  $p = 1.000$ ), Thr<sub>556</sub> to Ser ( $\chi^2 = 0.895$ ;  $p = 1.000$ ), and Asn<sub>562</sub> to Tyr ( $\chi^2 = 4.338$ ;  $p = 0.072$ ), associated with a high level of beta-lactam resistance in acquired multidrug-resisting *H. pylori* [19] were insignificant in this population (Table 3). Additionally, other novel amino acid substitutions mentioned previously [13] in selected Amx<sup>R</sup> transformants comprising Thr<sub>540</sub> to Ile, Ser<sub>542</sub> to Arg, Thr<sub>555</sub> to Ser, and Asn<sub>561</sub> to Tyr were unchanged in this study. The results showed that several mutated positions had statistical significance between amoxicillin-sensitive and amoxicillin-resistant samples and were completely different from amino acid positions reported to be related to amoxicillin resistance published previously [15]. These mutations are adjacent to the second and third PBP motifs, which were observed only in Amx<sup>R</sup> *H. pylori* colonies after transformation [13].

Table 3  
Relationships between amino acid substitution and amoxicillin susceptibility

Position	Amino acid	Genotype	Resistant	Sensitive	$\chi^2$	<i>p</i> value
315	Glu	mt	9	20	2.312	0.128
	Lys	wt	8	41		
366	Leu	mt	15	16	19.055	< 0.001
	Phe	wt	4	50		
406	Ala	mt	1	3	0.017	1.000*
	Glu	wt	18	63		
414	Arg	mt	15	9	31.056	< 0.001*
	Ser	wt	4	57		
417	Thr	mt	0	1	0.291	1.000*
	Ser	wt	19	65		
Ins 464-465	Glu/Asn	mt	11	17	6.898	0.009
	-	wt	8	49		
469	Met	mt	4	2	7.304	0.021*
	Val	wt	15	64		
473	Val	mt	16	17	21.223	< 0.001*
	Phe	wt	3	49		
474	Val	mt	0	5	1.529	0.583*
	Ala	wt	19	61		
479	Glu	mt	13	58	4.060	0.044
	Asp	wt	6	8		
504	Asn	mt	14	53	0.387	0.534
	Asp	wt	5	13		
508	Asn	mt	6	16	0.414	0.520
	Asp	wt	13	50		
509	Ile	mt	6	12	1.586	0.208

*Note:* Coinfection samples were excluded from the analysis; (\*) Fisher's exact test was applied; (wt) wild-type; (mt) mutant (amino acid is different from strain 26695).

Position	Amino acid	Genotype	Resistant	Sensitive	$\chi^2$	<i>p</i> value
	Val	wt	13	54		
515	Ile	mt	10	41	0.554	0.457
	Met	wt	9	25		
543	Arg	mt	5	17	0.002	0.961
	Ser	wt	14	49		
556	Ser	mt	0	3	0.895	1.000*
	Thr	wt	19	63		
562	Tyr	mt	3	2	4.338	0.072*
	Asn	wt	16	64		
589	Gly	mt	19	62	1.208	0.571*
	Ser	wt	0	4		
593	Ala	mt	4	12	0.080	0.784*
	Thr	wt	15	54		
595	Ser	mt	3	12	0.058	1.000*
	Gly	wt	16	54		
Ins 595-596	Gly/Ser/Ala	mt	14	21	10.356	0.001
	-	wt	5	44		
596	Ile/Ala	mt	1	1	0.878	0.403*
	Val	wt	18	64		

*Note:* Coinfection samples were excluded from the analysis; (\*) Fisher's exact test was applied; (wt) wild-type; (mt) mutant (amino acid is different from strain 26695).

## Phylogenetic analyses of the *pbp1A* sequences

Phylogenetic analyses were performed on all *pbp1A* gene fragment sequences to determine whether the sequences with identified mutations were in the same group of sequences related to amoxicillin resistance. Trees were constructed from 85 sequences based on the *pbp1A* amplification fragments trimmed to 879 bp by the Tamura-Nei genetic distance model and the neighbour-joining tree building method (Geneious Prime® 2021.1.1). This primary phylogenetic analysis suggested that the *pbp1A* sequences obtained in the present study might belong to different sequence groups. The sequences from which *H. pylori* isolates showed resistance to amoxicillin could be combined into two groups with close relatedness to those from which *H. pylori* isolates were sensitive to amoxicillin, except for samples A003,

A075, A138 and A163. These two groups (a, b) also shared the most recent common ancestor of the *pbp1A* gene (Figure 3).

Table 4 shows that groups (a) and (b) had very similar amino acid substitution and insertion profiles and were quite different from the reference sequences at the seven observed positions. There were four novel strains from our samples presenting single amino acid substitutions at Leu<sub>366</sub> and Val<sub>473</sub>, as well as amino acid insertions at Glu/Asn<sub>464-465</sub> and Ser/Ala/Gly<sub>595-596</sub>. The other two amino acid replacements (Met<sub>469</sub> and Glu<sub>479</sub>) also appeared uniquely in our data when compared to the references. However, the presence of specific substitutions and insertions was present not only in the resistant samples but also in the sensitive samples.

