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# Determination of *Helicobacter pylori* virulence by analysis of the *cag* pathogenicity island isolated from Iranian population

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## Abstract

**Background—**The *cag* pathogenicity island (PAI), which can divide into two parts: *cag*I and *cag*II, is the most well-known virulence factor of *Helicobacter pylori*.

**Aims—**We investigated the association between genetic variations within the *cag* PAI (*cagA* and *cagE* in the *cag*I and *cagT* in the *cag*II) and clinical outcomes in Iranian population.

**Subjects—**A total of 231 patients including 182 patients with gastritis, 41 with peptic ulcer and 8 with gastric cancer.

**Methods—**The presences of the *cagA, cagE* and *cagT* genes were measured by polymerase chain reaction and the results were compared with clinical outcomes and gastric histology.

**Results—**The *cagA, cagE* and *cagT* genes were found in 154 (66.7%), 90 (39.0%) and 70 (30.3%) of clinical isolates. At least 144 (62.3%) strains possessed partially deleted *cag* PAI (e.g., 69 [29.9%] strains were *cagA*-positive, but *cagE* and *cagT*-negative).

**Conclusion—**The simple gene as well as the combination of the genes in the *cag* PAI appeared not to be useful markers to predict *H. pylori*-related diseases in Iranian population. The genomic sequences of the *cag* PAI in Iranian strains might be considerably different from those in other geographic locations.

### Keywords

*Helicobacter pylori*; *cag* pathogenicity island; gastroduodenal diseases; Iran

## Introduction

*Helicobacter pylori* is an important pathogen for gastroduodenal diseases. In most cases, *H. pylori* infection causes an asymptomatic chronic gastric inflammation and is also the cause of

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severe gastroduodenal diseases in some infected persons, including chronic atrophic gastritis, peptic ulcers, and gastric adenocarcinoma (1).

Albeit the pathogenesis of *H. pylori* infection is not well understood, several virulence factors have been proposed and the best studied is the *cag* pathogenicity island (PAI) which is approximately 40 kilobase pair region (2). The *cag* PAI encodes a type IV secretion system, by which CagA is delivered into host cells (3-7). After the delivery into gastric epithelial cells, CagA is mainly tyrosine-phosphorylated at Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs located in the 3′ region of *cagA* gene (8).

The *cag* PAI can divide into two parts: the upstream *cag*II region and the downstream *cag*I region (2). The *cagA* gene is located in the most downstream portion of *cag*I and is known as a marker for the *cag*I region. The *cagA*-positive strains are reported to be related to severe clinical outcomes, especially in Western countries (9-12). In contrast, most previous studies reported that there were no relationship between *cagA* status and clinical outcomes in Iranian population (13-18). The *cagE* gene is a homolog of the *ptlF* and *virB4* genes of *Bordetella pertussis* and *Agrobacterium tumefaciens*, respectively, and is also located in the *cag*I, and is necessary to induce interleukin (IL)-8 from gastric epithelial cells (2). In previous studies, the *cagE* gene was reported to be a better marker for the *cag*I region than the *cagA* gene, and the *cagE* gene was more useful gene in discriminating between *H. pylori* strains causing different rates of disease progression than the *cagA* gene (19). CagT protein is found in the pilus of the type IV secretion system and the *cagT* gene has been reported to be a marker of the *cag*II region (20). The *cagT*-positive strains are also reported to be related to severe clinical outcomes (20;21).

These results indicate that investigating not only the *cagA* status, but also the *cagE* and *cagT* status should be necessary for understanding the roles of *cag* PAI in clinical outcomes; however there is currently no report investigating the importance of the *cagE* and *cagT* status on clinical outcomes in Iranian population. We therefore aimed to investigate the association between genetic variations within the two parts of the *cag* PAI (*cagA* and *cagE* in the *cag*I and *cagT* in the *cag*II) and clinical outcomes in Iranian population.

## Methods

### Population Studied

The patient group consisted of individuals coming to the Taleghani and Mehrad Hospitals in Tehran, Iran for investigation of dyspeptic symptom between April 2007 and January 2008. The study was explained to the 311 patients who received endoscopy by members of the local *H. pylori* research group. All eligible patients meeting inclusion criteria were entered.

