Prevalence of Helicobacter pylori vacA, cagA, cagEl, cagE2, dupA and oipA Genotypes in Patients With Gastrointestinal Diseases

Hossein Masoumi Asl1, Ali Badamchi2, Shima Javadinia3, Siamak Khaleghi4, Leila Tehraninia5, Samaneh Saedi5, Azardokht Tabatabaei1

1 Research Center of Pediatric Infectious Diseases, Institute of Immunology and Infectious Diseases, Iran University of Medical Sciences, Tehran, Iran
2 Children’s Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
3 Department of Lung, Firoozabadi Hospital, Iran University of Medical Sciences, Tehran, Iran
4 Department of Gastroenterology, Rasool Akram Hospital, Iran University of Medical Sciences, Tehran, Iran

Abstract - Helicobacter pylori (H. pylori) is a bacterium that resides in the human stomach, which is associated with gastroduodenal diseases. We investigate the prevalence of cagA, vacA, oipA, cagEl, cagE2, and dupA genotypes in H. pylori isolated from patients with Gastric ulcer, duodenal ulcer, and Gastric Cancer. Collected 74 samples from the Gastroenterology Unit of the Rasool Akram Hospital were included in this study. Gastric disorders were identified by endoscopy. Gastric cancer was further confirmed by histopathology. H. pylori were detected by the urease test. Subsequently, DNA was extracted from gastric tissue of the subjects with the CLO-test yielded positive results. In general, 74 patients with a mean age of 53.45 years (Range 22 to 86-year-old), including 45 men and 29 women, were studied. Among 74 H. pylori-positive patients, 70 (94.5%) patients were positive for the cagA gene. About 95.8% (23/24) of the patients with gastric carcinoma were dupA positive and VacA gene (91.8%). The oipA genotype was detected in 71 (96%) of H. pylori positive samples. This gene was more common in patients with gastritis rather than cancer group. Also, 97.2% of 74 H. pylori isolates were cagE2-positive. In 25 patients with PUD, the occurrence percent of cagA+/VacA+, cagA+/Vac−, cagA−/VacA+ and cagA−/VacA− genotypes were found 80%, 12%, 4.2% and 4.2% respectively. The results of the present study suggest that a high prevalence of virulent factors could contribute to the risk of developing gastroduodenal diseases.

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Keywords: Helicobacter pylori; Vacuolating cytotoxin gene A (vacA); Cytotoxin-associated gene A (cagA); Cytotoxin-associated gene E1 (cagEl); Cytotoxin-associated gene E2 (cagE2). Duodenal ulcer promoting gene A (dupA)

Introduction

Helicobacter pylori (H. pylori) is a human-specific pathogen that infects approximately 50% of the population worldwide. The way of infection for H. pylori is forcefully based on person-to-person transmission and fecal-oral and oral-oral routes (1).

The infection implicates several medical conditions responsible for 90% of the gastric cancer cases, such as chronic gastritis, gastric ulcers, duodenal ulcers, gastric cancer, and peptic ulcer disease (2). The prevalence rates of infection vary greatly in the world. In developed countries, prevalence rates of infection among children have been shown to range from 1.8% up to 65%, and the epidemic range of infection in developing countries is higher than in developed countries and up to 90% (3,4). In Iran, we observe different prevalence rates of H. pylori infection (5).

A unique trait infection is a permanence, which causes prolonged active inflammation, including the influx of neutrophils. Flagella and urease activity of H. pylori cause colonization in the gastric mucosa (6). The adhesive interaction of H. pylori and cellular receptors help the gastric mucosa infection, which is caused by...
tissue damage by the secretion of virulence factors (such as vacA, cagA, dupA, and oipA genes). Analysis of genomic variation is useful for epidemiological studies in H. pylori. Many virulence-associated genes have an essential role in infection, such as vacuolating cytotoxin gene A (vacA), cytotoxin-associated gene A (cagA), and duodenal ulcer promoting gene A (dupA) (7). The vacA gene encodes the vacuolating cytotoxin A, produced by approximately 50% of the H. pylori strains (8). In human vacA, increase the risk of developing gastric cancer by inhibition of T-cell proliferation and activation of proinflammatory response (9). CagA is produced by approximately 50 to 60% of the H. pylori strains. The attendance of cagA correlated with duodenal ulceration and gastric cancer (9). CagA is part of Pathogenicity Island (cag-PAI) that is related to the virulence and pathogenicity of the H. pylori strain (9,10). cagE is another cag-PAI gene. This gene is associated with more virulent H. pylori strains; several studies have described an association between cagE and gastritis, duodenal ulcer, and peptic ulcer diseases (11).

