



Determination of *Helicobacter pylori* virulence-associated genes in duodenal ulcer and gastric biopsies

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Received: 01 Feb 2017

Published: 17 Dec 2017

Abstract

Background: *Helicobacter pylori* (*H. pylori* or Hp) has been strongly associated with the peptic ulcer diseases, chronic gastritis, ulcers, and gastric cancer. Genes associated with pathogenicity have been designated for *H. pylori*, and some of them appear to be related to more severe clinical consequences of the infection. The present study was conducted to determine *cagA*, *vacA*, *cagE*, *iceA1*, *oipA*, and *iceA2* genes in *H. pylori* strains isolated from gastroduodenal patients, who referred to Shariati hospital in Tehran, Iran.

Methods: Gastric biopsy specimens were collected during endoscopy from patients, who referred to the Shariati hospital in Tehran, Iran during January and November 2015. After isolation of *H. pylori* from the biopsy culture, genomic DNA was extracted and subsequently used to identify *H. pylori* and virulence genes using specific primers.

Results: The isolation rate of *H. pylori* strains was 65.7% (169/257). The frequency of *cagA*, *vacA*, *cagE*, *iceA1*, *oipA*, and *iceA2* was 143 (% 84.6), 169 (100%), 131 (77.5%), 97 (57.3%), 89 (52.6%), and 72 (42.6%), respectively.

Conclusion: In this study, a significant difference was observed between investigated genes and strains isolated from PUD and GC patients ($p < 0.05$).

Keywords: *Helicobacter pylori*, Iran, Virulence factors, Multiplex PCR, Duodenal ulcers

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Cite this article as: Saeidi Y, Pournajaf A, Gholami M, Hasannejad-Bibalan M, Yaghoubi S, Khodabandeh M, Emadi B, Ferdosi-Shahandashti E, Rajabnia R. Determination of *Helicobacter pylori* virulence-associated genes in duodenal ulcer and gastric biopsies. *Med J Islam Repub Iran*. 2017 (17 Dec);31:95. <https://doi.org/10.14196/mjiri.31.95>

Introduction

Helicobacter pylori (*H. pylori* or Hp) is a gram-negative, spiral-shaped, motile, and microaerophilic bacterium that colonizes in the human stomach, however, it may be present in other parts of the body (1). This organism is responsible for a remarkable number of diseases including acute and chronic gastritis, peptic or duodenal ulcer, and gastric cancer (GC) (2). Several virulence factors have been proposed for Hp infections including urease, flagella, adhesions, vacuolating cytotoxin (*VacA*), duodenal ulcer promoting gene (*dupA*), outer inflammatory protein (*OipA*), cytotoxin-associated gene (*CagA*), and *IceA* (induced by

contact with epithelium) (3,4). The *cag* pathogenicity island (*cag* PAI) is a 40-kb DNA insertion element, which encodes CagA. Expression of CagA was found to be intensely related to peptic ulceration (4, 5). Thus, the *cag* PAI is a good criterion for Hp virulence determination. Hp strains are commonly divided into 2 *CagA*-positive and *CagA*-negative groups, depending on the presence or absence of the final *cag* PAI gene product (6). The *cagE* gene, located on *cag* PAI, is a Hp virulence determinant associated with more severe illness consequences. Isolates that possess *cagE* induce greater chemokine response in vitro

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↑What is “already known” in this topic:

Virulence markers of Hp play an important role in gastrointestinal disorders in Iranian patients. Several Hp virulence-associated genes have been reported to be associated with gastroduodenal patients in Iran. In this study, we assessed the relationship between Hp genotype and gastroduodenal pathology.

→What this article adds:

Despite a significant difference between investigated genes and strains isolated from PUD and GC patients ($p < 0.05$), further work is required to clarify the roles of Hp virulence factors in the development of gastroduodenal diseases.

