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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Helicobacter pylori cagA, *vacA*, and *iceA* genotypes and clinical outcomes: a cross-sectional study in central Vietnam

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Abstract

Helicobacter pylori is the most common cause of gastroduodenal diseases. The concept that *cagA*-positive *H. pylori* is a risk factor for gastric cancer appears to be true only for *H. pylori* strains from Western countries. Other virulent genes may have a synergistic interaction with *cagA* during pathogenesis. This study aims to investigate *H. pylori cagA, vacA,* and *iceA* prevalence, genotypes, and their association to clinical outcomes in Vietnamese patients. The *cagA* status and *vacA* and *iceA* genotypes were determined using the PCR technique on DNA extracted from gastric biopsies of 141 patients with gastroduodenal diseases. After performing molecular analysis for *cagA*, *vacA*, and *iceA* genes, samples with mixed *H. pylori* strains, positivity, or negativity for both *cagA* and *cag*PAI-empty site, or unidentified genotypes were excluded. Finally, 107 samples were examined. The presence of the *cagA, vacA,* and *iceA* genes were detected in 77.6%, 100%, and 80.4% of cases, respectively. Notably, *cagA*(+) with EPIYA-ABD, *vacA* s1i1m1, *vacA* s1i1m2, *iceA1*, and *iceA2* accounted for 73.8%, 44.9%, 33.6%, 48.6%, and 31.8% of cases, respectively. Four *iceA2* subtypes (24-aa, 59-aa, 94-aa, and 129-aa variants) were found, with the 59-aa variant the most prevalent (70.6%). The *cagA*(+)/*vacA*s1i1m1/*iceA1* and *cagA*(+)/*vacA*s1i1m2/*iceA1* combinations were found in 26.2% and 25.1% of cases, respectively. A multivariable logistic regression analysis was performed, after adjusting for age and gender, with the gastritis group was used as a reference control. Statistically significant associations were found between the *vacA* s1i1m2 genotype, the *iceA1* variant, and the *cagA*(+)/*vacA*s1i1m2/*iceA1* combination and gastric cancer; the adjusted ORs were estimated as 18.02 (95% CI: 3.39–95.81), 4.09 (95% CI: 1.1–15.08), and 16.19 (95% CI: 3.42–76.66), respectively. Interestingly, for the first time, our study found that *vacA* s1i1m2, but not *vacA* s1i1m1, was a risk factor for gastric cancer. This study illustrates the genetic diversity of the *H. pylori cagA, vacA,* and *iceA* genes across geographical regions and contributes to understanding the importance of these genotypes for clinical outcomes.

Keywords Gastric cancer · Gastritis · Peptic ulcer · s1i1m1 · s1i1m2

Introduction

Helicobacter pylori (*H. pylori*) is a spiral-shaped gram-negative bacterium that colonizes the gastric mucosa of more than half of the population worldwide. This bacterium is a leading cause of gastroduodenal diseases. Notably, *H. pylori* has been classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) since 1994 [1]. The pathogenesis of *H. pylori* infection is associated with their toxins, the most well-studied of which are the virulence factors cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) [2, 3].

The *cagA* gene, located at the 3'-end of the *cag* pathogenicity island (*cag*PAI), encodes the CagA protein, which is prevalent in 60–70% of *H. pylori* worldwide [4, 5]. It is thought that *cagA*-positive *H. pylori* strains are a risk factor

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for gastric cancer (GC). However, this appears to be true only for *H. pylori* strains in Western countries, where the *cagA*-positivity rate for *H. pylori* is only about 40% [6]. At the same time, regardless of gastroduodenal disease, *H. pylori* strains in East Asian countries exhibit extremely high rates of possessing the *cagA* gene, up to 90–95% [4, 7, 8]. Therefore, the *cagA* gene is not the only biological indicator for assessing the clinical outcome caused by *H. pylori* [9].

The *vacA* gene, which encodes the VacA protein, is found in all *H. pylori* strains. This vacuolating cytotoxin plays an important role in apoptosis and the proinflammatory response [7]. The diversity of *vacA* genotypes causes differences in cytotoxic activity between specific *H. pylori* strains [10, 11]. The signal region, which has two alleles (s1 and s2), the middle region, which has two alleles (m1 and m2), and the intermediate region, which has two alleles (i1 and i2), are the three main parts of the *vacA* gene [12, 13]. The *H. pylori* strains with the *vacA* s1m1 genotype exhibit the highest vacuolating activity, higher than the *vacA* s2m2 strains [10]. The toxicity of the *vacA* s1m2 strains is determined by the combination of alleles i1 or i2, with *vacA* s1i1m2 strains being vacuolating and *vacA* s1i2m2 strains being non-vacuolating [12].

Although *H. pylori* strains with both the *cagA* gene and vacuolating toxin-producing *vacA* genotypes are commonly found in patients with gastroduodenal diseases, a significant number of individuals infected with such *H. pylori* strains remain asymptomatic [14, 15]. Several other *H. pylori* virulence genes have been studied. Peek, in particular, discovered the *iceA* gene (induced by contact with epithelium gene A), whose transcription was induced by *H. pylori*'s adherence to the gastric epithelium. This gene has two major allelic variants, *iceA1* and *iceA2* [14]. A number of studies have considered the role of the *iceA* gene in conjunction with the *cagA* gene and the *vacA* gene in the pathogenesis of *H. pylori* [2, 16, 17]. The current study aimed to investigate the prevalence of *H. pylori cagA, vacA,* and *iceA*, the genotypes and their association to clinical outcomes in Vietnamese patients.