The first disease-specific virulence factor is dupA because of its ability to enhance the risk for gastric. However, infections with H. pylori dupA-negative strains can increase the risk for duodenal ulcer, but it reduces the chance of occurrence for gastric (12). Studies show an association between the dupA gene and high IL-8 production from gastric epithelial cells that causing dominant gastritis (13). Outer membrane inflammatory protein A, The outer inflammatory protein (OipA), is an outer membrane protein-specific H. pylori. This protein has special functions, including adhesion and pH regulation (13). OipA is a major virulence factor of H. pylori, which is associated with peptic ulcer and enhanced inflammation by increased interleukin-8 secretion (14).

This study aimed to investigate the frequency of cagA, vacA, oipA, cagE1, cagE2 and dupA genotypes in H. pylori isolated from patients with Gastric ulcer, duodenal ulcer, and Gastric Cancer.

Materials and Methods

Patients

Sampling was performed from 74 patients with gastroduodenal diseases referred to Rasool Akram Hospital in Tehran from March to September. These patients underwent standard gastric endoscopy. At the time of sampling, patients had not received any proton pump inhibitor drugs or antibiotics for at least two weeks before. The clinical features of the patients recruited are presented in Table 1.

Table 1. Demographic and clinical characteristics of patients

| Result of endoscopy | Male (N=45) | Female (N=29) | Total (N=74) |
|---------------------|------------|--------------|-------------|
| Age (mean±SD)       | 56±17.8    | 48±14.5      | 53.45±15.7  |
| Gastric ulcer       | 10(22.22%) | 15(51.4%)    | 25(33.8%)   |
| Duodenal ulcer      | 15(33.33%) | 10(34.5%)    | 25(33.8%)   |
| Gastric Cancer      | 20(44.45%) | 4(13.8%)     | 24(32.4%)   |

Biopsy extract

Three gastric biopsies from the gastric antrum or body (1 sample for histological examination, 1 sample for CLO test, and 1 for PCR) were obtained from patients after obtaining their informed consent. This protocol was approved by the Rasool Akram Hospital Ethics Committee. H. pylori infection was evaluated by Urease Test, histology as well as polymerase chain reaction (PCR). Gastric ulcer (GU), duodenal ulcer (DU), and Gastric Cancer (GC) diagnosis were identified by endoscopy, and gastric cancer diagnosis was further confirmed by histopathology. Gastritis was defined as histological gastritis in the absence of peptic ulcer or gastric malignancy. All endoscopy and histology results evaluated and confirmed by a specialist (15).

The urease test (CLO test)

One biopsy extract samples from the antrum were used for the detection of H. pylori by the urease test. A fragment is placed in a tube containing urea indole to detect urease activity, which shows the presence of the bacteria in the biopsy. The positive result is interpreted by the color change of urea-indole from orange to pink or red after incubation at 37° C for 24 h.

DNA extraction and H. pylori genotyping

H. pylori DNA was extracted from gastric tissue in patients with CLO-test yielded positive results. DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, USA) according to the manufacturer’s instructions in Table 2.
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Data analyses

All results are expressed as frequency and percentage as appropriate. Fisher's exact test or the Chi-square test was used for analyzing categorical data. A *P* of less than 0.05 was considered statistically significant. The data analysis was performed using the SPSS software version 24.

