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

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**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 are required 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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**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.
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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 \textit{cagE} (9, 10). Also, \textit{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 (\textit{vacA}) is desirable for rapid, reliable, and accurate detection of Hp-infections (13). Moreover, \textit{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, \textit{iceA}, like \textit{vacA}, is present in all Hp strains. This gene has 2 \textit{iceA1} and \textit{iceA2} allelic variants (14, 15). \textit{IceA1} is upregulated during Hp-gastric epithelium contact, and there is an important association between the presence of the \textit{iceA1} allele and peptic ulcer disease (PUD) (16). Hp \textit{iceA1} genotype enhances mucosal interleukin-8 (IL-8) expression and acute inflammation (17). \textit{IceA2} gene has no homology to any known gene and the function is not clear. An epidemiological study found an association between \textit{iceA} gene and its alleles with gastric and/or duodenal ulcer and GC in different geographic areas (18). Outer inflammatory protein (\textit{OipA}) is one of the porin proteins and responsible for pathogen-host interaction. The protein was originally recognized as a pro-inflammatory response, according to the fact that \textit{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 \textit{cagA}, \textit{cagE}, \textit{vacA}, \textit{oipA}, \textit{iceA1}, and \textit{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 nonduplicate 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 of the 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 Staurt’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 (Biogene 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 \textit{vacA} gene was detected to confirm Hp in gastric biopsies. Multiplex PCR was performed for amplification of \textit{vacA}, \textit{cagA}, \textit{cagE}, \textit{iceA1}, \textit{iceA2}, and \textit{oipA} genes in a volume of 0.6 µL (0.5 µg) of 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) (AmpliCo, Denemark), and 4.4 µL ddH2O. Amplification was performed in a thermal gradient cycler (Eppendorf Co., Germany). The cycling conditions were as follows: 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 for 30 seconds.

Table 1. Oligonucleotide primer sequences used in the study

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer sequence (5'→3')</th>
<th>Amplicon size (bp)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cagE}</td>
<td>F: 5'- TTGAAAACCTTCAAGGAGTATGAGAAGC-3'</td>
<td>500</td>
<td>21</td>
</tr>
<tr>
<td>R: 5'- GCCGATATGCAAATGAGCCGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{cagA}</td>
<td>F: 5'- AATACACCAAGGCCTCCAAAG-3'</td>
<td>298</td>
<td></td>
</tr>
<tr>
<td>R: 5'- TTGTTGCGCTGCCGTCTCTC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{iceA1}</td>
<td>F: 5'- GTGTTTTTTACCAAAAGATCT-3'</td>
<td>247</td>
<td>23</td>
</tr>
<tr>
<td>R: 5'- CTATAGCAGCCTTCTTGTGCA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{iceA2}</td>
<td>F: 5'- GTGTGCTTGGTTTTTAAATGA-3'</td>
<td>120</td>
<td>24</td>
</tr>
<tr>
<td>R: 5'- GTCTAATACCCCAAGATTAAA-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{VacA}</td>
<td>F: 5'- GCGGATATGCAAATTAGGCGGC-3'</td>
<td>678</td>
<td>24</td>
</tr>
<tr>
<td>R: 5'- CAATCGTGTGGTCTGGGAGC-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{oipA}</td>
<td>F: 5'- GTTTTTGTATGCGATGGAATTT-3'</td>
<td>401</td>
<td>25</td>
</tr>
<tr>
<td>R: 5'- GTGACATCTTCTATGGCCTT-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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 gastrointestinal 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 microbiobical 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 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 bioips 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 cag PAI 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 cag PAI 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 cag PAI 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 iceA1 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

<table>
<thead>
<tr>
<th>Gastroduodenal pathology</th>
<th>No. (%) of positive for H. pylori</th>
<th>Distribution of virulence factors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric ulcer</td>
<td>87 (51.4)</td>
<td>cagA 75 (86.2)</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>36 (21.3)</td>
<td>cagE 23 (63.8)</td>
</tr>
<tr>
<td>Gastric cancer</td>
<td>5 (2.9)</td>
<td>iceA1 2 (40)</td>
</tr>
<tr>
<td>Gastritis</td>
<td>30 (17.7)</td>
<td>iceA2 25 (83.3)</td>
</tr>
<tr>
<td>Duodenitis</td>
<td>11 (6.7)</td>
<td>oipA 4 (24.8)</td>
</tr>
<tr>
<td>Total</td>
<td>169 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Relationship between the Hp genotype and gastroduodenal pathology

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of patients with gastroduodenal pathology (%)</th>
<th>No. of patients with gastroduodenal pathology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA+/oipA+</td>
<td>20 (22.9)</td>
<td>6 (16.6)</td>
</tr>
<tr>
<td>cagE+/oipA+</td>
<td>21 (24.1)</td>
<td>7 (19.5)</td>
</tr>
<tr>
<td>cagA+/oipA+/iceA1+</td>
<td>10 (11.5)</td>
<td>6 (13.8)</td>
</tr>
<tr>
<td>cagK+/oipA+/cagE+/iceA1+</td>
<td>6 (6.9)</td>
<td>5 (13.8)</td>
</tr>
<tr>
<td>Others</td>
<td>30 (34.6)</td>
<td>12 (33.4)</td>
</tr>
<tr>
<td>Total</td>
<td>87 (100)</td>
<td>36 (100)</td>
</tr>
</tbody>
</table>
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assessement 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 Yamakoa 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 declare that they have no competing interests.

References
31. Podzorski RP, Podzorski DS, Wuerth A, Tolia V. Analysis of the