Simultaneous Detection of *CagA* and *CagE* of *Helicobacter pylori* Strains Recovered from Iranian Patients with Different Gastroduodenal Diseases

M Douraghi 1,2, M Mohammadi 2, *MH Shirazi 1, A Oghalaie 2, S Saberi Kashani 2, MA Mohagheghi 3, M Eshagh Hosseini 4, H Zeraati 5, M Esmaïli 2, M Babaei 2, N Mohajerani 2

1Dept. of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Iran
2Helicobacter pylori Research Group, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran
3Cancer Research Center, Tehran University of Medical Sciences, Iran
4Endoscopy Unit, Amiralam Hospital, Tehran University of Medical Sciences, Iran
5Dept. of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Iran

(Received 11 Nov 2008; accepted 21 May 2009)

**Abstract**

**Background:** To assess the status of two representative genes of *cag* PAI i.e *cagA* and *cagE* of *Helicobacter pylori* strains infecting Iranian patients suffered from various clinical outcomes using one-step PCR.

**Methods:** A total of 120 *H. pylori* infected patients including non–ulcer dyspepsia, NUD (n = 81), peptic ulcer disease, PUD (n = 17), and gastric carcinoma, GC (n = 22) referred for endoscopy or gastric resection to AmirAlam Hospital or Cancer Institute from 2005 to 2008 were assessed. The status of *cagA* and *cagE* genes was determined by gene specific PCR.

**Results:** 84.2% and 90.8% of the tested strains were positive for *cagA* and *cagE*, respectively. 81.7% strains were positive for both *cagA* and *cagE* genes, whereas 8 (6.7%) were found double negative. The prevalence of *cagA* in GC patients (100%) was slightly higher than PUD patients (94.1%). All of GC cases were infected with *cagA*-positive strains. The same distribution pattern was indicated for *cagE* gene in GC and PUD patients. The *cagA*-positive strains were significantly associated with GC as compared with NUD (*P* < 0.05) but this association did not gain statistical significance when *cagE* gene was assessed.

**Conclusion:** The concurrent detection of *cagA/cagE* genes allowed rapid and specific clarification of *cag* PAI status. The strains with *cagA/cagE* genotype are predominant in Iran regardless of clinical outcome and create a distinct cluster pattern from those in the West and similar to those of East Asian countries. The current study also demonstrated that *cagE* gene can be explored as a better indication of *cag*-PAI in Iranian *H. pylori* strains.

**Keywords:** Helicobacter pylori, Cag PAI, Peptic ulcer, Gastric ulcer, Gastric carcinoma, Iran

**Introduction**

*Helicobacter pylori* infect more than half the human population worldwide. Infection of gastric mucosa with *H. pylori* results in different disease outcomes including gastritis, peptic ulcer disease, and gastric adenocarcinoma (1-3).

Various studies have demonstrated that bacterial virulence factors, host genetic and environmental factors contribute to the development of disease (4). The most studied virulence marker of *H. pylori* is cytotoxin-associated gene pathogenicity-island (*cag* PAI) which is horizontally transferred from one strain to another. *Cag PAI* is a 40-kb gene locus in the chromosomal glutamate racemase gene with a 35% G+C content distinguishing it from of the rest of the bacterial genome with a 39% G+C content (5). *cag* PAI contains 31 genes, six of which encode type IV secretion system and are involved in *H. pylori*-host cell interactions and pathogenesis (6,7). *CagA* and *CagE* are the representative genes of *cag* PAI; *cagA* gene is located in the right half of the *cag* island affecting host cells following delivery into the gastric epithelial cells, whereas

*Corresponding author: Tel: +98 21 66112379, E-mail: mhshirazi@sina.tums.ac.ir*
the cagE gene is located upstream of cagA, and encodes a protein involved in the process of interleukin 8 expression and translocation of cagA (8, 9). Only 1/2 to 2/3 of Western isolates carry the cag PAI gene cluster. This is in contrast to East Asian strains, nearly all of which carry this gene cluster. The severity of H. pylori-related disease is correlated with the presence of the cag PAI. Infection with cag PAI-positive H. pylori is associated with duodenal ulcer, gastric mucosal atrophy and gastric cancer (10-12). Therefore, the presence and the integrity of the cag island appears to be a critical factor in the interaction between H. pylori and its host resulting in a variety of subsequent GI disorders. In this study, we aimed to assess the status of two representative genes of cag PAI i.e cagA and cagE of H. pylori strains infecting Iranian patients suffering from various clinical outcomes using one-step PCR.

Material and Methods

Subjects
This cross-sectional study conducted for 120 H. pylori infected patients with upper gastrointestinal disorders referred for endoscopy to Amir Alam Hospital or gastric resection to Cancer Institute of Tehran University of Medical Sciences, Iran between 2005-2008. The subjects consisted of 81 non-ulcer dyspepsia (NUD), 17 peptic ulcer disease (PUD), and 22 gastric cancer (GC) patients. Informed consent was obtained from every patient prior to sample collection which was performed according to standard protocols approved by the local ethical committee.

