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Evaluating the Genetic Diversity of *Helicobacter pylori* Isolates in Patients Suffering from Gastritis

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ABSTRACT

Backgrounds: *Helicobacter pylori* infections vary in severity and virulence in different populations for various reasons. There are different *H. pylori* strains with varying degrees of virulence. The genetic diversity of *H. pylori* strains in gastritis patients in different areas has not been well understood. This study aimed to evaluate the prevalence rate and different genotypes of *H. pylori* strains in clinical specimens of patients with gastritis in Ilam, Iran.

Materials & Methods: Saliva and gastric biopsy samples were collected from 81 patients (55 males and 26 females in the age range of 20 to 90 years) referring to Ilam medical centers. After DNA extraction, the prevalence of *H. pylori* as well as *vacA, cagA,* and *ureC* genes was evaluated using PCR, and then each *vacA*-positive sample was further evaluated for *m1m2* and *s1s2* variants.

Findings: The *cagA* and *vacA* genes were found in 27 (71%) and 36 (94.7%) *H. pylori*-positive samples, respectively. The *cagA* gene was detected in patients with gastric pain (44.4%) and anorexia (18.51%). Also, the results showed that the *vacA s2m2* genotype and *m2* allele were present in 32.9% of *H. pylori* isolates. Moreover, *s2m2* and *s1m2* genotypes were detected in 42.1 and 26.3% of *vacA*-positive samples, respectively. The lowest frequency was related to the *m1* allele (17.18%).

Conclusion: This study results indicate a plausible relationship between the presence of some genotypes of *H. pylori* and the progression of gastritis, suggesting these markers as promising biomarkers to predict the disease severity.

Keywords: *Helicobacter pylori*, Gastritis, Genotyping, *vacA*, *cagA*, PCR.

CITATION LINKS

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Introduction

Helicobacter pylori (*H. pylori*) is a microaerophilic Gram-negative bacterium that produces urease [1, 2]. *H. pylori* could spread worldwide; this bacterium could colonize the gastric mucosa of 50% of the world's population and cause prolonged infections in the gastric and gut mucosa [1, 3]. The prevalence of *H. pylori* varies from 25% in developed countries to over 90% in developing countries [1]. It could cause upper gastrointestinal diseases such as chronic gastritis, peptic ulcer disease, gastric/lymphoid margin mucosa-associated (MALT) lymphoma, and gastric lymphoma. Recently, it has been suggested that *H. pylori* may be associated with extra-intestinal diseases, such as immune thrombocytopenic purpura, anemia, and vitamin B12 deficiency [3]. Various studies have been conducted to evaluate the role of *H. pylori* virulence factors in the pathogenicity of this bacterium [4]. The prevalence of cytotoxin-associated gene pathogenicity island (*cag PAI*), which could be considered as a marker for the presence of gastric diseases, is one of the significant health problems worldwide. The *cag PAI* encodes a type IV secretion system, which transmits the *cagA* into host gastric epithelial cells. The association between *H. pylori* and gastric cancer or gastritis has been well established [5]. Those infected with *cagA*-positive pathogens may experience higher levels of interleukin-8 and mucosal inflammation and may be at higher risk of gastric ulcers and gastritis. Moreover, the vacuolating cytotoxin A (*vacA)* gene of *H. pylori* produces a piercing toxin that could damage epithelial cells. This gene is present in most *H. pylori* strains and has two variable segments. The *s* region located at the 5'-end of the gene encodes a signal peptide and exhibits the *s1* or *s2* allele. Also, the *m* (middle) region exists in the form of the *m1* or *m2* allele [6]. Piercing cytotoxin production is related to a mosaic combination of allelic variants of *s* and *m* regions. Strains encoding *vacA s1* are associated with gastric ulcer disease. The mosaic composition of the *vacA* gene with specific genotypes is associated with different outcomes, especially duodenal ulcer disease. Upon entering the host cell, *cagA* could disrupt signaling pathways by phosphorylation-dependent mechanisms, resulting in abnormal proliferation, motility, and skeletal alteration in gastric epithelial cells. Furthermore, the polymorphic *vacA* gene has different types and exerts varying degrees of destructive cytotoxic activity. There are significant variations in the signal (*s1* or *s2*) and middle regions (*m1* or *m2*) of *vacA*. Strains harboring *vacA s1m1* are highly toxic, those harboring *vacA s2m2* are nontoxic, and those harboring *vacA s1m2* are often intermediate. The interaction of *vacA*-activated toxin with specific cellular receptors induces a cascade and consequently triggers some cellular events, such as induction of large cytoplasmic vacuoles, mitochondrial damage, release of cytochrome C, inhibition of T-lymphocyte activation, and interference with antigen presentation. Strains harboring *vacA s1m1* and *s1m2* are associated with gastric ulcers. There is a considerable relationship between *vacA s1m1*-encoding strains and gastritis [7], and the combination of mosaic alleles of *s, m,* and *i* regions in *vacA* is related to host cell specificity and cytotoxicity degree [8]. *H. pylori* strains that carry the *cagA* gene are more prone to induce inflammation, ulcers, gastritis, and cancer than those that do not carry this gene [9, 10].