Materials and methods

Patients

This cross-sectional study was conducted at the University of Medicine and Pharmacy Hospital, Hue University, Vietnam, between June 2019 and February 2022. Patients with dyspepsia symptoms, who underwent esophagogastroduodenoscopies at the Centre of Gastroenterology and Endoscopy and were diagnosed with gastroduodenal diseases, were recruited for the study. At least two gastric mucosa biopsy specimens were obtained from the corpus and antrum to determine *H. pylori* infection by rapid urease test (RUT). Subsequently, DNA was extracted from these biopsy specimens to confirm the *H. pylori* positivity and genotyping the *H. pylori cagA, vacA*, and *iceA* genes by polymerase chain reaction (PCR) assays. *H. pylori* infection was diagnosed if both the RUT and PCR (with primers specific for the *ureC* gene) assays were positive [18]. With respect to patients who had neoplastic suspicious lesions, additional gastric mucosa biopsy specimens were obtained for histopathological examination to confirm gastric cancer. As a result, 141 patients with *H. pylori*-positive gastroduodenal diseases were recruited for initial study samples. After performing molecular analysis for *H. pylori cagA*, *vacA*, and *iceA* genes, samples with mixed *H. pylori* strains, positivity or negativity for both *cagA* and *cag*PAI empty sites, or unidentified genotypes (for example *vacA* s? or *vacA* m?), were excluded. 107 samples were ultimately evaluated regarding their association with clinical outcomes (Fig. 1).

DNA extraction

Gastric mucosa biopsy specimens were kept at -20 °C in tubes containing TE buffer solution at a pH of 7.5 (Promega Corp., Madison, WI, USA) until performing the DNA extraction. Biopsy specimens were ground in 600 µL of Nuclei lysis solution (Promega Corp., Madison, WI, USA) added 17.5 µL of proteinase K, 20 mg/mL (Thermo Scientific, Wilmington, USA), before being extracted using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). The DNA concentration was determined using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, Wilmington, USA).

Genotyping *H. pylori cagA, vacA,* and *iceA* genes

The *cagA* gene is polymorphic, so the *cagA* status was identified by two PCR assays, the first using forward primer cag2 and reverse primer CAGTR previously described by Rudi and Yamaoka [19, 20] and the second using primers cag5c-F and cag3c-R developed by Chattopadhyay [21]. Primers cag2 and CAGTR are specific for the region flanking the *cagA*-EPIYA motif, so the PCR reaction yielded products with different sizes (450–550 bp) depending on the different *H. pylori* strains. The PCR reaction using primers cag5c-F and cag3c-R yielded products of 350 bp in size. All DNA samples were subjected to PCR assay using primers Luni1 and R5280 specific for the "*cag*PAI empty site" and yielded a 550-bp product [22]. The samples that were positive with one of two PCR assays (using primers cag2 and CAGTR and/or primers cag5c-F and cag3c-R) and negative with the *cag*PAI empty site were confirmed as true *cagA*-positive *H. pylori* samples, and vice versa. The *cagA*-EPIYA motif was identified by four single PCR reactions using cag2 forward

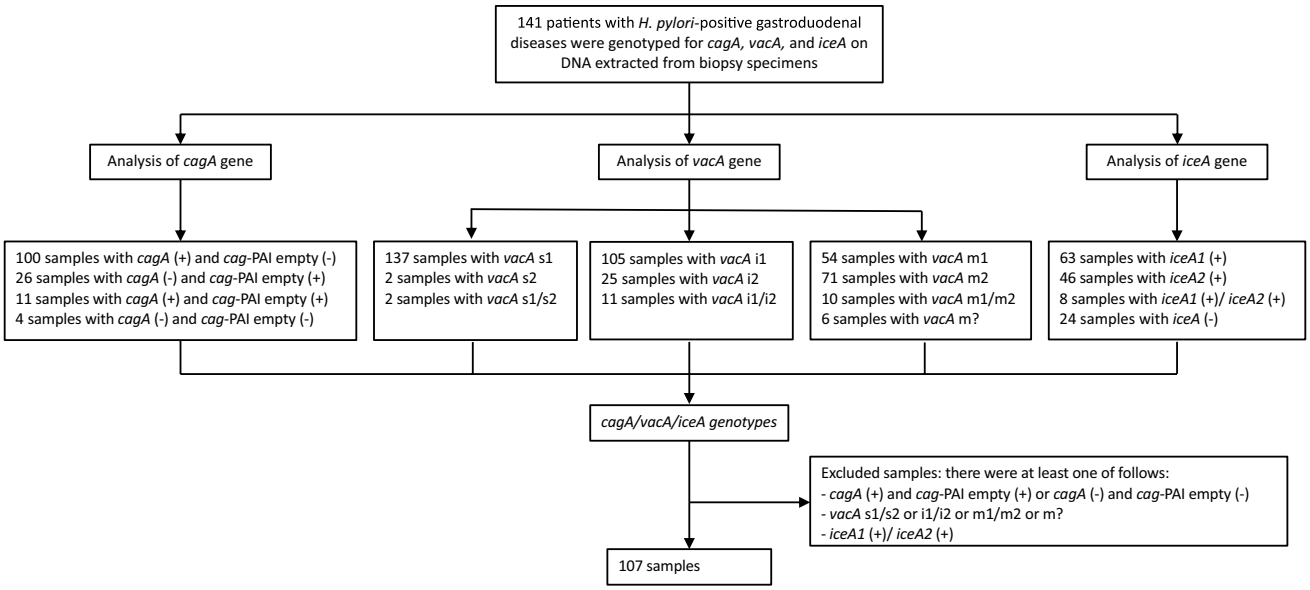

Fig. 1 Flowchart of recruitment. The flowchart above shows the findings of molecular analysis of the *cagA, vacA,* and *iceA* genes in 141 *H. pylori* clinical strains. After discharging inappropriate samples, 107 were eventually assessed for their association with clinical outcomes

primer and reverse primers as cagA-P1C, cagA-P2TA, cagAWest, and cagAEast specific for EPIYA-A, -B, -C, and -D, respectively [23, 24]. The EPIYA-A, -B, -C, and -D motifs were identified based on the sizes of specific amplicons, as 172 bp, 216 bp, 402 bp, and 416 bp, respectively.

A multiplex PCR assay was performed for genotyping the *vacA* sm of the *H. pylori* strains using the *vacA* s1/s2 allele-specific primers (VA1-F, VA1-R) and the *vacA* m1/m2 allele-specific primers (VAG-F, VAG-R) [10, 21, 25]. Samples without amplification with VA1-F and VA1-R primers or VAG-F and VAG-R primers in the multiplex PCR reaction were followed by a simplex PCR assay using the same specific primers. *H. pylori vacA* sm genotypes were determined based on the sizes of specific amplicons, in particular 259-bp and 286-bp for *vacA* s1 and s2, respectively, and 567-bp and 642-bp for *vacA* m1 and m2, respectively.