Table 4

Overview of amino acid differences in PBP1A proteins occurring in clinical samples with resistance and sensitivity to amoxicillin.

Sequence groups	E-test for Amoxicillin <sup>b</sup>	Clinical samples	Amino acid position <sup>a</sup>							
			366	414	Ins1 <sup>c</sup>	469	473	479	Ins2 <sup>d</sup>	
Group (a)	0.047–0.125	A283, A407, A105, A186, A146, A207, A271, A142, A481	Leu	Ser Arg	Glu	Val	Val	Glu	Ser Gly	
	0.190–1.000	A132, A009, A234, A482, A102, A100	Leu	Ser Arg	Lys Glu -	Val Met	Val	Glu Asp	Ser Gly Val	
Group (b)	0.032–0.125	A119, A312, A404, A334	Leu	Ser Arg	Glu	Val	Val	Glu	Ser Gly	
	0.190–2.000	A147, A124, A277, A353, A162, A298, A255, A005, A141, A182	Leu	Arg	Lys Glu -	Val Met	Val	Glu Asp	Ser Gly Val -	
26695	Sensitive	-	Phe	Ser	-	Val	Phe	Asp	-	
Hagenberg	Resistant	-	Phe	Arg	-	Val	Phe	Asp	-	

<sup>a</sup> Positions of amino acid differences are given to the relative start point of the *pbp1A* gene of *H. pylori* Hagenberg (AF479617); <sup>b</sup> mg/l; <sup>c</sup> Amino acid inserted between positions 464 and 465; <sup>d</sup> Amino acid inserted between positions 595 and 596.

## Discussion

Amoxicillin resistance in *H. pylori* is one of the greatest concerns of clinicians because anti-*H. pylori* regimens often consist of amoxicillin in addition to other antibiotics and proton pump inhibitors [20, 21]. Recent studies have suggested that amoxicillin resistance in *H. pylori* results from alterations in PBP1A [12, 13, 15, 22]. One of the main purposes of this study was to investigate the molecular mechanism of amoxicillin resistance in *H. pylori* strains collected from endoscopic biopsies in Vietnam.

The amoxicillin-resistant *H. pylori* proportion discovered in this study (25.7%) was similar to the finding by Saniee *et al.* in Iran (27.1%;  $p = 0.423$ ) in 2018 [23] but significantly higher than that in other studies, such as Zerbetto *et al.* in Argentina (7.6%,  $p < 0.001$ ) in 2017 [15], Manal *et al.* in Egypt (18.3%;  $p = 0.035$ ) in 2018 [24], Ortis *et al.* in Central America (10%;  $p < 0.001$ ) in 2019 [25], Azzaya *et al.* in Mongolia (11.9%;  $p < 0.001$ ), Aumpan *et al.* in Cambodia (0%;  $p < 0.001$ ) in 2020 [26, 27], Calinga-Ponce *et al.* in Mexico (1.8%;  $p < 0.001$ ) and Li *et al.* in China (0%;  $p < 0.001$ ) in 2021 [28, 29]. Compared to previous studies in Vietnam, the amoxicillin resistance proportion has been trending up significantly, for instance, from 0% ( $p < 0.001$ ) in 2013 [30], 1.1% ( $p < 0.001$ ) in 2015 [8], 10.4% ( $p < 0.001$ ) in 2016 [9], 15% ( $p = 0.002$ ) in 2019 [3] and to 25.7% in 2020 for this study. Although the previous local studies were different from each other regarding geographical areas, sample sizes, study period and antimicrobial testing methods, an increasing proportion of amoxicillin resistance in *H. pylori* in Vietnam has been generally demonstrated, suggesting that this is a serious emerging threat to the success of amoxicillin-based regimens. The high rate of amoxicillin-resisting *H. pylori* in our study might be explained by the fact that the combination of amoxicillin and clavulanate potassium is often empirically prescribed for various infectious diseases [31]. The incorrect use of antimicrobials can accelerate the selection of drug-resistant strains [23].

Multiple strains of *H. pylori* can coinfect the same patient [32]. The coinfection detected by *vacA* genotype in our study presented a rate of 12.3%. A similar proportion in terms of coinfection determination was obtained when confirmed by random amplified polymorphic DNA (RAPD) fingerprinting (12.5%;  $p = 0.500$ ) [32] or through *vacA* and *iceA* genotyping (11.0%;  $p = 0.255$ ) [33]. Therefore, coinfection needs to be excluded before evaluating the relationship between *H. pylori* genotypes and other factors, such as disease status and clinical symptoms, to maintain accuracy. Moreover, coinfection could undermine the success of eradication therapy and should be considered when interpreting the results of antimicrobial susceptibility tests [32].