Objectives: Genotypes of *H. pylori* and inflammatory reactions triggered by this infection vary across different nationalities. Therefore, the present study attempted to evaluate different genotypes among *H. pylori* strains isolated from saliva and gastric

biopsy specimens of patients with gastritis in Ilam, Iran.

Materials and Methods

Patients: The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR. SBMU. MSP.REC.1398.33). To analyze the presence of *vacA, cagA,* and *ureC* genes*,* gastric biopsy and salvia specimens were taken from 81 patients (including 55 males and 26 females in the age range of 20 to 90 years) referring to Ilam medical centers. Written consent was obtained from these individuals.

Extraction of genomic DNA: DNA was extracted from saliva and gastric biopsy specimens using GeneAll®kit (Seoul, South Korea). The quality of DNA samples was evaluated by Nano-Drap (Thermo Scientific NanoDrop 2000 Spectrophotometer, USA). All DNA samples were stored at -20 °C until used for further evaluation.

Polymerase chain reaction (PCR): Bio Intellectica PCR was used to detect the selected genes (*ureC, cagA,* and *vac*A) in *H. pylori* isolates using specific primers (Table 1). *H. pylori* ATCC700392 (strain 26695) was used as a positive control. The accuracy of DNA extraction was evaluated by detecting the *β*-globin gene. The frequency of *m1m2* and *s1s2* variants among *vacA*-positive strains was then evaluated accordingly. In this study, PCR reactions were performed in a final volume of 25 µL, containing 12.5 µL of master mix, 1 µL of forward primers, 1 µL of reverse primers, 5.5 µL of distilled water, and 5 µL of DNA.

To detect *ureC* and *cagA* genes, the following PCR thermal cycling conditions were used: a pre- denaturation step at 95 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, 56.5 ºC for 30 s, and 72 ºC for 45 s and a final extension step at 72 °C for 5 min. Also, the *vacA* PCR program was as follows: an initial denaturation step at 95 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 30 s, 53 ºC for 30 s, and 72 ºC for 45 s and a final extension step at 72 °C for 5 min. Moreover, multiplex PCR was used to detect *s2m2* and *s1m2* genotypes in *vacA*-positive isolates under the following conditions: a pre-incubation step at 94 ºC for 3 min, followed by 34 cycles of denaturation at 94 ºC for 30 s, 56.5 ºC for 30 s, and 72 ºC for 45 s and a final extension step at 72 °C for 5 min. Final PCR products were visualized by electrophoresis on 1.5% agarose gels.

Statistical analysis: Data were analyzed by SPSS software Version 22. Pearson's Chi-square test (X^2 test) and ANOVA test were used to evaluate the relationships between *vacA* and *cagA* genotypes of *H. pylori* and

Table 1) PCR primers used for amplification of *ureC*, *cagA*, and *vacA*, and their genotypes

DNA Region	Primer Sequence	PCR Products Size (bp)	References
ureC (glmM)	F: AAGCTTTTAGGGGTGTTAGGGGTTT R: AAGCTTACTTTCTAACACTAACGC	294	39
cagA	F:ATAATGCTAAATTAGACAACTTGAGCGA R: AGAAACAAAAGCAATACGATCATTC	128	40
vacA	F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	259	40
vacA -s1 or *s2*	F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	*S1*:259 *S2*:286	40
vacA -m1 or *m2*	F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG	*M1*:567 *M2*:642	40

Table 2) Relationship between *cagA* and *vacA* alleles

		cagA			
***vacA* Alleles**	**Category**	**Yes(Positive) Count (%)**	**No(Non-Positive) Count (%)**	**Test**	***P*-Value**
S1	No (non-positive)	16 (25.4)	47 (74.6)	Chi-square	.005
	Yes (positive)	11 (61.1)	7 (38.9)		
S2	No (non-positive)	13 (20.6)	50 (79.4)	Chi-square	<.001
	Yes (positive)	14 (77.8)	4 (22.2)		
M1	No (non-positive)	21 (30.0)	49 (70.0)	Chi-square	.108
	Yes (positive)	6 (54.5)	5 (45.5)		
M2	No (non-positive)	8 (14.3)	48 (85.7)	Chi-square	< .001
	Yes (positive)	6 (24.0)	19 (76.0)		