Bacterial strains
H. pylori strains were primarily isolated from two antral biopsy specimens. Tissue samples were homogenized and plated onto H. pylori Specific Pepton Agar (HPSPA) medium (13). Incubation was performed at 37° C in micro-aerophilic atmosphere (10% CO2, 5% O2 and 85% N2) up to 5 d. Identification was based on morphology under Gram staining and biochemical tests including urease and catalase tests. One single colony was expanded and collected for further studies.

DNA extraction
Briefly, bacterial genomic DNA was extracted by incubating bacterial pellets of one single colonies in 50mM NaOH at 100° C for 20 min, followed by 10 min incubation in 1M Tris-HCl, pH 7.5. The supernatants containing genomic DNA were used as the template for PCR amplification (14).

PCR assays
The ureC gene was primarily amplified to confirm the identity of the isolated H. pylori strains in accordance with published reports (15). Amplification of conserved regions of cagA and cagE genes were performed simultaneously using specific primers (Table 1). Every PCR was repeated to confirm the results. Amplification of the target genes was carried out in a total volume of 20 µl containing 2 µl of 10×PCR buffer (Fermentas, Lithuania), 1.5 mM of MgCl2, 0.5 µl of each primer (25 pM/µl), 0.2 mM of deoxynucleotide, 0.5 U of Taq Polymerase. The cycling programs, was preceded by 4 min at 94° C and consisted of 35 cycles of 94° C for 50 s, 54° C for 50 s and 72° C for 50 s, followed by a final extension step at 72° C for 4 min. PCR products were electrophoresed in 2.0% agarose gels and visualized by staining with ethidium bromide under UV light. H. pylori 26695 strain was used as a reference strain for every assay (14).

Statistical Analysis
Data were analyzed using SPSS (version 11.5) program. For univariate analysis, χ2 test and Fisher’s exact test were used. A P value of ≤0.05 was considered statistically significant.

Results

Confirmation of H. pylori identity
All of the 120 DNA samples were amplified with the ureC-specific oligonucleotide primers and yielded a 294-bp PCR product confirming the identity of H. pylori thereby confirming presence of H. pylori.

**Distribution of cagA and cagE genes**

One hundred one (84.2%) of the 120 tested strains were amplified using gene-specific primers for conserved region of cagA and were thus considered cagA-positive. Only 19 (15.8%) of H. pylori-infected patients regardless of disease category were cagA-negative. The cagE gene was detected in 90.8% (109/120) of H. pylori strains. Eleven (9.2%) of the studied strains were not amplified using primers designed for detection of cagE and were thus reported as cagE-negative strains.

**Coexistence of cagA and cagE**

Ninety eight (81.7%) strains were positive for both cagA and cagE genes, whereas 8 (6.7%) were found double negative. The presence of the cagA and cagE genes was strongly linked (P< 0.001) with a trivial number of strains (2.5%) possessing cagA positive/cagE negative genotype. A cagA negative/cagE positive genotype was detected in eleven (9.2%) strains. The distribution of different cagA/cagE genotypes in relation to clinical outcome was as indicated in Table 2.

**Association between cagA/cagE and clinical outcomes**

Distribution of H. pylori cagA/cagE positive and negative strains in patients with different clinical diagnosis is as depicted in Fig. 3. The prevalence of cagA in GC patients was slightly higher than (100%) that in PUD patients (94.1%). All of GC cases were infected with cagA-positive strains. The same distribution pattern was indicated for cagE gene in GC and PUD patients. More than half of NUD cases (77.8%) also harbored cagA-positive strains. Accordingly, 87.7% of NUD cases were infected with cagE-positive H. pylori strains. CagA-positive strains were significantly associated with GC as compared with NUD (P< 0.05) but this association did not gain statistical significance with cagE gene.

### Table 1: Primer sequences and DNA amplification conditions for each selected region

<table>
<thead>
<tr>
<th>Amplified region</th>
<th>Primer</th>
<th>Primer sequence</th>
<th>Size of amplified fragment (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UreC</td>
<td>HP-F</td>
<td>5'-GGA TAA GCT TTT AGG GGT GTT AGG GG-3'</td>
<td>294</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td>HP-R</td>
<td>5'-GCT TAC TTT CTA ACA CTA ACG CGC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CagA</td>
<td>F1</td>
<td>5'-GATAACAGGCAAGCTTTTGAGG-3'</td>
<td>349</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td>B1</td>
<td>5'-GCGTCAAAATAATTCACAAGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CagE</td>
<td>cagE-F</td>
<td>5'-TTGAAAACCTTCACGGATAGGCAGGC-3'</td>
<td>508</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cagE-R</td>
<td>5'-GCCTAGCGTAATATGCACCATTACCC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2: Coexistence of cagA/cagE in relation to clinical outcome