Inclusion criteria included patients with *H. pylori* infection proven by the culture of *H. pylori*, patients with the absence of non-gastrointestinal chronic medical conditions and the absence of contraindications to upper gastrointestinal endoscopy, patients providing informed consent, and patients with willingness to complete a standardized data-collection form. Exclusions included patients with upper gastrointestinal bleeding, patients who had received non-steroidal anti-inflammatory drugs, steroids or proton pump inhibitors at least three months prior to endoscopy, patients who had received any antibiotics at least one month prior to endoscopy, and patients who had received previous treatment for *H. pylori* infection. Informed consent was obtained from all patients and the protocol was approved by the hospital ethics committee.

The clinical presentations recognized were *H. pylori*-related gastritis, gastric ulcers (GU), duodenal ulcers (DU), and non-cardiac gastric adenocarcinoma. Peptic ulcers were identified

endoscopically. Gastric cancers were confirmed histologically and all were of the distal type and advanced. Gastritis was defined as histological gastritis without peptic ulcer diseases or gastric malignancy, or erosive esophagitis.

### Histology

Three biopsy specimens were taken from the greater curve of the antrum; two were used for histological examination and one for *H. pylori* culture. Biopsy specimens for histology were fixed overnight in buffered formalin, embedded in paraffin, cut to 3-μm thickness, and stained with hematoxylin-eosin (H&E) for routine histological evaluation. The slides were blindly evaluated by two expert gastrointestinal pathologists. The degree of gastric mucosal inflammation (mononuclear cell infiltration), polymorphonuclear cell infiltration, glandular atrophy, and intestinal metaplasia were classified according to the Updated Sydney System (22).

### Preparation of Genomic DNA

Gastric biopsy specimens for culture were kept in transport medium consisting of thyoglycolate with 1.3 g/L agar (Merck Co, Humbuerg, Germany) and 3% yeast extract (Oxoid Ltd., Basingstoke,. UK) and brought to the laboratory on the day of endoscopy. The specimens were cultured on Brain Heart Infusion agar with 10% (v/v) sheep blood and Campylobacter selective supplement (Merck Co, Humbuerg, Germany). The cultured plates were incubated at 37°C for 3 to 5 days under microaerobic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$) in $CO_2$ incubator ((Innova-Co 170; New Brunswick Scientific). The organisms were identified as *H. pylori* by colony morphology, Gram staining and positive reactions to oxidase, catalase, and urease activities. The identified *H. pylori* was then subcultured to single colonies for DNA extraction. DNA extraction from *H. pylori* isolates was performed using QIAamp tissue DNA extraction kit (Qiagen, Hilden, Germany).

### Polymerase chain reaction amplification analysis

PCR analyses were carried out to determine the presence or absence of *cagA*, *cagE*, and *cagT* genes. The *glmM (ureC)* gene was used as controls for detecting *H. pylori* DNA. Primers sequences used in this study were listed in Table 1 (23;24). All PCR mixtures were performed in a total volume of 25 μL containing 10 × PCR buffer, 500 nM of each primer, 2 mM $MgCl_2$; 200 μM each dNTP, 1.5 U Taq DNA polymerase, and 200 ng DNA sample. The total volume was made up with autoclaved Milli-Q water (Eppendorf AG 22331, Hamburg, Germany). Amplification conditions were optimized in thermocycler as follows: initial denaturation for 5 min at 94°C was followed by 30 cycles of denaturation at 93°C for 1 min, annealing at 58°C, 57°C, and 60°C for *cagA* and *glmM*, *cagE* and *cagT* respectively for 30 seconds and extension at 72°C for 1 min. After a final extension at 72°C for 10 min, PCR products were visualized by electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and examined under UV illumination. *H. pylori* 26695 DNA was used as a positive control for *cag* PAI-positive strain.

### Data Analysis

Variables such as gender (male or female), mean age, frequencies of the *cagA*, *cagE* and *cagT* genes (negative or positive) were evaluated. Statistical differences in demographic characteristics among the different disease groups were determined by one-way ANOVA or the chi-square test. The univariate association between genotype status and clinical outcomes was quantified by the chi-square test and student t-test. The effects of the *cagA*, *cagE* and *cagT* status on the risk for developing gastric cancer and peptic ulcer in patients were expressed as odds ratios (ORs) with 95% confidence intervals (CIs) with reference to gastritis alone or mild gastritis alone subjects adjusted by age and sex. Analyses were performed using Sigma

Stat for Windows V2.03 (SPSS, Chicago, IL). A $p$ value of <0.05 was accepted as statistically significant.