| Table 2. primers used in PCRs |
|-------------------------------|
| Gene | Sequence (5'-3') | Temperature annealing |
|-----|-----------------|----------------------|
| CagA | CTAACGAAAACATTGGACC | 45 |
|     | GTTATTTTGCTGTTAGCTTG |
| VacA | CAATCTGCATCAATCAAAGG | 47 |
|     | GGTCTCAAAATAATTECCAGG |
| dupA | AACACCGTGCAGGACAAATCCC | 56 |
|     | TGGTTTCTACTGACAGAGCC |
| OipA | CCATGAAAAAAGCTCTGTAT | 43 |
|     | GCCCTTTTTACCTCTCGTCAA |
| cagE1 | CAATGAGGAGCTTTGAGGTTGTA | 48 |
|     | AGACATGCAAAAAAGGTAT |
| cagE2 | TGCTGATACGATTAGAGA | 48 |
|     | TAGTCCCTTATGATGAT |

Results

Seventy-four patients with a mean age of 53.45 years (Range 22 to 86 years old), including 45 men and 29 women, were studied (Table 1).

Relationship between *H. pylori* virulence factors and clinical outcomes

Among 74 *H. pylori* positive patients, 70 patients were positive for the cagA gene (94.5%) (Table 3). The majority of patients with cagA genotype had Gastric disorder (23/74, 31%), Peptic ulcer was found only in 32.4% (24/74) of patients and percent of patients with Gastric cancer was (23/74, 31%), but differences could not reach statistically significant (Table 3). Almost all patients were positive for the VacA gene (91.8%); there was no relationship between the vacA gene and clinical outcomes (Table 4).

| Table 3. A variety of gastrointestinal diseases, Virulence Factors of *H. pylori* and Clinical Outcomes (*P*<0.05 is considered significant) |
|-----------------------------------------------|
| Genotype | Gastric ulcer 25(%) | Duodenal ulcer 25(%) | Gastric cancer 24(%) | *P* |
|---------|---------------------|-----------------------|---------------------|------|
| CagA-   | 2(2.7%)             | 1(1.3%)               | 1(1.3%)             | 0.7  |
| CagA+   | 23(31%)             | 24(32.4%)             | 23(31%)             |      |
| VacA-   | 3(4%)               | 2(2.7%)               | 1(1.3%)             | 0.6  |
| VacA+   | 22(29.7%)           | 23(31%)               | 23(31%)             |      |
| dupA-   | 1(1.3%)             | 1(1.3%)               | 1(1.3%)             | 0.9  |
| dupA+   | 24(32.4%)           | 24(32.4%)             | 23(31%)             |      |
| OipA-   | 1(1.3%)             | 1(1.3%)               | 1(1.3%)             | 0.9  |
| OipA+   | 24(32.4%)           | 24(32.4%)             | 23(31%)             |      |
| CagE1-  | 0                   | 0                     | 0                   | 0.9  |
| CagE1+  | 25(33.8%)           | 25(100%)              | 24(100%)            |      |
| CagE2-  | 1(1.3%)             | 1(1.3%)               | 0                   | 0.9  |
| CagE2+  | 24(32.4%)           | 24(32.4%)             | 24(32.4%)           |      |

(*P*<0.05 is considered significant)

| Table 4. Relationship between two genes; vacA gene with cagA gene |
|---------------------------------------------------------------|
| Genotype/N (%) | Positive 70 | cagA gene | Negative 4 | *P* |
| vacA gene | Positive 68(%) | 65(95.6%) | 3(4.4%) | 0.8 |
|           | Negative 6(%)  | 5(83.3%) | 1(16.7%) |

*P*<0.05 is considered significant

About 96 % (24/25) of *H. pylori* strains of patients with gastritis, 96 % (24/25) from those with duodenal...
ulcer, and 95.8% (23/24) of the patients with gastric carcinoma were dupA positive (Table 3). There was no significant difference in the prevalence of dupA and CagA genotypes between studied groups, suggesting an association with the development of disease in this population (Table 5).

The oipA genotype was detected in 71 (96%) of H. pylori positive samples. This gene was more common in patients with gastritis rather than cancer group (Table 3).

In total, 100% of 74 collected H. pylori isolates were cagE1-positive (Table 3). There was no relationship between cagA and cagE genotypes status. Of the, 70 isolates of 74 cagE1-positive isolates were cagA positive, and 4(5.4%) isolates were cagA-negative (not significant) (Table 6).

