Genetic diversity in the *Helicobacter pylori* cag pathogenicity island and effect on expression of anti-CagA serum antibody in UK patients with dyspepsia

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**Abstract**

**Aims**—To investigate variation within the cag pathogenicity island (PAI) of *Helicobacter pylori* isolated from patients with dyspepsia in mid-Essex, and to evaluate the effect on expression of anti-CagA antibody.

**Methods**—Sixty two isolates of *H pylori* cultured from gastric biopsies were screened by specific PCR assays for the presence of cagA and other gene markers (cagD and cagE, and virD4) in the cag PAI. An enzyme linked immunosorbent assay (ELISA) kit (Viva Diagnostica helicobacter p120) was used to test for anti-CagA IgG antibody in matching sera. Isolates were also genotyped by vaculating cytotoxin polymerase chain reaction (PCR) analysis, and tested for absence of the complete cag PAI (empty site PCR assay).

**Results**—Forty one of the *H pylori* isolates had a cag PAI containing cagA. One strain had no cagA but other cag PAI loci were present, whereas the remaining 20 strains had no detectable cag PAI markers. Anti-CagA IgG antibody was detected in 34 sera by the ELISA assay, and when compared with the cag PAI genotype of the infecting strain, accuracy, sensitivity, and specificity were 92%, 87%, and 100%, respectively. The seven discrepant or borderline strains in the ELISA were all vacA s1 but differed in other genotypic markers.

**Conclusions**—The cag PAI was widely distributed in *H pylori* from patients with dyspepsia in mid-Essex who had different gastric pathologies. Infection with a strain having an uninterrupted cag PAI was associated with the presence of anti-CagA antibody in most patients. Discrepant ELISA results, mostly for elderly patients with duodenal ulcers, were attributed to cagA associated variation, particularly to the presence of mixed cagA+/cagA− cell variants in the infecting strain population. Tests for anti-CagA serum antibody were unreliable for predicting severity of clinical disease associated with *H pylori* infection in this series of patients.

**Materials and methods**

**Bacterial strains and growth conditions**

The 62 isolates of *H pylori*, for which matching patient sera were available, were obtained between 1996 and 1998 from gastric biopsies of patients (39 men, age range 29.8–75 years; and 23 women, age range 27.5–77.8 years; mean 52.3 years) in mid-Essex undergoing routine endoscopy for a range of gastroduodenal symptoms. Patients were excluded from the
study if they had previously received acid suppression treatment, *H. pylori* eradication treatment, or were current users of non-steroidal anti-inflammatory drugs. Patient groups based on endoscopic diagnosis before treatment were broadly as follows: the peptic ulcer group (13 patients); and the non-ulcer dyspeptic group with diagnoses of gastritis and/or duodenitis (16 patients), oesophagitis (two patients), and endoscopically normal mucosa including normal oesophagus, stomach, and duodenum (nine patients).

The biopsy specimens were homogenised in Griffith's tubes, inoculated on to 10% (vol/vol) Columbia blood and selective (DENT) agars (Oxoid, Basingstoke, UK) and incubated at 37°C under microaerophilic conditions (5% O₂, 5% CO₂, 2% H₂, 88% N₂) for five days. After primary isolation, all isolates of *H. pylori* were cultured for two to three days on 10% Columbia blood and selective (DENT) agars (Oxoid). One unit Taq polymerase (Life Technologies, Paisley, UK) was added and PCR was performed in a Sprint thermal cycler (Hybaid Ltd, Ashford, UK).

Details of the primers and PCR conditions for the various assays have been described elsewhere. Briefly, these were as follows. PCR conditions for the two assays for cagA (a marker for the right hand end of the cag PAI and for the cagI region) were as described previously for the F1/B1 primers and the D008/F008 primers. The assay for cagD and cagE (also referred to as part of the picAB operon, where picA comprises cagC and cagD, and picB is cagE), which is located within the cagI region upstream of cagA, was used as a second marker for that region. The primers were designed to amplify both cagD and cagE and to give a 2091 bp product based on the published sequences for NCTC 12455 (table 1). The PCR amplification comprised one cycle of 94°C for 110 seconds followed by 25 cycles of 94°C for 50 seconds, 54°C for 50 seconds, 72°C for 110 seconds, and finally 72°C for three minutes. The gene locus identified as a virD4 homologue (*Hp 524*) was selected as a marker for the cagH region, and two primers were designed to give a 1993 bp product based on the NCTC 12455 sequence (table 1). The PCR amplification conditions used comprised one cycle at 94°C for two minutes followed by 30 cycles at 94°C for one minute, 56°C for one minute, and finally 72°C for two minutes. The empty site (ES) assay was used to test for absence of the complete cag PAI. A 360 bp fragment was amplified with primers 2 and 25 specific for the genome regions flanking the left and right ends of the cag PAI insert location for *H. pylori* NCTC 12908, giving fragments of 259 bp for s1 and 329 bp for s2; and VAG-F and VAG-R for the m region, giving fragments of 570 bp for m1 and 645 bp for m2.