## Results

### Patients

*H. pylori* was isolated from 231 patients including 95 men and 136 women with mean age of 43.9 years old (14 to 71 years old). Clinical presentations included 182 patients with gastritis, 16 with DU, 2 with GU, 23 with both DU and GU, and 8 with gastric cancer. Since the number of patients with both DU and GU were predominant among patients with peptic ulcer and the number of patients with GU was small, we combined patients with peptic ulcer as peptic ulcer (PUD) group in the subsequent analyses. There were no age and gender differences among different clinical outcomes (data not shown).

### *cag*I markers

In 231 *H. pylori*-positive patients studied, 154 (66.7%) patients were infected with the *cagA*-positive strains (Table 2). The *cagA* gene was detected from 127 of 182 (69.7%) strains isolated from patients with gastritis, 22 of 41(53.6%) with peptic ulcer and 5 of 8 (62.5%) with gastric cancer. There was no significant relationship between the presence of the *cagA* gene and clinical outcomes both by univariate analyses (Table 2) and adjusted by age and sex (Table 3). We also classified the gastritis cases with no acute inflammation (polymorphonuclear cell infiltration), none to mild chronic inflammation (mononuclear cell infiltration) as well as no gastric atrophy/intestinal metaplasia as “mild gastritis”, and analyzed separately; however the prevalence of the *cagA* gene in strains isolated from patients with mild gastritis (76.5%: 26/34) was even higher than those with total gastritis as well as those with PUD and GC (Table 2), indicating that the *cagA* gene was not involved in the severity of gastritis. ORs adjusted by age and sex also showed that the presence of the *cagA* gene was independent of the risk for PUD and GC.

The *cagE* gene was found in 39.0% (90/231) of clinical isolates. The prevalence of the *cagE* gene was 39.0% (71/182) in strains from patients with gastritis and 43.9% (18/41) with peptic ulcers. Only one out of the 8 patients with gastric cancer (12.5%) was found to be *cagE*-positive. As similar to the *cagA* gene, there was no relationship between the presence of the *cagE* gene and clinical outcomes or the severity of gastritis both by univariate analyses (Table 2) and adjusted by age and sex (Table 3).

### *cag*II marker

The *cagT* gene as a *cag*II marker was present in 30.3% (70/231) of clinical isolates. The *cagT* gene was detected in 29.1% (53/182) of strains from patients with gastritis and in 36.6% (15/41) with peptic ulcer. The *cagT* gene was detected in 25% (2/8) of strains from gastric cancer patients. As similar to two *cag*I genes described above, there was no relationship between the presence of the *cagT* gene and clinical outcomes or the severity of gastritis both by univariate analyses (Table 2) and adjusted by age and sex (Table 3).

### Combination of *cag*I and II with clinical outcome

Coexistence of *cag*I and II (i.e., positive for *cagA*, *cagE* and *cagT*: triple-positive) were found in 17.3% (40 of 231) of the isolates (Table 2). These triple-positive strains were detected in 19.5% (8 of 41) of isolates from patients with peptic ulcer and 17.5% (32 of 182) from patients with gastritis. There were no patients with gastric cancer who were infected with triple-positive strains. Strains lacking both *cag*I and *cag*II (i.e., *cagA-/cagE-/cagT-*: triple-negative) were deleted in 20.3% (47 of 231) of isolates from patients including 18.7% (34 of 182) with gastritis,

26.8% (11 of 41) with peptic ulcer and 25% (2 of 8) with gastritis cancer (Table 2). At least 62.3% (144/231) of strains possessed the partially deleted *cag* PAI. However, there was no relationship between the combination of *cag*I and *cag*II and clinical outcomes or the severity of gastritis ($P < 0.05$).