<table>
<thead>
<tr>
<th>Clinical outcome</th>
<th>cagA/cagE status</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos/Pos</td>
<td>Pos/Neg</td>
</tr>
<tr>
<td>NUD</td>
<td>60 (74.1)</td>
<td>3 (3.7)</td>
</tr>
<tr>
<td>PUD</td>
<td>16 (94.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>GC</td>
<td>22 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>98 (81.7)</td>
<td>3 (2.5)</td>
</tr>
</tbody>
</table>
Fig. 1: The *ureC* gene amplification using specific oligonucleotide primers yielded a product of 294-bp typical to *H. pylori*. NC and M illustrate the negative control and 100 bp DNA marker respectively.

Fig. 2: The representative of simultaneous detection of *cagA* and *cagE* yielded 349 bp and 508 bp amplicon respectively. NC and M illustrate the negative control and 100 bp DNA marker respectively.

Fig. 3: Distribution of *cagA/cagE* in relation to clinical outcome.
Discussion

*H. pylori* is associated with diverse diseases such as chronic gastritis, peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric carcinoma (1-3). The occurrence of such diverse diseases is a multifactor process dependent on specific properties of the organism, host and environmental factors. Strain-specific diversity has been proposed to be involved in the organism’s ability to cause different diseases. Furthermore, significant geographical differences have been demonstrated among *H. pylori* strains around the world.

The *cag* PAI is a major virulence determinant in *H. pylori* and strains lacking this island induce less pathogenicity that strains harboring *cag* PAI (8). Both *cagA* and *cagE* genes constitute major segments of the *cag* PAI, thus detection of the former genes may indicate the integrity of the latter gene.

This study carried out the simultaneous amplification of representative genes of *cag* PAI including *cagA* and *cagE* of Iranian *H. pylori* strains yielding distinguishable PCR products of distinct sizes. The concurrent detection of mentioned genes allowed rapid and specific clarification of *cag* PAI status. In addition, this one step PCR assay is cost and time efficient and recommended for routine laboratory use.

Analysis of *cagA* gene in patient with NUD, PUD, or GC revealed that it is present in 84.2% of examined strains regardless of the clinical status. The distribution of *cagA*-positive *H. pylori* strains in Iran is higher than Western countries such as the Netherlands (67%) and is closer to some geographical regions such as Ethiopia (79%), Brazil (94%), Korea (97%) and Japan (95%) (18-22). The variations in the prevalence of *cagA* gene in different countries may be due to distinct dyspeptic populations and geographical heterogeneity of *H. pylori* isolates. Although the possession of *cagA* is associated with GC when compared to NUD, due to the uniform distribution of *cagA* in all other disease categories detection of *cagA* alone can not be considered as a discriminative marker for a specific clinical outcome. Accordingly, in other Asian countries where the majority of *H. pylori*-infected individuals harbor *cagA*-positive strains, associations of *cagA* status and diseases are not observed (23, 24).

In the present study, 109 of 120 (90.8%) of strains possessed the *cagE* gene and its prevalence was higher among subjects with DU and GC as compared to NUD patients. *CagE* is more prevalent in Iranian *H. pylori* strains than that of Turkey (59.3%), United States (62%), Malaysia (59%) and is closer to Brazil (89.3%) and India (85.4%) (25-29). Although distribution of *cagE* was higher in GC and DU than NUD cases, the possession of *cagE* gene was not significantly associated with clinical pictures, a finding which is in contrast to previous study reporting a significant association between the *cagE* gene and DU (30).

The current study demonstrated that *cagE* gene can be explored as a better marker of *cag*-PAI in Iranian *H. pylori* strains. Previous studies in Japanese populations (22) and French isolates (31) also confirmed the application of *cagE* as a superior marker for *cag* PAI detection. This finding is in accordance with the observation of Ikenoue et al., who suggested that the *cagE* gene is a more precise marker of an intact *cag* island than are other *cag* genes (32). A study in Brazil also demonstrated that the frequency of *cagE* gene was higher than that of *cagA* and reported the former gene as a more accurate marker of *cag* PAI (33). Nevertheless, our study is in agreement with the study by Modena who showed no association between *cagE* and disease outcomes (34).

In conclusion, the majority of *H. pylori* strains in Iran are *cagA/cagE* double positive regardless of clinical outcome and construct a different pattern cluster from those in the West and similar to East Asian countries. Furthermore, this study introduces *cagE* as a more accurate marker of *cag* PAI gene cluster.
Acknowledgements
The authors declare that there is no conflict of interests.

References