The genotype of *vacA* i was determined by two separate PCR assays. The forward primer VacF1 was used in both assays, whereas reverse primers C1-R and C2-R were used for assays identifying *vacA* i1 and i2, respectively [12]. The amplicon sizes for the i1 and i2 alleles were 426 bp and 432 bp, respectively.

Two separate PCR assays with primers iceA1-F5 and iceA1-R4 and primers iceA2-F6 and iceA2-R5 specific for *iceA1* and *iceA2* alleles, respectively, were used for genotyping the *iceA* gene [16]. The *iceA1* allele yielded a 247-bp product, whereas the *iceA2* allele yielded a 124-bp product corresponding to the absence of the 105-bp sequence encoding 35 amino acids, or yielded 229-bp, 334-bp or 439-bp products depending on whether the presence of the 105-bp sequence was present or repeated twice or three times, respectively. Depending on the number of amino acids (aa) in the encoded sequence, these *iceA2* variants are referred to as 24-aa, 59-aa, 94-aa, and 129-aa variants [26].

Each PCR reaction was performed in a total volume of 25 µL, including 12.5 µL GoTaq Green MasterMix 2X (Promega Corp., Madison, WI, USA), 10 pmol each forward and reverse primer, 200 ng of DNA, and nuclease-free water. The positive controls were DNA samples extracted from previous *H. pylori* isolates which were identified genotypes in our laboratory and the negative control was nuclease-free sterile water. The thermal cycle was as follows: initial denaturation at 95 °C for 5 min; 30 cycles including denaturation at 94 °C for 1 min, annealing at 53 °C for *cagA*, the *cag*PAI empty site, and *iceA* genotyping, at 50 °C for EPIYA motifs, at 52 °C for *vacA* sm genotyping, at 55 °C for *vacA* i genotyping, extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. The primer sequences are presented in Table 1. The PCR reactions were performed in the Agilent SureCycler 8800 (Agilent Technologies, Malaysia). The amplification products of *cagA*, the *cag*PAI empty site, the EPIYA motifs, *vacA* i, *iceA1*, and *iceA2* were electrophoresed on 1% agarose gel, whereas the products of *vacA* sm were electrophoresed on 2% agarose gel (Fig. 2). SafeView (Applied Biological Materials Inc. (abm), Canada) served as DNA staining.

Statistical analysis

The IBM SPSS Statistics Version 20 software was used to analyze the data. The chi-square test or Fisher's exact test (if more than 20% of expected frequencies were smaller than 5) was used to assess the linkage of genotypes, as well as the

Table 1 Primer sequences were used for genotyping *cagA, vacA, iceA* in this study

Primers	Nucleotide sequence (5'-3')	Reference
Identifying cagA gene		
cag5c-F	GTTGATAACGCTGTCGCTTC	[21]
cag3c-R	GGGTTGTATGATATTTTCCATAA	[21]
cag2F	GGAACCCTAGTCGGTAATG	[19]
CAGTR	GCTTTAGCTTCTGAYACYGC	[20]
Confirming the cagPAI empty site		
Luni1	ACATTTTGGCTAAATAAACGCTG	[22]
R5280	GGTTGCACGCATTTTCCCTTAATC	[22]
Genotyping cagA-EPIYA motif		
cagA-P1C	GTCCTGCTTTCTTTTTATTAACTTKAGC	[23]
cagA-P2TA	TTTAGCAACTTGAGTATAAATGGG	[23]
cagAWest	TTTCAAAGGGAAAGGTCCGCC	[24]
cagAEast	AGAGGGAAGCCTGCTTGATT	[24]
Genotyping vacA sm by multiplex PCR		
VA1-F	ATGGAAATACAACAAACACAC	[10]
VA1-R	CTGCTTGAATGCGCCAAAC	[10]
VAG-F	CAATCTGTCCAATCAAGCGAG	[25]
VAG-R	GCGTCAAAATAATTCCAAGG	[25]
Genotyping vacA i		
VacF1	GTTGGGATTGGGGGAATGCCG	[12]
C1-R	TTAATTTAACGCTGTTTGAAG	[12]
C2-R	GATCAACGCTCTGATTTGA	[12]
Genotyping iceA		
iceA1-F5	GTGTTTTTAACCAAAGTATC	[26]
iceA1-R4	CTATAGCCASTYTCTTTGCA	[26]
iceA2-F6	GTTGGGTATATCACAATTTAT	[26]
iceA2-R5	TTRCCCTATTTTCTAGTAGGT	[26]