Based on the *H. pylori* genotype analysis, our data showed an association between the *vacA*<sup>m1</sup> genotype and gastric ulcers. This result suggests that individuals colonised with *vacA*<sup>m1</sup>-positive *H. pylori* strains are at an increased risk of developing gastric ulcers. These results confirmed the *vacA*<sup>m1</sup> genotype is associated with an increased risk of peptic ulcers, which has been reported by Nguyen *et al.* [34] and Trang *et al.* [35] in Vietnam. Conversely, Milad *et al.* revealed that the *vacA*<sup>m2</sup> genotype was significantly higher in patients with peptic ulcer disease than in patients with gastritis in Iran [36]. On the other hand, several previous studies published by Godoy *et al.* in Brazil and Loivukene *et al.* in Estonia did not find any association between virulence factors such as *vacA*<sup>m1</sup> genotype and clinical outcomes or bacterial resistance to metronidazole, although the coinfection by multiple strains has been well considered in

these studies [33, 37]. The difference in *cagA* and *vacA* genotype proportions as well as the association of these genotypes with the clinical outcomes might result from the fact that various populations have been evaluated with different ethnic groups and that a high genetic variability of strains in different countries exists [38, 39]. However, there is universal agreement regarding the role of the *vacA*<sup>m1</sup> genotype among studies carried out in Vietnam at different time points. In addition, we did not find any association between the virulence factors and resistance to amoxicillin.

Amino acid substitutions in the acyl transpeptidase domain of PBP1A are required for resistance to amoxicillin [12, 13]. However, other mechanisms could be involved in increasing the MIC value and contributing to the levels of high Amx<sup>R</sup> strains, such as decreasing membrane permeability due to altered porin proteins (HopC, HopH), increasing the activity of efflux pumps to eject antibiotics from the periplasm, or even reducing the binding of antibiotics to other PBPs, especially PBP2 [19, 40].

In this study, we discovered a high rate of resistance to amoxicillin. To identify the mutations of *pbp1A* responsible for amoxicillin resistance, sequences of the *pbp1A* gene were analysed. We found seven amino acid changes possibly linked to amoxicillin resistance in clinical samples. Among them, the Ser<sub>414</sub> to Arg substitution has been proven to be the main factor in amoxicillin resistance of the Hardenberg strain by site-directed mutagenesis [12] and it was also common in clinical Amx<sup>R</sup> strains by natural transformation [11, 13]. The Phe<sub>366</sub> to Leu alteration was reported to be present in the clinical Amx<sup>R</sup> strain SZ79 in combination with the Ser<sub>414</sub> to Arg substitution [13]. Phe<sub>473</sub> was recognised in strains sensitive to but less susceptible to amoxicillin but also in Amx<sup>R</sup> transformants in the absence of Ser<sub>414</sub> to Arg substitutions [15], while Val<sub>473</sub> was common in our Amx<sup>R</sup> samples in the context of Ser<sub>414</sub> to Arg changes. Although it has become clear that amino acid variations conferring resistance vary by the geographical origins of the strains [15], these data have confirmed the combination of amino acid substitutions or mutations in multiple loci to amoxicillin resistance [13].

To investigate the relatedness, as well as the combination of different mutations of *pbp1A* sequences in the resistance to amoxicillin, we created a phylogenetic tree of 85 obtained *pbp1A* gene fragments. The grouping of the *pbp1A* gene sequences suggested that there were other mechanisms in addition to the mutations in the *pbp1A* gene contributing to amoxicillin resistance in *H. pylori* in Vietnam. Other studies have also shown that distinct mechanisms of antimicrobial resistance also play important roles in the resistance to amoxicillin in *H. pylori* [13].

Our study had several limitations. First, resistant mutations were not identified directly from isolates but only from gastric biopsy specimens. We excluded coinfection cases from the data analysis, and this approach might not be ideal for identifying the molecular mechanisms related to amoxicillin resistance. Second, the role of other genes that could have synergistic effects in amoxicillin resistance could not be excluded. We did not evaluate coinfection by multiple *H. pylori* strains by fingerprinting methods such as random amplified polymorphic DNA (RAPD) or multilocus sequence typing (MLST). Last but not least,

resistance has not been confirmed to have a direct correlation with eradication efficacy in real-life practice.

However, the strength of this study is that it was conducted at one of the largest hospitals in southern Vietnam, which usually admits patients from Ho Chi Minh City and many nearby areas. This is the first study that reported mutations related to Amx<sup>R</sup> *H. pylori* in Vietnamese patients and it has identified some novel mutations, especially insertion mutations Glu/Asn<sub>464-465</sub> and Ser/Ala/Gly<sub>595-596</sub> in the *pbp1A* gene and other nearby mutations, which are likely specific to *H. pylori* strains in Vietnamese. Further studies are required to validate the role of these novel mutations in conferring amoxicillin resistance. In addition to the emerging prevalence of amoxicillin-resistant *H. pylori* strains, direct detection of *pbp1A* gene mutations from *H. pylori*-positive biopsy specimens may lead to novel diagnostic strategies for amoxicillin resistance determination and would be useful in clinical practice. More importantly, other mechanisms, such as the acquisition or expression of  $\beta$ -lactamase or changes in other proteins involved in cell wall synthesis, such as PBP2, PBP3, and PBP4, should also be evaluated.