than those without it (7, 8). This may be considered as a potential mechanism for altering disease outcomes after colonization with a Hp that contains *cagE* (9, 10). Also, *cagE* could be a risk factor for gastric wounds, may contribute to a good assessment of pathogenic potential of the Hp strains, and may also predict disease development in the gastric mucosa (11, 12). Hp-specific vacuolating cytotoxin gene (*vacA*) is desirable for rapid, reliable, and accurate detection of Hp-infections (13). Moreover, *vacA* is present in all Hp isolates and can promote multiple cellular activities and vacuolation including pore formation and cyt c release from mitochondria, which can cause cell death and attach to cell surface receptors, starting a proinflammatory reaction. Another virulence factor gene, *iceA*, like *vacA*, is present in all Hp strains. This gene has 2 *iceA1* and *iceA2* allelic variants (14, 15). *IceA1* is upregulated during Hp-gastric epithelium contact, and there is an important association between the presence of the *iceA1* allele and peptic ulcer disease (PUD) (16). Hp *iceA1*⁺ genotype enhances mucosal interleukin-8 (IL-8) expression and acute inflammation (17). *IceA2* gene has no homology to any known gene and the function is not clear. An epidemiological study found an association between *iceA* gene and its alleles with gastric and/or duodenal ulcer and GC in different geographic areas (18). Outer inflammatory protein (*OipA*) is one of the porin proteins and responsible for pathogen-host interaction. The protein was originally recognized as a proinflammatory response, according to the fact that *oipA* isogenic mutants decrease the release of IL-8 from gastric epithelial cells (19). Although the prevalence of Hp infection is high, as much as 70% of developing nations, only few people are affected by serious illnesses caused by this infection (20). This can be attributed to the participation of particular elements that contribute to the pathogenicity of this microorganism. Thus, the present study aimed at investigating the *cagA*, *cagE*, *vacA*, *oipA*, *iceA1*, and *iceA2* genes in Hp strains isolated from gastroduodenal biopsies in patients admitted to Shariati hospital in Tehran, Iran.

Methods

In this cross-sectional study, which was conducted during January and November 2015, a total of 257 nonduplicative and nonreiterative biopsy samples were collected by gastroenterologists from each untreated patient, who underwent upper gastroduodenal endoscopy in Shariati hospital in Tehran, Iran. Shariati hospital is one the most equipped teaching therapeutic centers affiliated to Tehran University

of Medical sciences (TUMS), with more than 857 beds. In total, 257 patients (132 males and 125 females) aged 18 to 91 years, with an average age of 53.1 years and clinical manifestations of abdominal pain or burning, nausea, vomiting, frequent burping, bloating, and weight loss, were enrolled in the study. Those who received Hp eradication therapy protocol or anti-Hp therapy, bismuth-containing regimens, proton pump inhibitors (PPIs), nonsteroidal anti-inflammatory drugs (NSAIDs) or H₂-receptor antagonists within 4 weeks prior to the project were excluded. Informed consent was obtained from all contributors. Two gastric mucosal biopsy samples (1 antrum and 1 corpus biopsy specimens) were obtained from each patient. Samples were used for culture and molecular examination. All samples were immediately placed in Stuart's medium and transferred to a microbiology laboratory within 2 hours and kept at 4°C. After homogenization with an electric tissue homogenizer (Ultraturrax, Iena, Germany), the biopsy specimens were streaked in the Campylobacter agar plates (Merck Co., Germany) supplemented with 10% defibrinated sheep blood and a set of specified antibiotics comprising 5 mg/L trimethoprim, 10 mg/L vancomycin, 5 mg/L cefsulodin, and 5 mg/L amphotericin B. Each plate was incubated at 37°C for 5 to 7 days under microaerophilic conditions (85% N₂, 10% CO₂ and 5% O₂). All suspected grown colonies were identified as Hp based on conventional microbiological and biochemical tests. All isolates were kept in the Brain Heart Infusion Broth (Merck Co., Germany) with 20% glycerol and stored at -70 °C for further DNA extraction. Then, genomic DNA was extracted using the ExiProgen™ Bacteria Genomic DNA Kit (Bioneer Co., Korea) in accordance with the manufacturer's instructions. The quality and quantity of the extracted DNA were evaluated using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific; Wilmington, DE, USA). The *vacA* gene was detected to confirm Hp in gastric biopsies. Multiplex PCR was performed for amplification of *vacA*, *cagA*, *cagE*, *iceA1*, *iceA2*, and *oipA* genes in a volume of 0.6 µL (0.5 µg) of template genomic DNA and then added to a final volume of 25µL PCR reaction mixture containing 2.0 µL of 10× PCR buffer, 0.9 µL MgCl₂ (50 mM), 0.5 µL dNTPs (10 mM), 1.2 µL of each primer, 0.7 µL of Taq DNA polymerase (5 U/µL) (Amplicon Co., Denmark), and 4.4 µL ddH₂O. Amplification was performed in a thermal gradient cyler (Eppendorf Co., Germany). The cycling conditions were as follow: initial denaturation at 95°C for 4 minutes, 31 cycles with denaturation at 95°C for 44 seconds, annealing at 51°C for 45 seconds, extension at 72°C