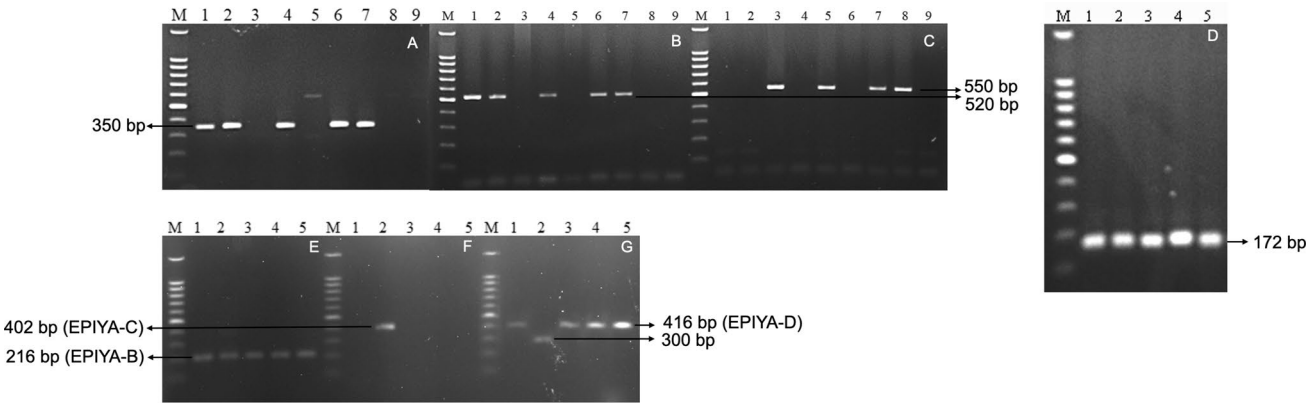

Fig. 2 Agarose gel electrophoresis image of the PCR products. Lane M: 100 bp DNA ladder (Promega Corp., Madison, WI, USA). 2A, 2B, and 2C: Amplication products of *cagA* gene using primers cag5c-F and cag3c-R, primers cag2 and CAGTR, and amplication products using primers Luni1 and R5280 specific for the "*cag*PAI empty site", respectively; lanes 1, 2, 4, 6: *cagA* (+) and *cag*PAI empty site (-); lanes 3, 5, 8: *cagA* (-) and *cag*PAI empty site (+); lane 7: *cagA* (+) and *cag*PAI empty site (+); lane 9: *cagA* (-) and *cag*PAI empty site (-). 2D, 2E, 2F, and 2G: Amplication products of EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D, respectively; lanes 1, 3, 4, 5: EPIYA-ABD subtype; lane 2: EPIYA-ABC subtype. 2H: Amplication products of *vacA* sm, lanes 1, 7: s1; lanes 2, 5, 6, 13: s1m1; lanes 3, 4, 8–11, 14–16: s1m2; lane 12: s2m2. 2I and 2 J: Amplication products of *vacA* i1 and i2, respectively; lanes 1–3, 7, 8, 11: i2; lanes 4–6, 12, 13: i1; lanes 14: i1/i2. 2 K and 2L: Amplication products of *iceA1* and *iceA2*, respectively; lane 1: *iceA1* (+) / *iceA2* (+) with 59-aa variant; lane 2: *iceA1* (-) / *iceA2* (+) with 94-aa variant; lane 3: *iceA1* (+) / *iceA2* (+) with 24-aa variant; lane 4: *iceA1* (-) / *iceA2* (+) with 129-aa variant; lanes 5, 7: *iceA1* (+) / *iceA2* (-); lane 6: *iceA1* (-) / *iceA2* (+) with 59-aa variant; lane 8: *iceA1* (+) / *iceA2* (+) with 59-aa and 94-aa variants; lane 9: *iceA1* (-) / *iceA2* (-)

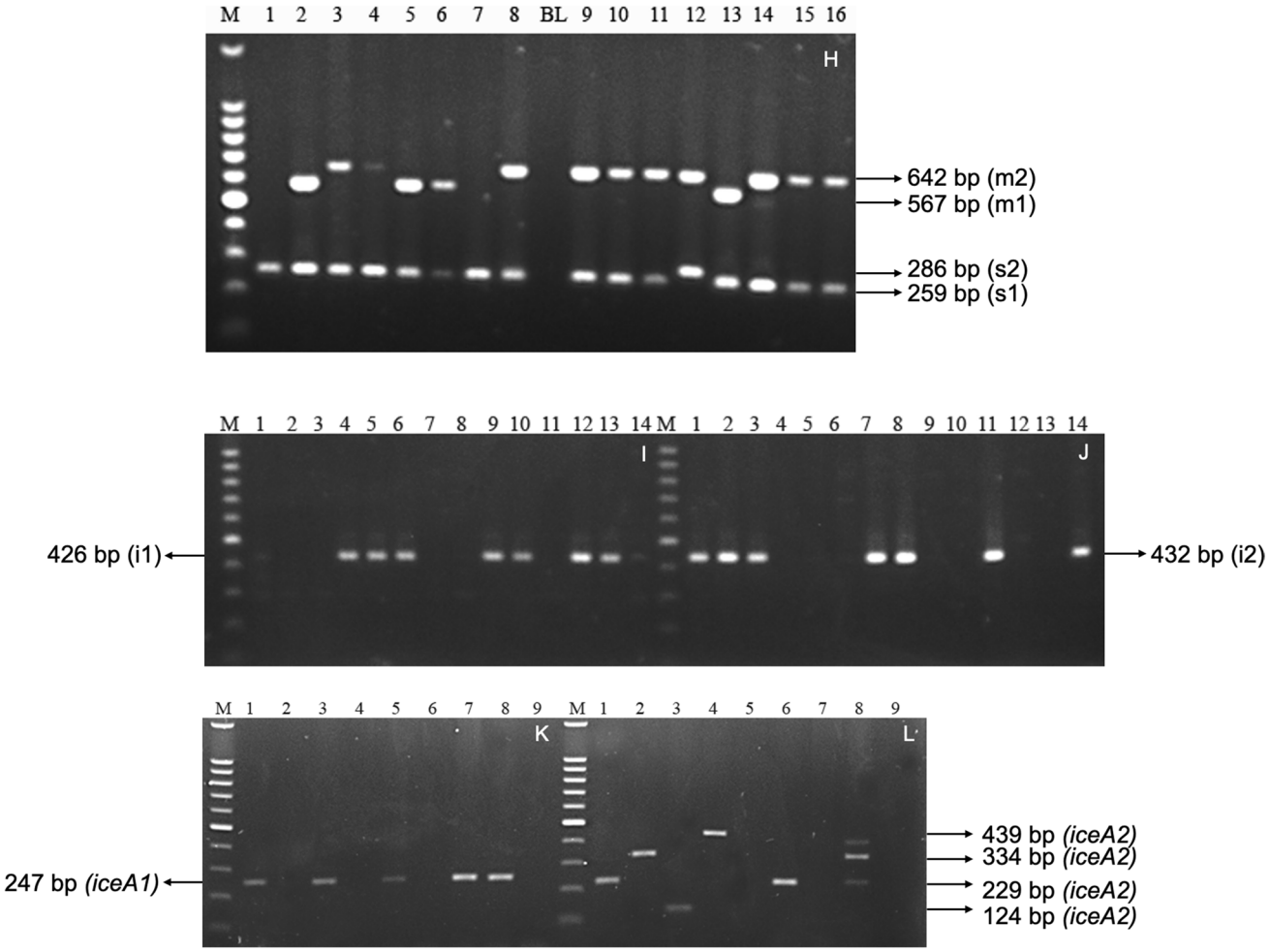

Fig. 2 (continued)

differences in distribution of genotypes between clinical outcomes. After adjusting for age (less than 40 years old; equal to or more than 40 years old) and gender (male; female), a multivariable logistic regression analysis was performed in order to assess the associations between genotypes and clinical outcomes. The adjusted odds ratio (aOR) and 95% confidence interval (CI) were estimated to identify the strength of the associations. *P*-values less than 0.05 were considered statistically significant.