## Conclusion

Our study has identified new mutations that are statistically associated with amoxicillin resistance and demonstrated the importance of the detection of amoxicillin-resistant *Helicobacter pylori* in clinical practice because of the emergence of these strains in Vietnam. However, further studies should be carried out to identify additional mechanisms contributing to amoxicillin resistance in *H. pylori*.

## Declarations

### Acknowledgements

We highly appreciate support from University Medical Center 1 & 2 in Ho Chi Minh City.

### Author's contributions

TTT, ATN and NMC designed the experiment. TTT, MAT, LHQ, KTT, NQL, VVU, MNQV collected gastric biopsies and performed clinical diagnosis. ATN, DTHP, UTHN, ANTD, DTN and KDN conducted the experiment, analysed the samples and performed data analysis. ATN carried out data interpretation and produced the first draft of the manuscript. DTQ, TLT and VTN contributed to drafting and editing the manuscripts critically. All the authors have read and approved the final version of the manuscripts.

### Funding

This study was supported by a research grant (reference number: 51/2019/HĐ-QPTKHCN) from the Department of Science and Technology Ho Chi Minh City (DOST HCMC) Vietnam.

### Availability of data and materials

The detail data and materials available on request (anh.nt@umc.edu.vn).

## Ethics approval and consent to participate

The study was approved by the ethics committees of the University of Medicine and Pharmacy at Ho Chi Minh City (reference number: 374/ĐHYD-HĐĐĐ, signed on 30/07/2019). All participants provided written informed consent. This work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments in Humans.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Wroblewski LE, Peek RM, Jr., Wilson KT. *Helicobacter pylori* and gastric cancer: factors that modulate disease risk. Clin Microbiol Rev. 2010;23(4):713–39.
2. Rahman R, Asombang AW, Ibdah JA. Characteristics of gastric cancer in Asia. World J Gastroenterol. 2014;20(16):4483–90.
3. Khiem VV, Thang DM, Hai TM, Duat NQ, Khanh PH, Ha DT, Binh TT, Dung HDQ, Trang TTH, Yamaoka Y. Management of antibiotic-resistant *Helicobacter pylori* infection: Perspectives from Vietnam. Gut Liver. 2019;13(5):483–497.
4. Quach DT, Vilaichone RK, Vu KV, Yamaoka Y, Sugano K, Mahachai V. *Helicobacter pylori* infection and related gastrointestinal diseases in Southeast Asian countries: An expert opinion survey. Asian Pac J Cancer Prev. 2018;19(12):3565–3569.
5. Lee YC, Chiang TH, Chou CK, Tu YK, Liao WC, Wu MS, Graham DY. Association between *Helicobacter pylori* eradication and gastric cancer incidence: A systematic review and meta-analysis. Gastroenterology. 2016;150(5):1113–1124 e5.
6. Malfertheiner P, Megraud F, O'Morain CA, Gisbert JP, Kuipers EJ, Axon AT, Bazzoli F, Gasbarrini A, Atherton J, Graham DY, Hunt R, Moayyedi P, Rokkas T, Rugge M, Selgrad M, Suerbaum S, Sugano K, El-Omar EM. Management of *Helicobacter pylori* infection-the Maastricht V/Florence Consensus Report. Gut. 2017;66(1):6–30.
7. Dang NQH, Ha TMT, Nguyen ST, Le NDK, Nguyen TMT, Nguyen TH, Pham TTH, Tran VH. High rates of clarithromycin and levofloxacin resistance of *Helicobacter pylori* in patients with chronic gastritis in the south east area of Vietnam. J Glob Antimicrob Resist. 2020;22:620–624.
8. Phan TN, Santona A, Tran VH, Tran TN, Le VA, Cappuccinelli P, Rubino S, Paglietti B. High rate of levofloxacin resistance in a background of clarithromycin- and metronidazole-resistant *Helicobacter pylori* in Vietnam. Int J Antimicrob Agents. 2015;45(3):244–8.
9. Quek C, Pham ST, Tran KT, Pham BT, Huynh LV, Luu NB, Le TK, Quek K, Pham VH. Antimicrobial susceptibility and clarithromycin resistance patterns of *Helicobacter pylori* clinical isolates in