Table 3) Relationship between *cagA* and *vacA* genotypes

		cagA			
***vacA* Genotype**	**Category**	**Yes (Positive) Count (%)**	**No (Non-Positive) Count (%)**	**Test**	***P*-Value**
S1M1	No (non-positive)	23 (31.5)	50 (68.5)	Chi-square	.292
	Yes (positive)	4 (50)	4 (50)		
S2M1	No (non-positive)	25 (32.1)	53 (67.9)	Fisher's exact test	.256
	Yes (positive)	2 (66.7)	1 (33.3)		
S1M2	No (non-positive)	20 (28.2)	51 (71.8)	Chi-square	.009
	Yes (positive)	7 (70.0)	3 (30.0)		
S2M2	No (non-positive)	15 (22.7)	51 (77.3)	Chi-square	< .001
	Yes (positive)	12 (80.0)	3 (20.0)		

Table 4) Relationship between *ureC* and different conditions

	ureC			
Variables	**Yes (Positive) Count (%)**	**No (Non-Positive) Count (%)**	**Test**	***P*-Value**
Epigastritis	17 (44.7)	29 (67.4)		
Anemia	6 (15.8)	4 (9.3)		
Weight loss	4 (10.5)	2 (4.7)	Fisher's exact test	.036
Early satiety	6 (15.8)	5 (11.6)		
Anorexia	1 (2.6)	2 (4.7)		
Lung metastasis	2 (5.3)	1 (2.3)		
Chest pain	2 (5.3)	0 (0.0)		

clinical symptoms of patients. Experimental data were expressed as the mean (± stane dard deviation) of three independent assays. *P*-values less than .05 were considered significant.

Findings

This study investigated the presence and genotypes of *H. pylori* in saliva and gastric biopsy specimens of 55 male and 26 female patients in the age range of 20 to 90 years. The results showed that 44 out of 81 patients suffered from epigastric pain during gastroscopy. Moreover, *ureC* (294-bp) was detected in 38 (46.9%) samples, representing the presence of *H. pylori*. Also, *cagA* and *vacA* genes were detected in 27 (71%) and 36 (94.7%) *H. pylori*-positive samples, respectively (Figure 1 and 2).

Anorexia (18.51%) and gastric pain (44.4%) were the most frequent symptoms associated with strains encoding *cagA*. Furthermore, the prevalence of *vacA s2m2* genotype and *m2* allele (32.9%) was high among *H. pylori* strains isolated. Among the *vacA*-positive samples, the prevalence of *s2m2* and *s1m2* genotypes was 42.1 and 26.3%, respectively, and the frequency of *s1* and *s2* alleles was 50%. The *m1* allele had the lowest frequency (17.18%). It was found that the majority of people with *H. pylori* infection were in the age range of 46 to 60 years. The relationships between *cagA* and *vacA* alleles and genotypes are presented in Tables 2 and 3, respectively. Table 4 demonstrates the relationship between *ureC* and different conditions.

Discussion

H. pylori is one of the most genetically diverse bacterial species. There is a relationship between *H. pylori* genotypes and geographic distributions and the severity of gastric disease after infection [11-13].

In 1994, the World Health Organization (WHO) announced *H. pylori* as a carcinogen [14, 15]. This bacterium, which often causes chronic gastritis and gastric malignancies, is one of the most important human pathogens. About half of the world's population is infected with *H. pylori*. There are many theories about the pathophysiological mechanism of *H. pylori* in gastritis, but none of which are conclusive. This may be due to the genetic diversity of *H. pylori* species. Chronic gastritis, peptic ulcer disease, mucosa-asso-

ciated lymphoid tissue (MALT) lymphoma, and gastric lymphoma are considered among the consequences of this bacterial infection. Although more than half of the world's population is infected with *H. pylori*, this infection has no signs in most cases. Moreover, in terms of the prevalence of cancer-related deaths, gastric cancer is the third most common cause of cancer-related mortality [16, 17]. Thus, it is vital to investigate the prevalence and genetic diversity of this bacterium.

In this study, we investigated the prevalence and genotypes of *H. pylori* strains isolated from saliva and gastric biopsy specimens of patients with gastric problems in Ilam. The results indicated that *cagA* was positive in 44.4% of patients with gastric pain symptoms. Moreover, clinical signs of abdominal pain were predominant in patients with *H. pylori* infection, which is similar to other studies performed in the same city [18-20].