### ELISA FOR DETECTION OF SERUM IgG ANTIBODIES TO CAGA PROTEIN

Serum samples were obtained from blood, taken at the time of endoscopy, from the 62 patients and were stored at −80°C until serological testing was performed. An ELISA using a highly purified p120 antigen of *H. pylori* (Viva Diagnostica Helicobacter p120 ELISA; supplied by Axis-Shield, Dundee, UK) was performed according to the manufacturer's instructions to detect anti-CagA serum IgG. The cut off values for the ELISA as recommended by the manufacturer were: < 5 units, negative; 5–7.5 units, borderline; > 7.5 units, positive. Control sera provided with the kit were used for semiquantitative analysis.

### VACUOLATING CYTOTOXIN GENOTYPING

The strains of *H. pylori* that were negative or borderline in the CagA ELISA were genotyped based on the signal (s1 and s2) and mid (m1 and m2) regions of the vacuolating cytotoxin gene (*vacA*). Genotyping was performed using previously described PCR conditions and the following primers: VA1-F and VA1-R for the s region, giving fragments of 259 bp for s1 and 289 bp for s2; and VAG-F and VAG-R for the m region, giving fragments of 570 bp for m1 and 645 bp for m2.
Helicobacter pylori cag PAI and CagA expression

Table 2: Serology results with the CagA ELISA for serum from Helicobacter pylori infected individuals.

<table>
<thead>
<tr>
<th>H pylori cag PAI genotype: status of infecting strain</th>
<th>Number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>cagA+ (cagDE/virD4 not tested)</td>
<td>34</td>
</tr>
<tr>
<td>cagA+ (cagDE/virD4 not tested)</td>
<td>0</td>
</tr>
<tr>
<td>cagA−/cagDE− (virD4 not tested)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
</tr>
</tbody>
</table>

ELISA, enzyme linked immunosorbent assay; PAI, pathogenicity island.

Results

Cag PAI GENOTYPES

The 62 isolates of H pylori, each from a different patient, were tested for the presence of the cag PAI using the panel of PCR assays, and the results are shown in table 2. A hierarchical scheme of assays was used to minimise the number of individual PCR tests, because it was established previously that a positive cagA result was a reliable predictor of positivity for cagD and cagE (picAB).

Overall, 34 strains were shown to have cagA with one or both of the two cagA assays. Seventeen of those strains were negative with the F1/B1 primers but were positive with the D008/F008 primers. Strains of H pylori that were confirmed negative for cagA in the above assays were also for the presence of cagD and cagE, which are key components of the cagI region of the cag PAI. No strains with the cagA−/cagDE+ genotype were identified.

Strains of H pylori that were negative in the above PCR assays were then tested using the virD4 assay, which provided a marker of the cagII region. One positive strain appeared to have a diverged cag PAI, in which the two markers (cagA and cagDE) for the cagI region had been deleted, although the cagII region indicated by virD4 was conserved. Because the remaining 20 strains of H pylori were negative in the above PCR assays, it may be that they have neither a complete nor a partial cag PAI. However, owing to the large size (about 40 kb) of the cag PAI, the absence of these specific markers did not necessarily indicate its complete absence. Such cagA−/cagDE−/virD4− strains were the second most common group and a feature of 20 of 62 of all strains in our series of patients. The distribution of the H pylori cagA+ and cagA− strains according to the patient disease categories were as follows: 12 strains, peptic ulcer group; 19 strains, oesophagitis group; 16 strains, gastritis group; 14 strains endoscopically normal mucosa; and one strain, gastric cancer. The relative numbers of strains with the cagA positive genotype were similar in each disease group.

DETECTION OF ANTI-CAGA ANTIBODY AND ASSOCIATIONS WITH CAG PAI GENOTYPE

The 62 human sera that matched the strains of H pylori isolated from gastric biopsies before treatment were investigated. When tested for the presence of anti-CagA antibody by ELISA, a total of 34 patients showed CagA positivity, with two additional sera giving borderline results. Twenty one sera gave a negative ELISA result. Figure 1 shows the distribution of semiquantitative values obtained in the ELISA assay.

Table 2 lists the serology results with the CagA ELISA for Helicobacter pylori CagA enzyme linked immunosorbent assay. Each point represents one strain. A, negative zone; B, borderline zone; C, positive zone; and D, strong positive zone.