- Vietnam. F1000Res. 2016;5:671.
10. Matteo MJ, Granados G, Olmos M, Wonaga A, Catalano M. *Helicobacter pylori* amoxicillin heteroresistance due to point mutations in PBP-1A in isogenic isolates. J Antimicrob Chemother. 2008;61(3):474–7.
  11. Rimbara E, Noguchi N, Kawai T, Sasatsu M. Mutations in penicillin-binding proteins 1, 2 and 3 are responsible for amoxicillin resistance in *Helicobacter pylori*. J Antimicrob Chemother. 2008;61(5):995–8.
  12. Gerrits MM, Schuijffel D, van Zwet AA, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG. Alterations in penicillin-binding protein 1A confer resistance to beta-lactam antibiotics in *Helicobacter pylori*. Antimicrob Agents Chemother. 2002;46(7):2229–33.
  13. Gerrits MM, Godoy AP, Kuipers EJ, Ribeiro ML, Stoof J, Mendonca S, van Vliet AH, Pedrazzoli J, Jr., Kusters JG. Multiple mutations in or adjacent to the conserved penicillin-binding protein motifs of the penicillin-binding protein 1A confer amoxicillin resistance to *Helicobacter pylori*. Helicobacter. 2006;11(3):181–7.
  14. Paul R, Postius S, Melchers K, Schafer KP. Mutations of the *Helicobacter pylori* genes *rdxA* and *pbp1* cause resistance against metronidazole and amoxicillin. Antimicrob Agents Chemother. 2001;45(3):962–5.
  15. Zerbetto De Palma G, Mendiondo N, Wonaga A, Viola L, Ibarra D, Campitelli E, Salim N, Corti R, Goldman C, Catalano M. Occurrence of mutations in the antimicrobial target genes related to levofloxacin, clarithromycin, and amoxicillin resistance in *Helicobacter pylori* isolates from Buenos Aires City. Microb Drug Resist. 2017;23(3):351–358.
  16. Tran VH, Ha TMT, Le PTQ, Phan TN, Tran TNH. Characterisation of point mutations in domain V of the 23S rRNA gene of clinical *Helicobacter pylori* strains and clarithromycin-resistant phenotype in Central Vietnam. J Glob Antimicrob Resist. 2019;16:87–91.
  17. Blanchard TG, Nedrud JG. Laboratory maintenance of *Helicobacter* species. Curr Protoc Microbiol. 2012;Chapter 8:Unit8B 1.
  18. Trung TT, Minh TA, Anh NT. Value of CIM, CLO test and multiplex PCR for the diagnosis of *Helicobacter pylori* infection status in patients with gastritis and gastric ulcer. Asian Pac J Cancer Prev. 2019;20(11):3497–3503.
  19. Kwon DH, Dore MP, Kim JJ, Kato M, Lee M, Wu JY, Graham DY. High-level beta-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob Agents Chemother. 2003;47(7):2169–78.
  20. Liyen Cartelle A, Uy PP, Koehler TE, Yap JEL. Persistent *Helicobacter pylori* infection: An insight to the limitations of current clinical practice. Cureus. 2020;12(12):e12309.
  21. Gisbert JP. Empirical or susceptibility-guided treatment for *Helicobacter pylori* infection? A comprehensive review. Therap Adv Gastroenterol. 2020;13:1756284820968736.
  22. Okamoto T, Yoshiyama H, Nakazawa T, Park ID, Chang MW, Yanai H, Okita K, Shirai M. A change in PBP1 is involved in amoxicillin resistance of clinical isolates of *Helicobacter pylori*. J Antimicrob