Table 1. Oligonucleotide primer sequences used in the study

Target Gene	Primer sequence (5'→3')	Amplicon size (bp)	Ref
<i>cagE</i>	F: 5'- TTGAAAACCTCAAGGATAGGATAGAGC-3' R: 5'- GCCTAGCGTAATATCACCATTACCC-3'	500	21
<i>cagA</i>	F: 5'- AATACACCAACGCTCCAAG-3' R: 5'- TTGTTGGCGCTTGCTCTC-3'	298	22
<i>iceA1</i>	F: 5'- GTGTTTTTAACCAAAGTATC-3' R: 5'- CTATAGCCASTCTCTTTGCA-3'	247	23
<i>iceA2</i>	F: 5'- GTTGTCGTTGTTTTAATGAA-3' R: 5'- GTCTTAAACCCACGATTAATA-3'	120	24
<i>VacA</i>	F: 5'- GCCGATATGCAAATGAGCCGC-3' R: 5'- CAATCGTGTGGGTCTGGAGC-3'	678	24
<i>oipA</i>	F: 5'- GTTTTTGATGCATGGGATTT-3' R: 5'- GTGCATCTCTTATGGCTTT-3'	401	25

for 62 seconds, and final extension at 72°C for 5 minutes. The amplified products were visualized by electrophoresis in 1.5% agarose gels stained with Gel Red™. Table 1 summarizes the primer sequences and the expected size of products. Positive and negative controls were evaluated with test samples on each run.

Ethics statement

The bacterial isolates analyzed in this study were collected by gastroenterologists from each untreated patient, who underwent upper gastroduodenal endoscopy in Shariati hospital in Tehran, Iran. Furthermore, all patient identifiers had been previously removed and data anonymity was assured (No.1395.9221133207).

Results

The presence of Hp infection in the participants was determined by microbiological culture examination and *vacA* gene polymerase chain reaction (PCR). Our patients were categorized into the following 3 groups based on the clinical data: 123 (72.78%) peptic ulcer diseases (PUD; duodenal and gastric ulcers), 41 (24.2%) non-ulcer dyspepsia (NUD; gastritis and duodenitis), and 5 (2.9%) GC (Table 2). Of 257 biopsy samples, 169 (65.7%) Hp strains were obtained. The distribution analysis of the Hp isolates revealed that most isolates (N; 87, 51.4%) were recovered from the gastric ulcer and the lowest isolates (N; 5, 2.9%) from GC. The highest and lowest prevalence of virulence-associated genes were detected in patients with gastric ulcer and GC, respectively. The highest frequency (N; 87, 51.4%) of virulence genes in the biopsy specimens obtained from gastric ulcer was 84.6% (*cagA*), 77.5% (*cagE*), 57.3% (*iceA1*), 42.6% (*iceA2*), and 52.6% (*oipA*), respectively (Table 2). The highest and lowest genotypes were *cagE/oipA* (N; 42, 24.8%) and *cagA/oipA/cagE/iceA1* (N; 15, 8.8%) (Table 3).

Discussion

Using culture-based method and PCR-amplified *vacA* genes, we found that of the 257 biopsy samples, 169 were

positive for Hp. Moreover, Hp strains were detected in patients with gastric ulcer (N; 87; 51.4%) significantly more frequently than in those with other gastroduodenal disorders. The *cagA* results (84.6%) in our study are similar to those obtained in Mexican (86%) and Japanese studies (90%) (26, 27). These data are in contrast with those of other studies in other countries including Pakistan (56%), Iraq (71%), India, and Bangladesh (70%) (28). The *cagPAI* encoded effector proteins for the type IV secretion system (T4SS of TFSS) mediate construction of CagA peptidoglycan and probably other bacterial factors into host cells cytoplasm, which are the chief cause of inflammation. A previous study found that *cagA/E/M/T* of *cagPAI* was used as a valuable marker for the risk of PUD. In our study, the prevalence of the *cagA* gene in strains isolated from patients with gastric ulcer was 85% (74 of 87). The *cagE* gene is located on the *cagPAI* and involves in the creation of the bacterial transport system and IL-8 production in gastric epithelial cells. In our study, the *cagE* gene was present in 131 of the 169 strains (77.5%). The prevalence of *cagE* was found in 70%, 81.6%, and 39% of Hp isolates in Malaysia, India, and China, respectively (29). In Tiwari et al. study, *cagE* was found in a larger proportion of the ulcer group (92.5%) compared with the NUD group (77.5%) (30). In studies of Podzorski et al. (31) and Li et al. (32), the frequency of *cagE* gene was 62% and 99%, respectively. The *iceA* gene has 2 allelic forms: *iceA1* and *iceA2*. The *iceA1* has been described to be the prevalent gene in many studies, while *iceA2* is a prevalent allele in others (33). The *iceA1* gene was most frequent in this study (57.9%). A Brazilian study reported a rate of 90.1% for the *iceA2* (34). In contrast, A Mexican study reported a rate of 9% for the *iceA2* allele (35). In East Asia, the *iceA1* genotype has been reported to be predominant (76%), while *iceA2* is predominant in Portugal and Colombia (36). These differences in *iceA* gene rates in several countries are strongly suggestive of its geographical distribution. Our study results revealed that all Hp strains (100%) were positive for *vacA* gene. According to other studies in Iran, the *vacA* s1 and s1m1 genotypes are also the dominant genotypes among Iranian patients with a high rate (90%) (37). However, the collective