Results

General characteristics of the study population

This study included 107 patients with *H. pylori*-positive gastroduodenal diseases. Gastritis comprised 57% (n = 61) of these cases, gastric ulcer (GU) 15% (n = 16), duodenal ulcer (DU) 14% (n = 15), and GC 14% (n = 15) of the total cases. In terms of gender, 47.7% were males and 52.5% were females. The mean age was 47.0 ± 17.2 years, with 36 patients being younger than 40 years old (see Table 2).

The present investigation comprised a sample of 107 patients who were diagnosed with gastroduodenal diseases positive for H. pylori. Gastritis comprised 57% (n = 61) of these cases, gastric ulcer (GU) 15% (n = 16), duodenal ulcer (DU) 14% (n = 15), and GC 14% (n = 15) of the total cases.

H. pylori cagA, *vacA*, and *iceA* genotypes

The overall distribution of the *cagA*, *vacA*, and *iceA* genotypes among the *H. pylori* strains is presented in Table 3. The presence of the *cagA*, *vacA*, and *iceA* genes were detected in 77.6%, 100% and 80.4% of the cases, respectively. The majority of *cagA*-positive *H. pylori* strains carried the

Table 2 Demographic and clinical characteristics of 107 patients with gastroduodenal diseases

Characteristics	Results
Mean age (years) ± SD	47.0 ± 17.2
< 40 years old	36 (33.6)
≥ 40 years old	71 (66.4)
Gender, n (%)	
Male	51 (47.7)
Female	56 (52.3)
Gastroduodenal diseases, *n* (%)	
Chronic gastritis	61 (57.0)
Peptic ulcers	16 (15.0)
Duodenal ulcers	15 (14.0)
Gastric cancer	15 (14.0)

SD: standard deviation

EPIYA-ABD motif, while only four strains exhibited the EPIYA-ABC motif.

Regarding the *vacA* genotypes, the s1i1m1 and s1i1m2 were most frequent, accounting for 44.9% and 33.6%, respectively. The s1i2m2 genotype was found as 19.6%, while the s2i2m2 genotype was very rare (1.9%). The *vacA* s1 was found to be predominant compared to the *vacA* s2, in 105 cases (98.1%) compared to two cases (1.9%). Similarly, the *vacA* i1 was more prevalent than the *vacA* i2, in 84 cases (78.5%) compared to 23 cases (21.5%). Moreover, the *vacA* m1 and m2 accounted for 44.9% and 55.1%, respectively ($p = 0.332$).

With respect to the *iceA* gene, the *iceA1* and *iceA2* accounted for 48.6% and 31.8%, respectively, while 19.6% of samples were negative for the *iceA* gene. Among the *iceA2*-positive *H. pylori* strains, the 59-aa variant was the most prevalent (70.6%), whereas the 129-aa variant was very rare (2.9%). The 24-aa and 94-aa variants were found in 14.7% and 11.8% of cases, respectively.

Our findings revealed that most *cagA*-positive *H. pylori* strains have *vacA* s1i1m1 and s1i1m2 genotypes, as well as *iceA1* or *iceA2* alleles, whereas most *cagA*-negative *H. pylori* strains have *vacA* s1i2m2 and s2i2m2 genotypes, with *iceA* negativity. *H. pylori* strains with *vacA* s1i1m1 genotypes are also more likely to have *iceA1* or *iceA2* alleles, whereas *vacA* s1i2m2 genotypes are more likely to have *iceA* negativity (Fig. 3). The combinations of more virulent variants, such as *cagA*(+)/*vacA*s1i1m1/*iceA1* and *cagA*(+)/*vacA*s1i1m2/*iceA1*, were found to be prevalent, at 26.2% and 25.1%, respectively. In addition, *cagA*(+)/*vacA*s1i1m1/*iceA*2 accounted for 17.8%. The combinations of less virulent variants, such as *cagA*(-)/*vacA*s1i2m2/*iceA*(-), was also found to have remarkable prevalence, namely 15.9% (Table 3).

Table 3 The distribution of *cagA, vacA* and *iceA* genotypes among *Helicobacter pylori* strains from 107 Vietnamese patients with gastroduodenal

Gene	Genotype	n	%
cagA	*cagA* (+) EPIYA-ABD	79	73.8
	cagA (+) EPIYA-ABC	4	3.7
	cagA (-)	24	22.4
vacA	*s1i1m1*	48	44.9
	s1i1m2	36	33.6
	s1i2m2	21	19.6
	s2i2m2	2	1.9
iceA	*iceA1*	52	48.6
	iceA2	34	31.8
	(24-aa)	*(5)*	*(14.7)**
	(59-aa)	*(24)*	*(70.6)**
	(94-aa)	*(4)*	*(11.8)**
	(129-aa)	*(1)*	*(2.9)**
	iceA (-)	21	19.6
Combination of *cagA, vacA,* and *iceA*	*cagA*(+)/*vacA* s1i1m1/*iceA*1	28	26.2
	cagA(+)/*vacA* s1i1m1/*iceA*2	19	17.8
	cagA(+)/*vacA* s1i1m1/*iceA*(-)	1	0.9
	cagA(+)/*vacA* s1i1m2/*iceA*1	23	21.5
	cagA(+)/*vacA* s1i1m2/*iceA*2	11	10.3
	cagA (+)/*vacA* s1i2m2/*iceA*2	1	0.9
	cagA(-)/*vacA* s1i1m2/*iceA*(-)	2	1.9
	cagA(-)/*vacA s1i2m2/iceA2*	3	2.8
	cagA(-)/*vacA* s1i2m2/*iceA*(-)	17	15.9
	cagA(-)/*vacA* s2i2m2/*iceA*1	1	0.9
	cagA(-)/*vacA* s2i2m2/*iceA*(-)	1	0.9
Total		107	100.0

*These percentages were calculated among 34 *iceA2*-positive *H. pylori* strains

Associations of *cagA*, *vacA*, and *iceA* genotypes with clinical outcomes

Overall, there was no statistically significant difference in the rate of *cagA*-positive *H. pylori* between the groups of gastroduodenal diseases (gastritis, GU, DU, and GC). In terms of the *iceA* genotype, neither the prevalence of *iceA1* nor of *iceA2* differed among the groups of gastroduodenal diseases. A statistically significant difference in *vacA* genotypes was found; specifically, *vacA* s1i1m1 (62.5%) was predominant in GU ($p = 0.037$) and *vacA* s1i1m2 (86.7%) was frequent in GC ($p < 0.001$). Regarding the combination of *cagA*, *vacA*, and *iceA* genotypes, we found a statistically significant difference in the rate of *cagA*(+)/*vacA*s1i1m2/*iceA*1 among the groups of gastritis, GU, DU, and GC; in particular, this combination was the most prevalent in GC patients, at 66.7%, $p < 0.001$ (Table 4).