In total, 97.2% of 74 H. pylori isolates were cagE2-positive. There was no relationship between cagA and cagE2 genotype. Of the 72 isolates that were cagE2-positive, 70 isolates were cagA positive, and two isolates were cagA-negative (not significant) of cagA-negative isolates, two isolates that were cagE2-positive (table 7).

According to Table7, In 25 patients with PUD, the occurrence percent of cagA+/VacA+, cagA+/Vac-, cagA-/VacA+ and cagA-/VacA- genotypes were found 80%, 12%, 4.2% and 4.2 respectively. In 22 patients with duodenal ulcers, the occurrence percent of desired genotypes were 88%, 8%, 0%, and 4.2 %, respectively. Occurrence percent of cagA+/VacA+, cagA+/Vac-, cagA -/VacA+ and cagA-/VacA genotypes in patients with cancer were 87.5%, 4.2%, 4.2% and 0% respectively. As shown, the difference between the occurrence percent of different genotypes of different diseases was significant (Table 8).

### Table 5. Relationship between two genes; dupA gene with cagA gene

| Genotype/N (%) | cagA gene |
|---------------|-----------|
| dupA gene     |           |
| Positive 72(%)| 69(95.8%) |
| Negative 2(%) | 1(50%)    |
|               | 3(4.2%)   |
|               | 1(50%)    |
| P             | 0.8       |

*P<0.05 is considered significant*

### Table 6. Relationship between two genes; cagE1 gene with cagA gene

| Genotype/N (%) | cagA gene |
|---------------|-----------|
| CagE1 gene    |           |
| Positive 74(%)| 70(94.6%) |
| Negative 0(%) | 0         |
|               | 4(5.4%)   |
| P             | 0.9       |

*P<0.05 is considered significant*

### Table 7. Relationship between two genes; cagE2 gene with cagA gene

| Genotype/N (%) | cagA gene |
|---------------|-----------|
| CagE2 gene    |           |
| Positive 72(%)| 70(97.2%) |
| Negative 2(%) | 0         |
|               | 2(2.8%)   |
|               | 2(100%)   |
| P             | 0.8       |

*P<0.05 is considered significant*

### Table 8. Frequency of selected genes in H. pylori strains

| Genotypes combinations | PUD (Gastric ulcer ) | Duodenal ulcer | Cancer | P |
|------------------------|----------------------|----------------|--------|---|
|                        | N (%)                | N (%)          | N (%)  |   |
| cagA+/VacA+            | 20(80)               | 22(88)         | 21(87.5)| 0.01|
| cagA+/VacA             | 3(12)                | 2(8)           | 1(4.2) |   |
| cagA-/VacA+            | 1(4.2)               | 0              | 1(4.2) |   |
| cagA-/VacA             | 1(4.2)               | 1(4.2)         | 0      |   |

*P<0.05 is considered significant*

### Discussion

H. pylori infection is prevalent worldwide. H. pylori is one of the most genetically diverse bacterial species which may be involved in the complex variety of gastroduodenal diseases in infected patients all over the
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world (16). In general, the prevalence is high in developing countries, and the infection is acquired at a young age. The outbreak of H. pylori infection is not lower in developed countries than in developing countries. For example, prevalence infection has reported more than 80% in Japan, Turkey, and Pakistan (17,18,19).

Latifi et al., Provide evidence that the frequency of gastrointestinal ulcer and gastric cancer is largely influenced by geographical conditions and ethnic groups that reflects historical interactions with external populations in Iran (16). The prevalence of this bacterium has been found 60-90%, indicating that Iran is a highly risky region for H. pylori infection. The prevalence of infection of our studied subjects was 82 %, indicating that our findings are consistent with previous reports in Iran (20,21,16).