## Discussion

The profile of *H. pylori cag* PAI genes shows great variability worldwide. It has been reported that selective pressure induced by the host immune defenses in long-term infection with *H. pylori* besides different geographic factors leads to high genetic diversity in *H. pylori* strains that can be affected on phenotypic characters linked to the gastroduodenal diseases (25). Studies especially from European countries showed that the presence of the *cagA* gene is associated with severe clinical outcomes and patients infected with the *cagA*-positive strains are associated with denser colonization of *H. pylori* and a more marked gastric inflammatory response than those with the *cagA*-negative strains (26;27); however these observation was not confirmed in East Asian countries where most strains possess the *cagA* gene (28;29). We could not find the relationship between the *cagA* status and clinical outcomes in Iranian population, which was in agreement with previous studies in Iran (14;17;18). The *cagA* gene is reported to be sub-typed based on the number of sequences of the repeat region in the 3′ region of the *cagA* gene (30-32), and the *cagA* gene with multiple repeats and/or East Asian type repeats are reported to be more virulent than that with fewer repeats and/or Western type repeats (30;31; 33). Further studies will be necessary whether the *cagA* sub-typing is involved in the development of clinical outcomes in Iranian population.

In previous studies, the *cagE* gene was reported to be a better marker for the *cag*I region than the *cagA* gene, and the *cagE* gene was more useful gene in discriminating between *H. pylori* strains causing different rates of disease progression than the *cagA* gene in Japanese (19). However, our data showed that the presence of the *cagA* gene could be used as a better marker for *cag*I region than that of the *cagE* gene, and we could not find the relationship between the *cagE* status and clinical outcomes in Iranian population. We also examined the *cagT* gene as a marker of the *cag*II region; however we could not find the relationship between the *cagT* status and clinical outcomes as similar to the *cagA* and the *cagE* genes. Overall, although the genes in *cag*I and *cag*II have been reported to be highly associated with the gastroduodenal diseases in some countries, the clinical outcomes of *H. pylori* infection is not reliably predicted by the three genes in *cag* PAI in Iranian population from Tehran Province. There are many factors such as host genetic factors, environmental factors, socio-economic status; irregular dietary habits besides *H. pylori* with *cag* PAI should contribute to the clinical outcomes of *H. pylori* infection.

Interestingly, at least 62.3% (144/231) of strains possessed partially deleted *cag* PAI. According to a previous study, the *cag* PAI genes were highly conserved in Japanese isolates, less conserved in European and African isolates, and very poorly conserved in Peruvian isolates and isolates from India (34). There is currently no information about the structures of the *cag* PAI in Iranian isolates. However, there was one report from Turkey (35) as a neighbor country of Iran that the structures of the *cag* PAI were poorly conserved. We therefore hypothesize that the structures of the *cag* PAI in Iranian strains might be similar to those of Turkish strains. The Iranian ancestral strains that including of intact *cag* PAI might undergo special condition in their gastric environments, and genetic rearrangements or DNA exchange might be occurred in the ancestral strains, which have evolved into genetically different subtypes. It is suggested that similar condition of life and diet in Iran and Turkey might affect on the selection of *H. pylori* with partial deleted *cag* PAI. Since we only used one set of PCR primers in each gene, there might yield false-negative results although the genes were present. However, we used well-known PCR primers used in other studies; therefore if this is the case,

we might conclude that the genomic sequences of the *cag* PAI, especially the *cagE* and *cagT* genes in Iranian strains should be considerably different from those in other geographic locations. Further studies using several sets of PCR primers, Southern blot hybridization and/or sequence analyses should be necessary to further investigating the roles of the *cag* PAI in Iranian strains.

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## Table 1

### PCR primers used in this study

| Gene | Primer designation | Sequence (5′-3′) | PCR product size (bp) | References |
|---|---|---|---|---|
| *glmM* | GlmM1-R<br>GlmM2-F | GCTTACTTTCTAACACTAACGCGC<br>GGATAAGCTTTTAGGGGTGTTAGGGG | 296 | (23) |
| *cagA* | CagA F1<br>CagA R1 | AACAGGACAAGTAGCTAGCC<br>TATTAATGCGTGTGTGGCTG | 349 | (23) |
| *cagE* | cagE F<br>cagE R | GTTACATCAAAAATAAAAGGAAGCG<br>CAATAATTTTGAAGAGTTTCAAACG | 733 | (24) |
| *cagT* | cagT F<br>cagT R | TCTAAAAAGATTACGCTCATA<br>CTTTGGCTGCATGTTCAAGTTGCC | 489 | (24) |