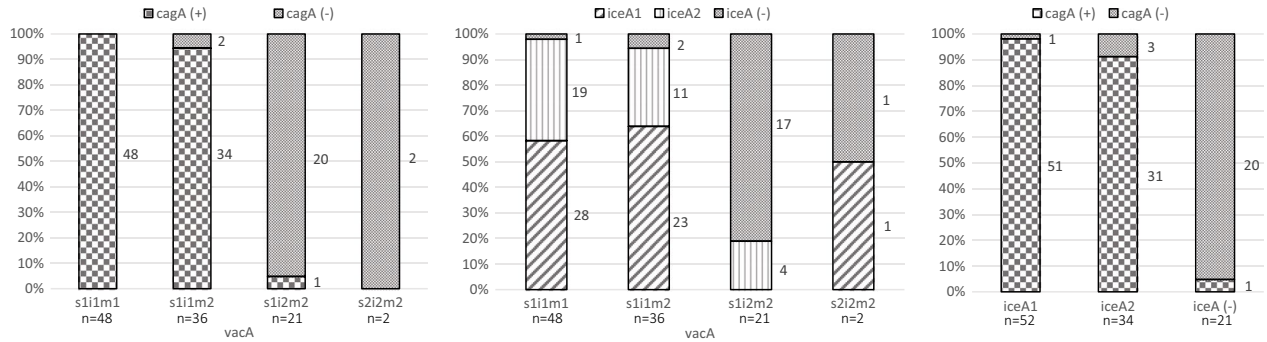

Fig. 3 The relationship of *cagA, vacA, and iceA* genotypes among 107 *Helicobacter pylori* strains without mixed or non-identified genotypes. The numbers on the graph are absolute values for *H. pylori* strain amounts. Statistical analysis revealed a significant association between *cagA, vacA,* and *iceA* genotypes, with *p*-values lower than 0.0001

However, a multivariable logistic regression analysis, after adjusting for age and gender, with the gastritis group used as a control group, found statistically significant associations between the *vacA* s1i1m2 genotype, the *iceA1* variant, and the *cagA(+)/vacA*s1i1m2/*iceA1* combination and GC. The adjusted ORs were estimated as 18.02 (95% CI: 3.39–95.81), 4.09 (95% CI: 1.11–15.08), and 16.19 (95% CI: 3.42–76.66), respectively (Table 5).

Table 4 The association of *cagA*, *vacA*, and *iceA* genotypes with gastroduodenal diseases

Genotypes	Gastritis n (%)	GU n (%)	DU n (%)	GC n (%)	*P*-value
cagA gene					
cagA (+)—EPIYA-ABD	41 (67.2)	14 (87.5)	11 (73.3)	13 (86.6)	0.272
cagA (+)—EPIYA-ABC	2 (3.3)	0	1 (6.7)	1 (6.7)	0.408
cagA (-)	18 (29.5)	2 (12.5)	3 (20.0)	1 (6.7)	0.209
vacA gene					
s1i1m1	28 (45.9)	10 (62.5)	8 (53.3)	2 (13.3)	0.037
s1i1m2	15 (24.6)	4 (25.0)	4 (26.7)	13 (86.7)	< 0.001
s1i2m2	17 (27.9)	2 (12.5)	2 (13.3)	0	0.064
s2i2m2	1 (1.6)	0	1 (6.7)	0	0.344
iceA gene					
iceA1 (+)	24 (39.3)	9 (56.2)	8 (53.3)	11 (73.3)	0.099
iceA2 (+)	20 (32.8)	6 (37.5)	5 (33.3)	3 (20.0)	0.741
iceA (-)	17 (27.9)	1 (6.2)	2 (13.3)	1 (6.7)	0.124
Combination of *cagA, vacA* and *iceA*					
cagA(+)/*vacA* s1i1m1/*iceA*1	16 (26.2)	6 (37.5)	5 (33.3)	1 (6.7)	0.191
cagA(+)/*vacA* s1i1m1/*iceA*2	11 (18.0)	4 (25.0)	3 (20.0)	1 (6.7)	0.615
cagA(+)/*vacA* s1i1m1/*iceA* (-)	1 (1.6)	0	0	0	N/A
cagA(+)/*vacA* s1i1m2/*iceA*1	8 (13.1)	3 (18.8)	2 (13.3)	10 (66.7)	< 0.001
cagA(+)/*vacA* s1i1m2/*iceA*2	6 (9.8)	1 (6.2)	2 (13.3)	2 (13.3)	0.789
cagA (+)/*vacA* s1i2m2/*iceA*2	1 (1.6)	0	0	0	N/A
cagA(-)/*vacA* s1i1m2/*iceA* (-)	1 (1.6)	0	0	1 (6.7)	N/A
cagA(-)/*vacA s1i2m2/iceA2*	2 (3.3)	1 (6.2)	0	0	N/A
cagA(-)/*vacA* s1i2m2/*iceA* (-)	14 (23.0)	1 (6.2)	2 (13.3)	0	0.108
cagA(-)/*vacA* s2i2m2/*iceA*1	0	0	1 (6.7)	0	N/A
cagA(-)/*vacA* s2i2m2/*iceA* (-)	1 (1.6)	0	0	0	N/A
Total	61	16	15	15	

N/A: Not applicable

Discussion

The *cagA*, *vacA*, and *iceA* genotypes are essential for *H. pylori* toxicity. However, the role of these genes in the pathogenesis of gastroduodenal disease is controversial. The main causes are the geographical differences in the prevalence of *H. pylori* virulence genes and genotypes, as well as the synergic interaction between these genes.