The results of this study also indicated that more than 70% of *H. pylori*-positive samples were *cagA* positive. This result is consistent with the results of some studies investigating the prevalence of *cagA* among *H. pylori*-positive samples; for example, Qiao et al. (2003) found that 87% of the samples were positive for the presence of the *cagA* gene, and this gene could be a cause of chronic inflammation or gastritis [17]. Also, the prevalence rate of *cagA* among Malay, Chinese, and Indian patients has been reported to be different (76.6, 86.4, and 86.8%, respectively) [17, 18]. In a study by Miernyk et al. (2011), 242 out of 286 patients (85%) were infected with *cagA*-positive *H. pylori* strains [21]. In another study in Cuba, 95 *H. pylori* isolates (73.2%) were *cagA* positive [22]. van Doorn et al. (1998) reported a tight relationship between *cagA* and gastric diseases [23]. Moreover, in a study in Iran, more than 70% of the samples were reported to be *cagA* positive [25]. In Mexico, the prevalence of *cagA* was found to be 39.2% [24]. All the studies reviewed above have indicated that the *cagA* gene is found at high levels in *H. pylori* strains, and the presence of this gene could be related to a more severe form of the disease and the disease progression towards gastric cancer.

Furthermore, about 95% of the strains isolated in this study carried the *vacA* gene. This result is similar to those reported in other studies where 95-100% of samples were positive for *vacA*. In a study in 2005, the *vacA* gene was positive in 69 out of 72 cases (95.8%) [25]. In another study, 83% of *H. pylori* strains harbored either *vacA s1m1* or *vacA s2m2* (223/269) [17]. In a study conducted by Amin and colleagues (2019), 100% of the samples were *vacA* positive [27]. According to a histopathology report from Colombia in 2019, 44.2% of gastric biopsy samples were positive for the presence of *vacA* gene [27]. In another study, the prevalence rate of *cagA* and *vacA* was reported to be 82.6 and 73.91% among patients with gastric cancer, respectively [28]. In another study, more than 80% of patients were *cagA* positive, and 91.3% were *vacA* positive [29].

The results showed that almost all *cagA*-positive *H. pylori* strains isolated in this study carried the *vacA s1m1*, *s1m2*, or *s2m2* genotype. The *vacA s2m2* genotype and *m2* allele were present in 32.9% of *H. pylori* isolates. Among the *vacA*-positive samples, *s2m2* and *s1m2* genotypes were detected in 42.1 and 26.3% of the samples, respectively, and the lowest frequency was related to the *m1* allele (17.18%). No significant association was found between clinical symptoms and *vacA* genotypes as well as between *cagA* and *vacA* genotypes, which may be due to the low number of samples. In addition, it was found that the prevalence of the *vacA s2* allele was the same as that of the *vacA s1* allele.

In another study in Kuwait, the prevalence of *vacA s1* and *s2* alleles in patients' samples was reported to be almost equal, consistent

with this study result. However, in several studies conducted in North African, *H. pylori* isolates have been reported to mainly carry the *vacA s2* gene [30- 32].

In only one study, a high percentage of Spanish and Portuguese *cagA*-positive *H. pylori* isolates harbored the *vacA s2m2* genotype (57%). However, other studies have not reported such a high percentage for *vacA s2m2* genotype among *cagA*-positive *H. pylori* isolates [31].

Most studies have shown a correlation between the *vacA s2m2* genotype and *cagA*-negative *H. pylori* strains [33], which was not confirmed in this study. The percentage of *vacA s2m2* genotype in this study was 32.9%, which is lower than those reported in other studies conducted in Australia (35%), Alaska (38%), and North Africa (57%) [23, 30, 34]. It should be noted that *s1m1* and *s2m2* genotypes have the highest and lowest cytotoxic activity, respectively [35-38], and *vacA s2m2* genotype has the lowest prevalence (0–57%) among all genotypes reported worldwide [20, 26, 30].

Conclusion

This study results reflect a relationship between some *H. pylori* genotypes and the progression of gastritis. A clear predominance of *s2m2* and *s1m2* genotypes was observed in this study.

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Ethical permissions: The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences (IR. SBMU. MSP.REC.1398.33).

Conflicts of interests: The authors declare that they have no competing interests**.**

Authors' contributions: B.H, MG, and S.KH designed the study and performed the molecular experiments. S.M and M.M performed the statistical analyses. M.D and M.G checked the results and drafted the manuscript. All authors read and approved the final version of the manuscript.

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Consent to participate: General information about the reason and method of conducting examinations was given to the patients, and a written consent form was obtained from all of them.

Availability of data and materials: The data supporting the findings of this study are available upon the request from the corresponding author.

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