- Chemother. 2002;50(6):849–56.
23. Saniee P, Hosseini F, Kadkhodaei S, Siavoshi F, Khalili-Samani S. *Helicobacter pylori* multidrug resistance due to misuse of antibiotics in Iran. Arch Iran Med. 2018;21(7):283–288.
  24. Diab M, El-Shenawy A, El-Ghannam M, Salem D, Abdelnasser M, Shaheen M, Abdel-Hady M, El-Sherbini E, Saber M. Detection of antimicrobial resistance genes of *Helicobacter pylori* strains to clarithromycin, metronidazole, amoxicillin and tetracycline among Egyptian patients. The Egyptian Journal of Medical Human Genetics. 2018.
  25. Ortiz V, Estevez-Ordonez D, Montalvan-Sanchez E, Urrutia-Argueta S, Israel D, Krishna US, Romero-Gallo J, Wilson KT, Peek RM, Dominguez R, Morgan DR. *Helicobacter pylori* antimicrobial resistance and antibiotic consumption in the low-resource Central America setting. Helicobacter. 2019;24(4):e12595.
  26. Azzaya D, Gantuya B, Oyuntsetseg K, Davaadorj D, Matsumoto T, Akada J, Yamaoka Y. High antibiotic resistance of *Helicobacter pylori* and its associated novel gene mutations among the Mongolian population. Microorganisms. 2020;8(7).
  27. Aumpan N, Vilaichone RK, Gamnarai P, Sanglutong L, Ratanachu-Ek T, Mahachai V, Yamaoka Y. Prevalence and antibiotic resistance patterns of *Helicobacter pylori* infection in Koh Kong, Cambodia. Asian Pac J Cancer Prev. 2020;21(5):1409–1413.
  28. Camorlinga-Ponce M, Gomez-Delgado A, Aguilar-Zamora E, Torres RC, Giono-Cerezo S, Escobar-Ogaz A, Torres J. Phenotypic and genotypic antibiotic resistance patterns in *Helicobacter pylori* strains from ethnically diverse population in Mexico. Front Cell Infect Microbiol. 2020;10:539115.
  29. Li J, Deng J, Wang Z, Li H, Wan C. Antibiotic resistance of *Helicobacter pylori* strains isolated from pediatric patients in Southwest China. Front Microbiol. 2020;11:621791.
  30. Binh TT, Shiota S, Nguyen LT, Ho DD, Hoang HH, Ta L, Trinh DT, Fujioka T, Yamaoka Y. The incidence of primary antibiotic resistance of *Helicobacter pylori* in Vietnam. J Clin Gastroenterol. 2013;47(3):233–8.
  31. Nguyen NV, Do NTT, Nguyen CTK, Tran TK, Ho PD, Nguyen HH, Vu HTL, Wertheim HFL, van Doorn HR, Lewycka S. Community-level consumption of antibiotics according to the AWaRe (Access, Watch, Reserve) classification in rural Vietnam. JAC Antimicrob Resist. 2020;2(3):dlaa048.
  32. Seo JW, Park JY, Shin TS, Kim JG. The analysis of virulence factors and antibiotic resistance between *Helicobacter pylori* strains isolated from gastric antrum and body. BMC Gastroenterol. 2019;19(1):140.
  33. Godoy AP, Ribeiro ML, Benvengo YH, Vitiello L, Miranda Mde C, Mendonca S, Pedrazzoli J, Jr. Analysis of antimicrobial susceptibility and virulence factors in *Helicobacter pylori* clinical isolates. BMC Gastroenterol. 2003;3:20.
  34. Nguyen TL, Uchida T, Tsukamoto Y, Trinh DT, Ta L, Mai BH, Le SH, Thai KD, Ho DD, Hoang HH, Matsuhisa T, Okimoto T, Kodama M, Murakami K, Fujioka T, Yamaoka Y, Moriyama M. *Helicobacter pylori* infection and gastroduodenal diseases in Vietnam: a cross-sectional, hospital-based study. BMC Gastroenterol. 2010;10:114.

35. Trang TT, Shiota S, Matsuda M, Binh TT, Suzuki R, Vilaichone RK, Mahachai V, Tshering L, Dung HD, Uchida T, Matsunari O, Myint T, Khien VV, Yamaoka Y. The prevalence of *Helicobacter pylori* virulence factors in Bhutan, Vietnam, and Myanmar is related to gastric cancer incidence. *Biomed Res Int*. 2015;2015:830813.
36. Milad SSA, Korosh A, Reza A, Ghorbanali R, Yousef M, Rana F, Mojtaba S, Fatemeh A. Frequency of virulence-associated genotypes of *Helicobacter pylori* and their correlation with clinical outcome and histological parameters in infected patients. *CelPress*. 2021;Heliyon 7:e07610.
37. Loivukene K, Kolk H, Maaros HI, Kasenomm P, Ustav M, Mikelsaar M. Metronidazole and clarithromycin susceptibility and the subtypes of *vacA* of *Helicobacter pylori* isolates in Estonia. *Scand J Infect Dis*. 2000;32(1):59–62.
38. Feliciano O, Gutierrez O, Valdes L, Fragoso T, Calderin AM, Valdes AE, Llanes R. Prevalence of *Helicobacter pylori vacA, cagA, and iceA* genotypes in Cuban patients with upper gastrointestinal diseases. *Biomed Res Int*. 2015;2015:753710.
39. Akeel M, Shehata A, Elhafey A, Elmakki E, Aboshouk T, Ageely H, Mahfouz M. *Helicobacter pylori vacA, cagA and iceA* genotypes in dyspeptic patients from southwestern region, Saudi Arabia: distribution and association with clinical outcomes and histopathological changes. *BMC Gastroenterol*. 2019;19(1):16.
40. Qureshi NN, Gallaher B, Schiller NL. Evolution of amoxicillin resistance of *Helicobacter pylori in vitro*: characterization of resistance mechanisms. *Microb Drug Resist*. 2014;20(6):509–16.