Table 2. Distribution of Hp virulence genes among patients with gastroduodenal pathology

Gastroduodenal pathology	No. (%) of positive for <i>H. pylori</i>	Distribution of virulence factors (%)				
		<i>cagA</i>	<i>cagE</i>	<i>iceA1</i>	<i>iceA2</i>	<i>oipA</i>
Gastric ulcer	87 (51.4)	74 (85)	75 (86.2)	61(70.1)	46 (52.8)	52(59.7)
Duodenal ulcer	36 (21.3)	28 (77.7)	23 (63.8)	17 (47.2)	16 (44.4)	20(55.5)
Gastric cancer	5 (2.9)	5 (100)	2 (40)	3(60)	1(20)	1(20)
Gastritis	30 (17.7)	29 (96.6)	25 (83.3)	13 (43.3)	6 (20)	15(50)
Duodenitis	11 (6.7)	7 (63.6)	6 (54.5)	3(27.2)	3(27.2)	1(9)
Total	169 (100)	143 (84.6)	131 (77.5)	97 (57.3)	72 (42.6)	89(52.6)

Table 3. Relationship between the Hp genotype and gastroduodenal pathology

Genotype	No. of patients with gastroduodenal pathology (%)					
	Gastric ulcer	Duodenal ulcer	Gastric cancer	Gastritis	Duodenitis	Total
<i>cagA⁺/oipA⁺</i>	20(22.9)	6(16.6)	1(20)	8(26.6)	2(18.1)	37(21.8)
<i>cagE⁺/oipA⁺</i>	21(24.1)	7(19.5)	1(20)	10(33.6)	3(27.4)	42(24.8)
<i>cagA⁺/oipA⁺/iceA1⁺</i>	10(11.5)	6(16.7)	2(40)	2(6.6)	2(18.1)	22(13.2)
<i>cagA⁺/oipA⁺/cagE⁺/iceA1⁺</i>	6(6.9)	5(13.8)	1(20)	2(6.6)	1(9)	15(8.8)
Others	30(34.6)	12(33.4)	0	8(26.6)	3(27.4)	53(31.4)
Total	87(100)	36(100)	5(100)	30(100)	11(100)	169(100)

assessment of the *vacA* genotype determined most of s1m2 genotypes in patients with PUD, which was remarkably correlated with the disease ($p < 0.05$) in other works (38). These variations may contribute to the varying prevalence of gastric diseases in these areas. We found that 52.6% of isolated strains contain *oipA* gene. These data are in contrast with those of Yamaoka et al. (39) and Kudo et al., who identified the *oipA* gene from 45.9% and 30% of studied Hp isolates, respectively (40). In most studies, the *oipA* gene was present in most strains. In contrast, there were many *oipA*-negative cases in our study.

Conclusion

These results revealed that the high number of Hp strains may be considered as highly virulent, as they possessed 3, 4, or even 5 of the virulence markers analyzed. In this study, a significant difference was observed between investigated genes and strains isolated from PUD and GC patients ($p < 0.05$). However, no significant difference was found in the NUD patients ($p = 0.08$). Although there was variation in the prevalence of the *cagA*, *cagE*, *iceA1*, *iceA2*, and *vacA* in HP strains isolated from patients in this study, this distribution was not statistically significant in our studied population.

Acknowledgment

The authors acknowledge Dr. Gholamreza Irajian for his opportune advises and contribution to this paper. We also greatly appreciate the assistance of the staff at the Gastroenterological Unit in Shariati hospital in Tehran, Iran, for their great support during this investigation.

Conflict of Interests

The authors declare that they have no competing interests.

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