In this study, the prevalence of H. pylori virulence factors from Rasool Akram Hospital in Tehran was tested and evaluated. It is estimated that 69% of the Iranian population currently suffers from H. pylori infection. The dominant genotypes in this study were the cagE1, followed by the cagE2, cagA, dupA, oipA (22) we show that these genotype variations modify the Clinical manifestations in H. pylori-infected patients. According to the results of studies on adults that identified an association between infection with cagA+ strains and peptic ulcer disease. However, subsequent studies have provided more inconsistent results. The current study demonstrated that the majority (94.5%) of the strains isolated from Iranian patients were cagA+ positive. Salih et al., (23), found that H. pylori infection is highly associated with DU (95.7%) and GU (87%). Differences in the applied methods of analysis might be the reason for such controversy. The cagA gene was reported in 73%, 55%, and 55% of H. pylori strains isolated from patients with NUD, PUD, and GC, respectively (24). The prevalence of cagA+ H. pylori differed from one geographic region to another, e.g., 97% in Korea (25), 90% in China (26), and 92% in Iran (27).

The researcher's view about the correlation between vacA genotypes and gastric diseases was different. For example, in Iran, Safavi et al., found no correlation between them (28), whereas Molaei et al., found that the s1a allele was associated with more severe inflammation (20). In Iran and Cuban strain, no association had been found (29,30).

About 94.5% of all isolates were cagA+ by PCR, which is in accord with the results of other studies from Europe (31). The majority of the patients with PUD (84%) were infected with cagA+ strains in contrast to strains that isolated from patients with gastritis only, in whom 67% of the H. pylori strains were cagA+ (31). dupA was described as H. pylori virulence marker linked with an increased risk for duodenal ulcer and decreased the risk for gastric carcinoma in Japan and Cuban.

In study Kobayashi (19) et al., all samples from an infant with and without duodenal ulcer were dupA+. Among the strains isolated from adults with gastritis (92.36%), duodenal ulcer (87.30%, P=0.30), and gastric cancer (87.65%, P=0.31) with dupA association were not observed. all samples from adults with and without duodenal ulcer were dupA+. In contrast to the results of Lu et al., (2005), dupA was not associated with duodenal ulcer and gastric carcinoma in our population (12).

Prevalence dupA gene in this study, 92.36% of H. pylori strains from adults with gastritis, 87.30% from those with duodenal ulcer, and 87.65% from the patients with gastric cancer were dupA positive. Lack of association between Helicobacter pylori infection with dupA+ strains and gastroduodenal diseases in Brazilian patients is shown. Association between dupA+ and duodenal ulcers was not observed in patients. Also, the presence of dupA+ was not associated with gastric cancer (32).

These discordant results may be explained by variations among strains isolated from different continents or ethnic groups. Since H. pylori has probably infected human beings since their origins, genetic drift may have happened during geographic isolation resulting in multiple populations and subpopulations that mirror ancient human colonization (33). Besides, DNA loss and rearrangement in the plasticity region are the rules, leading to diversity in gene content that may contribute to bacterial adaptation to the genetically different members of diverse ethnic groups in the human population (33).

Lu et al., reported that dupA is associated with an increased risk for DU, and protection against gastric atrophy and GC in Japan (12). In contrast, our results showed that dupA-positive H. pylori was detected not only in GU and DU patients (1/24) but also in GC patients (1/23), with no significant difference between these groups. The reason for this discrepancy is not clear, though it may be due to the limited number of subjects that were examined in the present study. Because of a shortage of patient's number, the present study should be recognized as a preliminary study. However, this study has presented further support for dupA as a negative marker of GC, Compliant with the study of Lu et al., (12). In this study, 96% of isolated strains contain an oipA gene, which is following the previous study that showed the oipA prevalence varies from 33% to 71% in the Iranian population based on the different ethnic
The importance of *cagE* gene presence can be observed by its high frequency in gastric cancer in India (100%) and Thailand (93.8%) populations (38,39). In addition, the *cagE* gene has proposed a good character for the integrity of cag-PAI than *cagA* (40). Therefore, our data confirm that the *cagA* gene is a good single marker of the pathogenicity of the island. However, it is suggested the use of both as markers for cag-PAI existence and also for the pathological importance of these genes.

Taken together, our results suggest that the high prevalence of virulent factors help to the risk of extending gastroduodenal diseases. We assume that the more virulence combination may be a trigger for chronic gastritis and a pioneer lesion of gastric cancer. This is the first study to disclose a high prevalence of the *oipA* gene in *H. pylori* isolates in Iran. Furthermore, this study discloses a high prevalence of the combination of *cagA*, *vacA*, *dupA*, and *oipA* genes.

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