In this study, we performed DNA extraction from gastric mucosa biopsy specimens in order to investigate *H. pylori* virulence genes. This approach avoided the bacteria culture process, which requires time and labor. We cleared our samples prior to analysis by discharging samples with mixed *H. pylori* strains, cases of positivity or negativity for both the *cagA* and *cag*PAI empty sites, or unidentified genotypes (in this study, only six samples had unidentified genotypes for *vacA* m). This mixture of multiple *H. pylori* strains had been observed in previous studies analyzing gastric mucosa biopsy specimens [21, 27], and even in culture isolates [22].

Prevalence of *cagA*-positive *H. pylori* strains

East Asian *H. pylori* strains were found to have a higher *cagA* gene prevalence (90–95%) than strains from other regions [7]. However, in the current study, this prevalence was only 77.6%. Our results were consistent with those of previous research studies conducted in Vietnam, such as Phan's study, which was conducted in Hue city between 2012 and 2014, and found a *cagA*-positive *H. pylori* prevalence of 84% (n = 88) [28]; and Nguyen's study, which was conducted in Ho Chi Minh city between 2016 and 2017, and found a *cagA*-positive *H. pylori* prevalence of 79.5% (n = 83) [29]. By contrast, the *cagA* prevalence was much higher in other regions of Vietnam, such as 99% (n = 96) in Daklak, 100% (n = 75) in Lao Cai [30], and 96.2% (n = 53) in Hanoi [31]. Regarding the EPIYA motif of *cagA*-positive *H. pylori*, our findings revealed that only four strains carried the EPIYA-ABC motif, with no EPIYA-C repetitions. This showed that patients in our region, a city in central Vietnam, were infrequently infected by Western-type *H. pylori* strains.

Although we discharged the samples that were positive with both PCR assays specific for the *cagA* gene and the *cag*-PAI empty site, as well as ones that were negative with both these PCR assays, this issue should be discussed, because it reveals specific genetic diversity with regard to the *cagA* gene and the *cag*PAI in Vietnamese *H. pylori* strains. The *cagA* gene is thought to be an indicator of the presence of *cag*PAI and the ability to produce the CagA protein; some studies have revealed a partial deletion of this island with a low prevalence of 5–10% [32]. In studies by Censini et al. and Maeda et al., the *cagA* gene was found in all *H. pylori* strains from Western countries and Japan that had a partial deletion of *cag*PAI [32, 33]. On the other hand, Nguyen demonstrated that 6 of the 12 Vietnamese *H. pylori* strains with partial deletion of *cag*PAI had a deleted part that included the *cagA* gene [34]. As a result, these strains are negative for both *cagA* and *cag*PAI empty site PCR assays. In the current study, 4 of 141 initial samples were negative for both the *cagA* gene and the *cag*PAI empty site. It is possible that *H. pylori* strains with partial deletion of *cag*PAI were present in our samples. Furthermore, the *cagA* point mutation could be a contributing factor to this situation [28]. The percentage of samples that were found to be negative for both *cagA* and *cag*PAI empty site PCR assays was very low at only 2.8% (4/141), so it did not significantly affect the prevalence of *cagA*-positive *H. pylori* in this study.

Table 5 Multivariable logistic regression analysis after adjusting by age and gender

Genotype	Gastritis	GC	aOR (95% CI)	*P*-value
cagA				
Yes	43	14	6.86 (0.8–58.61)	0.079
No	18	1	1	
vacA s1i1m2				
Yes	15	13	18.02 (3.39–95.81)	0.001
No	46	2	1	
iceA1				
Yes	24	11	4.09 (1.11–15.08)	0.035
No	37	4	1	
cagA(+)/*vacA* s1i1m2/*iceA*1				
Yes	8	10	16.19 (3.42–76.66)	< 0.001
No	53	5	1	
Total	61	15		

aOR: adjusted odds ratio

H. pylori vacA genotypes

The *vacA* gene, which encodes the VacA protein, a vacuolating cytotoxin, was detected in all *H. pylori* strains. This is a highly polymorphic gene that exhibits alternating activity depending on the genotype. Three specific regions of VacA that were extensively studied were the s- and i-regions on the p33 domain, as well as the m region on the p55 domain [35]. A 12-amino-acid segment found in VacA s2 but not in VacA s1 prevents vacuolating activity of the VacA s2 subtype, whereas the VacA s1 subtype exhibits this activity [35, 36]. It is known that the m region has cell-type specificity and plays a role in binding to epithelial cells [37–39]. Rhead et al. reported the i region for the first time in 2007, with two variants, i1 and i2, with the i1 form being more toxic than the i2 [12].

To identify the *vacA* s, m, and i genotypes, we performed multiplex and simplex PCR assays. All samples in the current study possessed the *vacA* gene. Our observations reveal that the *vacA* s1 subtype predominated. Previous research has found an extremely high prevalence of the *vacA* s1 genotype, at approximately 98–100%, in *H. pylori* strains infected Vietnamese patients with gastroduodenal diseases [2, 28–30]. By comparison, Hispanics, people of African origin, and others have a prevalence of only 65%, 80%, and 93%, respectively [2].

With respect to the *vacA* i genotype, the prevalence of *vacA* i1 in the current study was also high (78.5%); it was however lower than the prevalence of 91% found by the study of Phan et al. in Vietnam [28]. In contrast to the *vacA* s and i genotypes, the *vacA* m genotype was fairly evenly distributed in the m1 and m2 subtypes, $p = 0.332$. This distribution is consistent with previous findings in the Vietnamese population [28, 29]. However, a study on minor ethnic groups in Vietnam found a discrepancy, with m1 prevalence (65.5%) higher than that of m2 (33.3%) [30]. Regarding the combination of the *vacA* s, m, and i variants, our results reveal a predominance of the s1i1m1 and s1i1m2 genotypes, which could be due to selected advantage of higher toxicity. To date, there has been relatively little research on this intermediate region of *H. pylori* strains in Vietnam. Even recent studies published in 2018 and 2021 [29, 30] only investigated the *vacA* s and m genotypes in *H. pylori* strains in infected patients in Vietnam. Therefore, our findings on the *vacA* s, i, and m genotypes may contribute to elucidating the genetic diversity of *H. pylori* among the Vietnamese population.