## Figures

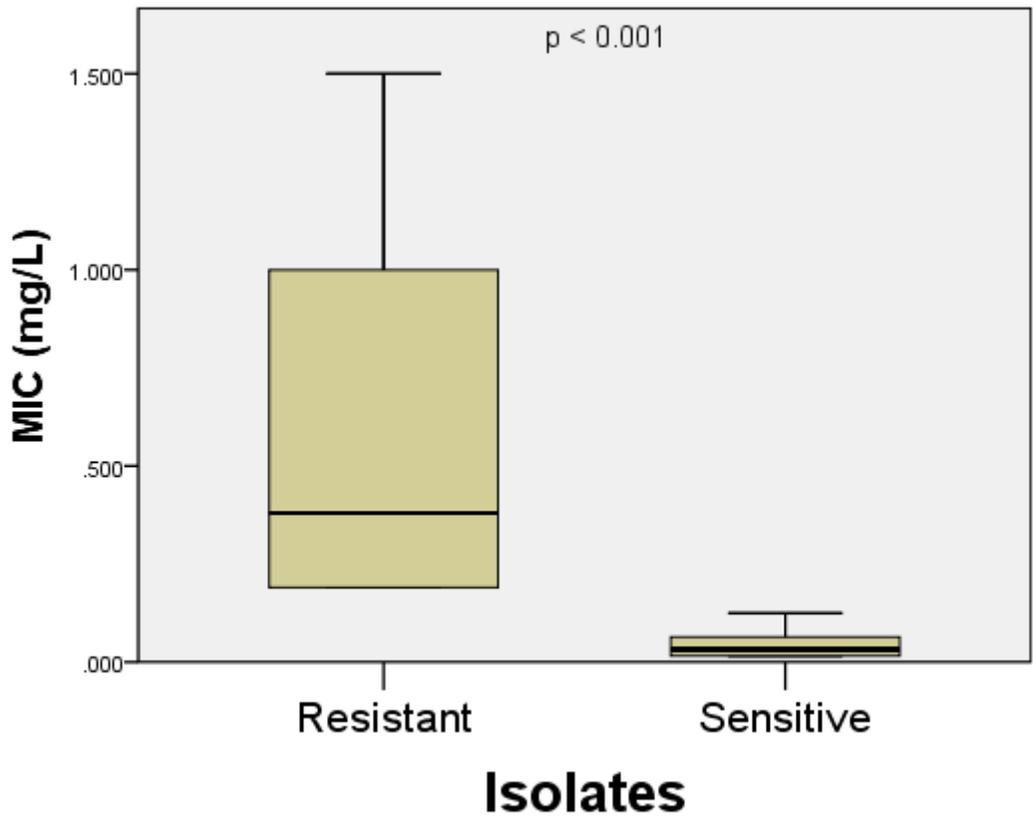


Figure 1

MIC range of isolates sensitive and resistant to amoxicillin

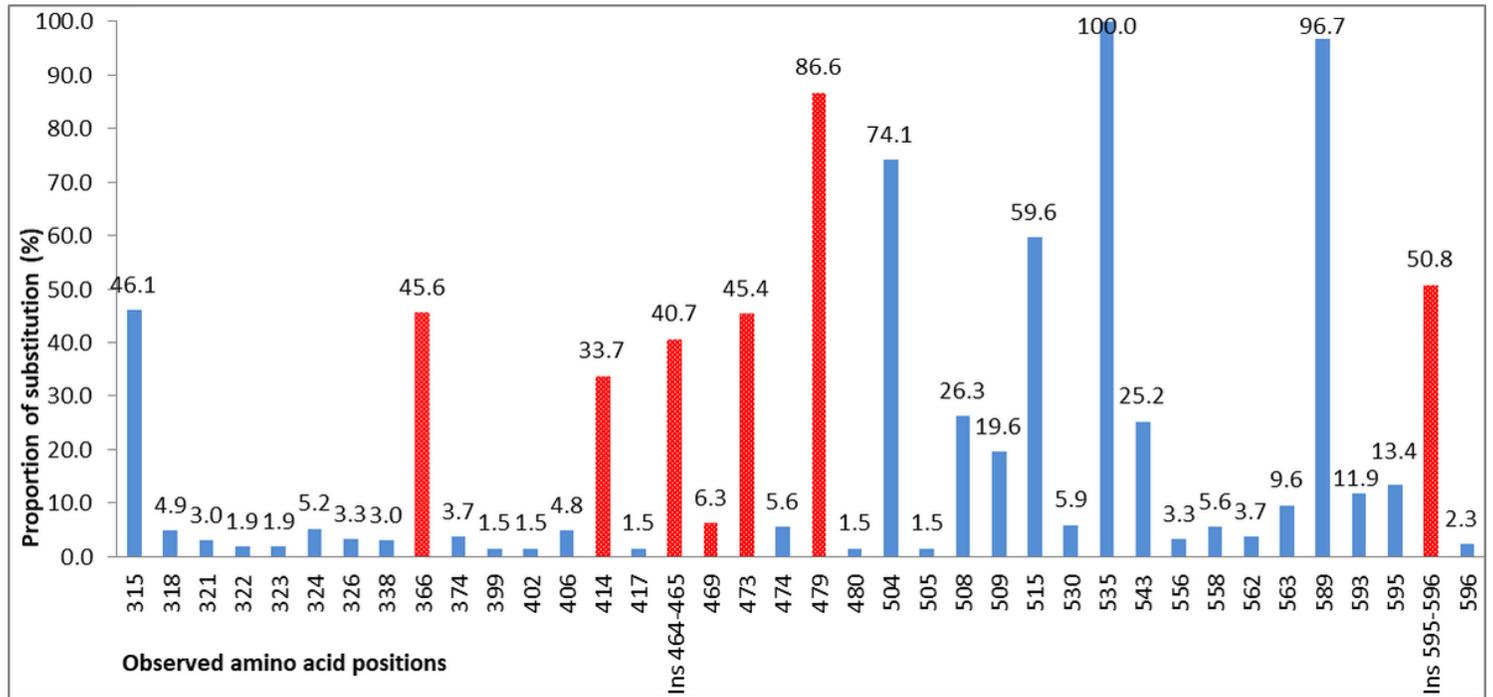


Figure 2

Amino acid substitution proportion of deduced PBP-1A discovered in the population compared to the AmxS *H. pylori* strain 26695. Red represents amino acid substitution differences with statistical significance from AmxS and AmxR samples by the E-test. A proportion lower than 1% was not included in the picture.