**Table 2**

**Clinical outcomes and the presence of different genes in *cag*I and *cag*II**

| | Gastritis (n = 182) | Mild gastritis (n = 34) | PUD (n = 41) | GC (n = 8) | Total (n = 231) |
|---|---|---|---|---|---|
| *cagA*+ | 127 (69.7%) | 26 (76.5%) | 22 (53.6%) | 3 (62.5%) | 154 (66.7%) |
| *cagE*+ | 71 (39.0%) | 13 (38.2%) | 18 (43.9%) | 1 (12.5%) | 90 (39.0%) |
| *cagT*+ | 53 (29.1%) | 12 (35.3%) | 15 (36.5%) | 2 (25.0%) | 70 (30.3%) |
| *cagA*+/*cagE*+/*cagT*+ (1) | 32 (17.5%) | 9 (28.1%) | 8 (19.5%) | 0 (0%) | 40 (17.3%) |
| *cagA*+/*cagE*-/*cagT*+ (2) | 12 (6.5%) | 2 (5.9%) | 2 (4.8%) | 1 (12.5%) | 15 (6.4%) |
| *cagA*-/*cagE*+/*cagT*+ (2) | 3 (1.6%) | 0 (0.0%) | 1 (2.4%) | 1 (12.5%) | 5 (2.1%) |
| *cagA*-/*cagE*-/*cagT*+ (2) | 6 (3.3%) | 1 (2.9%) | 4 (9.8%) | 0 (0.0%) | 10 (4.3%) |
| *cagA*+/*cagE*+/*cagT*- (3) | 24 (13.2%) | 3 (8.8%) | 6 (14.6%) | 0 (0.0%) | 30 (13.0%) |
| *cagA*-/*cagE*+/*cagT*- (3) | 12 (6.6%) | 1 (2.9%) | 3 (7.3%) | 0 (0.0%) | 15 (6.5%) |
| *cagA*+/*cagE*-/*cagT*- (3) | 59 (32.4%) | 12 (35.3%) | 6 (14.6%) | 4 (50%) | 69 (29.9%) |
| *cagA*-/*cagE*-/*cagT*- (4) | 34 (18.7%) | 6 (17.6%) | 11 (26.8%) | 2 (25%) | 47 (20.3%) |

Mild gastritis was defined as gastritis with no acute inflammation, none to mild chronic inflammation as well as no gastric atrophy/intestinal metaplasia.

*cagA*+: *cagA*-positive, *cagA*-: *cagA*-negative, *cagE*+: *cagE*-positive, *cagE*-: *cagE*-negative, *cagT*+: *cagT*-positive, *cagT*-: *cagT*-negative

(1) *cagA*+/*cagE*+/*cagT*+ = triple-positive

(2) *cagI*+/*cagII*+, but one of *cagI* gene was negative

(3) *cagI*+/*cagII*-

(4) all negative (*cagI*-/*cagII*-) = triple-negative

**Table 3**

**Age and sex-adjusted risks for PUD and GC in relation to *cag*I and *cag*II**

| | PUD (n = 41) | | | GC (n = 8) | | |
|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | **p value** | **OR** | **95% CI** | **p value** |
| vs. Gastritis patients (n = 182) | | | | | | |
| *cagA*+ vs. *cagA*- | 0.55 | 0.27-1.10 | 0.092 | 0.73 | 0.17-3.16 | 0.669 |
| *cagE*+ vs. *cagE*- | 1.27 | 0.64-2.55 | 0.465 | 0.22 | 0.03-1.82 | 0.159 |
| *cagT*+ vs. *cagT*- | 1.42 | 0.69-2.91 | 0.344 | 0.81 | 0.17-4.13 | 0.791 |
| vs. mild gastritis patients (n = 34) | | | | | | |
| *cagA*+ vs. *cagA*- | 0.36 | 0.13-1.13 | 0.066 | 0.39 | 0.06-2.36 | 0.303 |
| *cagE*+ vs. *cagE*- | 1.31 | 0.49-3.47 | 0.588 | 0.13 | 0.01-1.47 | 0.100 |
| *cagT*+ vs. *cagT*- | 0.92 | 0.34-2.49 | 0.869 | 0.522 | 0.08-3.40 | 0.497 |

Mild gastritis was defined as gastritis with no acute inflammation, none to mild chronic inflammation as well as no gastric atrophy/intestinal metaplasia.

*cagA*+: *cagA*-positive, *cagA*-: *cagA*-negative, *cagE*+: *cagE*-positive, *cagE*-: *cagE*-negative, *cagT*+: *cagT*-positive, *cagT*-: *cagT*-negative

OR: odds ratio adjusted by age and sex, CI, confidence interval