Table 3: Evaluation of ELISA for detecting anti-CagA antibody in sera of Helicobacter pylori infected patients.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>cagA+</th>
<th>cagA−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA test +</td>
<td>36</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>ELISA test −</td>
<td>5</td>
<td>21</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>21</td>
<td>62</td>
</tr>
</tbody>
</table>

Borderline results are included as positive results. Sensitivity is 36/41, or 87.8%; specificity is 21/21, or 100%; predictive value of a positive test is 36/36, or 100%; predictive value of a negative test is 21/26, or 80.8%; accuracy is 36 + 21/62, or 91.9%.

ELISA, enzyme linked immunosorbent assay.

Figure 1 The range of semiquantitative values for Helicobacter pylori CagA enzyme linked immunosorbent assay. Each point represents one strain. A, negative zone; B, borderline zone; C, positive zone; and D, strong positive zone.
Table 4  Features of Helicobacter pylori isolates and patients with sera giving negative or borderline results in the CagA ELISA assay

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain genotype</th>
<th>Patient age (years)</th>
<th>Clinical details</th>
</tr>
</thead>
<tbody>
<tr>
<td>CagA− ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H292</td>
<td>cagA+/s1m1/ES−*</td>
<td>35</td>
<td>DU</td>
</tr>
<tr>
<td>H293</td>
<td>cagA+/s1m2/ES+</td>
<td>55</td>
<td>Mild enteritis</td>
</tr>
<tr>
<td>H115</td>
<td>cagA+/s1m2/ES−*</td>
<td>56</td>
<td>GU</td>
</tr>
<tr>
<td>H177</td>
<td>cagA+/s1m2/ES−*</td>
<td>75</td>
<td>GU/DU</td>
</tr>
<tr>
<td>H298</td>
<td>cagA+/s1m2/ES−*</td>
<td>67</td>
<td>DU</td>
</tr>
<tr>
<td>Borderline CagA ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H6</td>
<td>cagA+/s1m2/ES−*</td>
<td>37</td>
<td>DU</td>
</tr>
<tr>
<td>H339</td>
<td>cagA+/s1m1/ES−*</td>
<td>68</td>
<td>Gastric neoplasia</td>
</tr>
</tbody>
</table>

Strain genotype: cagA+, strain gave positive result with F1/B1 primer pair but was positive with the F008/R008 primer pair; ES−*, non-specific product amplified as well as predicted amplicon; ES+, only non-specific product amplified.

DU, duodenal ulcer; ELISA, enzyme linked immunosorbent assay; GU, gastric ulcer.

Discussion

Serological detection of infection with a cagA ELISA strain of H pylori by means of an anti-CagA ELISA is the only non-invasive diagnostic test at present available for assessing strain virulence potential and possible disease risk. It is important, therefore, to establish the reliability of CagA serology as a predictive test for determining the cagA genotype of the infecting strain because various serological assays are now available.14 Our study was designed to determine the link between the composition of the strain cag PAI and the host response to exported CagA protein, as measured by anti-CagA IgG serum production. One possible problem in assessing such a serological test is the reliability of the cagA PCR assay because the correct design of primers is crucially important as a result of strain genomic diversity. Our results indicated that the F1/B1 primer pair resulted in a 50% underestimation of the number of strains containing cagA. In a previous study of H pylori from Italian patients, the problem of different sets of cagA primers giving different results was highlighted and attributed to divergence in the primer target sequences.14 It is important, therefore, to investigate other genes within the cag PAI to establish alternative markers of the degree of conservation.

For most of the 62 patients in our study, we found a direct correlation between the presence of the cagA gene in the infecting strain of H pylori and anti-CagA antibody seropositivity of the host, giving an accuracy of 91.9% for the CagA ELISA assay. The sensitivity of the assay was 87.8% because five patients were serologically CagA negative, even though the cagA gene and the rest of the cag PAI were conserved in the genomes of each of the infecting strains.
anti-CagA antibody in patient sera closely matched the infecting strain cag PAI genotype. False negative results (10% of sera) were best interpreted in the context of mixed infections, in particular cagA+/cagA− culture mixtures, as well as diversity within cagA, although limitations of the kit cannot be excluded because of some difficulties in interpreting borderline results. We conclude that the increasing availability of diagnostic tests, such as the CagA ELISA, provides the potential to make improvements in the management of H pylori infection in primary care. However, the results of such tests should be interpreted with caution in view of the fact that some negative ELISA results may lead to incorrect assumptions about the cagA status of the infecting H pylori strain. The ELISA appeared to be least reliable in older patients with more severe disease. Consequently, the value of predicting the clinical outcome of an H pylori infection based on cagA status alone must remain uncertain until the role of other possible determinants, such as host immunological and geographical status factors, as well as other H pylori strain specific virulence factors, have been clarified.

This work was supported in part by a three year award from the PHLS Central R & D Fund (1995–98).


3 Blaser MJ. Not all Helicobacter pylori strains are created equal should all be eliminated? Lancet 1997;349:1020–2.