H. pylori iceA1 and *iceA2* alleles

The *iceA* gene, which has two alleles known as *iceA1* and *iceA2*, was first identified by Peek et al. in 1998. The *iceA* transcription is induced by adherence to gastric epithelial cells, so IceA may contribute to the development of gastrointestinal pathogenesis within infected patients [14, 40]. Numerous studies on the prevalence of and association between *iceA1* and *iceA2* variants and clinical outcomes have been conducted. The *iceA1* allelic variant is more prevalent in Asian countries, while the *iceA2* allelic variant is more prevalent in Western countries [41].

The current study found that the prevalence of *iceA*-positive *H. pylori* is 80.4%, with *iceA1* predominating over *iceA2* in clinical *H. pylori* strains. Another study among the Vietnamese population has revealed similar results, with *iceA1* and *iceA2* indicating prevalence of 50% and 44%, respectively [31]. In Vietnam, the *iceA* gene of *H. pylori* has rarely been studied. The present study is the first to investigate the *iceA2* subtypes. We found four *iceA2* subtypes, namely the 24-aa, 59-aa, 94-aa, and 129-aa variants, with the 59-aa variant the most prevalent (70.6%). Most studies on the *iceA* gene have revealed two *iceA2* variants, 59-aa and 94-aa, with the 59-aa variant being predominant [2, 41]. The 24-aa variant has rarely been reported worldwide. It was found in only two out of 424 samples in a study by Yamaoka et al. [2], and in 5.8% of a study by Figueiredo et al. [26]. The current study highlights the higher prevalence (14.7%) of the 24-aa variant. The existence of the four variants demonstrates the genotypic diversity of *iceA2* among the Vietnamese population.

Combination of *cagA*, *vacA*, and *iceA* genotypes

Several studies have demonstrated the interaction of CagA and VacA. This property enables *H. pylori* to survive and cause damage to gastric epithelial cells without killing them [42, 43]. The combination of more virulent genetic variants of *H. pylori*, such as the *cagA*(+)/*vacA*s1/*iceA1* genotype, was demonstrated by van Doorn in *H. pylori* strains in the Netherlands [16], by Yamaoka in *H. pylori* strains in Japan and Korea [2], and by Fan in *H. pylori* strains in China [44]. The current study highlights that highly toxic genotypes tend to mix together in *H. pylori* genomes, with the *cagA*(+)/*vacA*s1i1m1/*iceA1* and *cagA*(+)/*vacA*s1i1m2/*iceA1* combinations being the predominant genotype. These combinations explain the ability of *H. pylori* strains to cause damage to gastric mucosa.

Associations of *cagA*, *vacA*, and *iceA* genotypes with clinical outcomes

In this study, we found no association between *cagA* status and gastroduodenal diseases. This is consistent with previous research on East Asian *H. pylori* strains, where *cagA* prevalence was high regardless of clinical outcomes [2, 9]. As previously stated, the *vacA* gene is present in all *H. pylori* strains and the *vacA* s-, i-, and m-region variants play different roles in the pathogenesis. Our study is one of only a few that looked at all three *vacA* regions of Vietnamese *H. pylori* strains. We discovered that the *vacA* s1i1m2 genotype, but not the *vacA* s1i1m1 genotype, is identified as a risk factor for GC when compared to a gastritis group as a control group. This is in contrast to several other studies that found the s1i1m1 to be a risk factor for GC [45, 46]. To the best of our knowledge, only one other study found s1cm2 to predominate in GC [47]. The s- and i-regions of VacA are required for vacuolating activity, whereas the m-region is cell-type specific [12]. According to Pagliaccia's research, the m2 variant is not toxic to HeLa cells, but it did exhibit vacuolating activity in cultured gastric cells [38]. This is consistent with the observation of a higher prevalence of the m2 variant in

cohorts with a high prevalence of GC, such as in our country and reported in the study by Wei et al. in China [47]. Furthermore, a study by González-Rivera et al. demonstrated that the i-region variants altered the ability of the *vacA* s1m2 subtype to vacuolate: the s1i1m2 subtype but not the s1i2m2 induced vacuolating activity [48]. Several studies have demonstrated a significant correlation between s1i1 and GC [49, 50]. Taken together, the above studies support our findings that the vacA s1i1m2 can be a risk factor for GC.

Since its discovery in 1998, the *iceA1* variant has been found to have a statistically significant high prevalence among patients with peptic ulcer disease (PUD) [14, 16]. Huang's meta-analysis of 22 studies indicated that infection with *iceA1*-positive *H. pylori* strains is a risk factor for PUD [51]. However, our study does not confirm this association. By contrast, we found *iceA1* allele increases the risk of GC but not of GU or DU. Various other studies have indicated an association between *iceA1* and GC in both Asian and Western populations [47, 49, 52, 53]. The current study found no association between *iceA2* and gastroduodenal diseases. Although both *iceA1* and *iceA2* alleles are expressed in gastric cells, *iceA1* expression induced inflammation via an increase in interleukin 8 [14, 16]. This clarifies the role of the *iceA1* allele in the pathogenesis of *H. pylori*. Although *cagA* (+) was not found to be a risk factor for GC in this study, the *cagA*(+)/*vacA* s1i1m2/*iceA1* combination was. This is consistent with the pathogenesis of *H. pylori*-induced gastroduodenal diseases, in which the interaction of several virulent genes plays an important role.

In conclusion, this study demonstrates the genetic diversity of the *H. pylori cagA*, *vacA* and *iceA* genes and contributes to elucidating the important roles of these genotypes for clinical outcomes. The *vacA* s1i1m2 and *iceA1* genotypes and the *cagA*(+)/*vacA*s1i1m2/*iceA1* combination are risk factors for GC. Our findings also reveal that Vietnamese *H. pylori* strains exhibit geographical differences.

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Data Availability The data that support the findings of this study are available from the corresponding author, TMTH, upon reasonable request.

Declarations

Ethics approval This study was approved by the Ethics Committee of the University of Medicine and Pharmacy, Hue University Vietnam (H2020-108). Each patient provided written informed consent.

Conflict of interest The authors declare no conflict of interest.

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