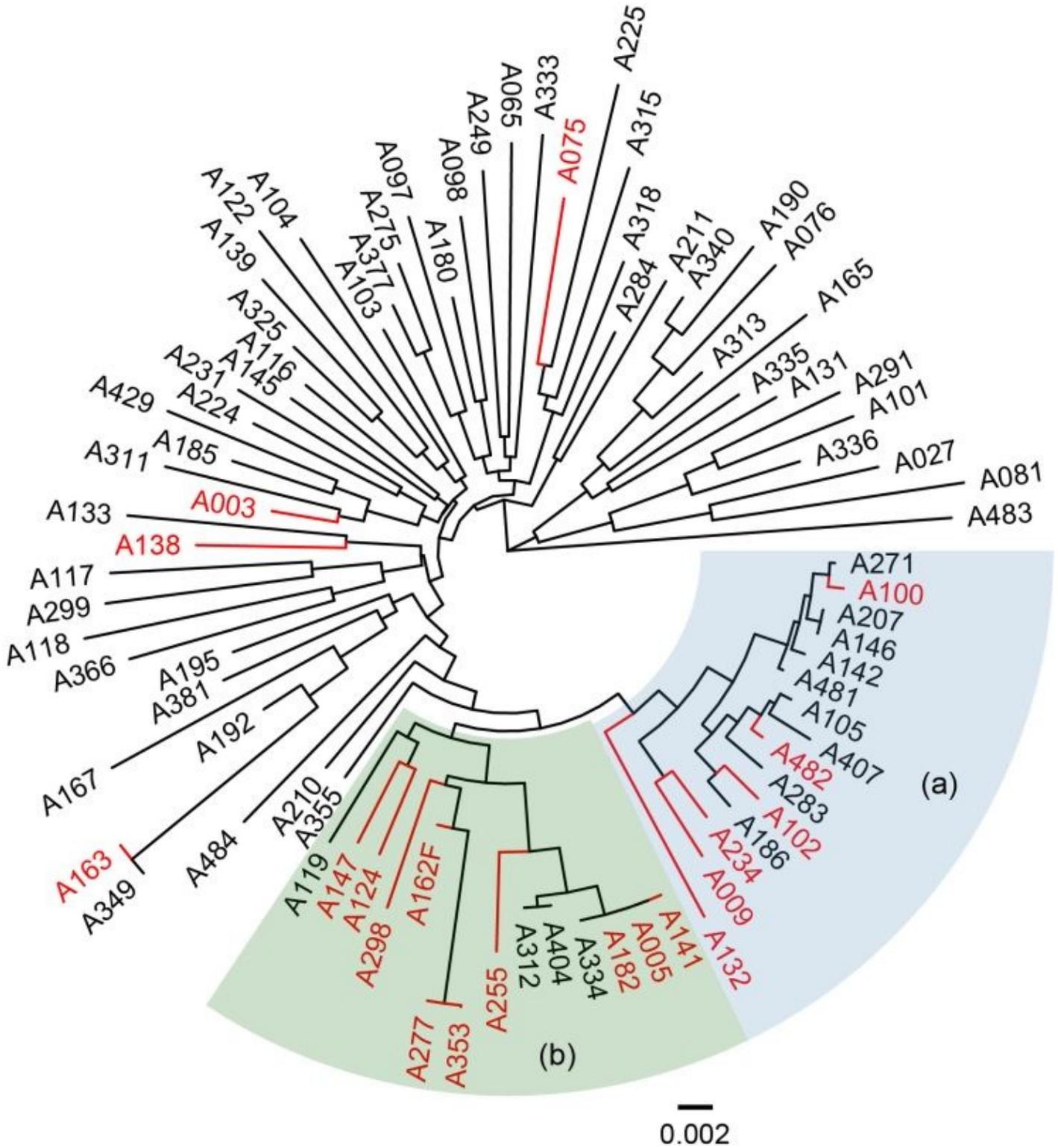


Figure 3

Phylogenetic tree of 85 sequences of the *pbp1A* gene obtained in this study. Scale bars indicate the numbers of nucleotide substitutions per site. Red nodes/tips represent *pbp1A* nucleotide sequences from the clinical specimens in which *H. pylori* isolates showed resistance to amoxicillin.