Association of CagA and VacA genotypes of Helicobacter pylori with Gastroduodenal Diseases.

Maha M El Gharbawy1, Magdy M Helmy2 and Iman M Imam3

Microbiology & Immunology 1, Tropical Disease 2 and Pathology 3 Departments, Faculty of Medicine, Alexandria University.

Helicobacter pylori (H. pylori) infects the majority of the population in developing countries. However, the rate of gastrointestinal complications has no parallel with the infection. In the present study our aim was to detect and type the cagA (cytotoxin associated gene) status and the vacA (vacuolating cytotoxin) genotypes directly from biopsy DNA, and to further define the relationship between H. pylori genotypes and gastroduodenal pathology. Antral gastric biopsies were obtained for molecular analysis and histopathological diagnosis from 105 patients with dyspeptic symptoms undergoing upper gastrointestinal endoscopy. H. pylori DNA, cagA status and vacA s and m types were detected by polymerase chain reaction (PCR). H. pylori DNA was detected in 100 (95.2%) of gastric biopsy specimens, of those, 43 (43%) were cagA positive and all (100%) were vacA positive. The vacA s2/m2 genotype was the most prevalent (54%) followed by s1/m2 (27%), then s1/m1 (16%) and 3% showed multiple genotypes. We found 90.5% and 75% of cases with peptic ulcer disease (PUD) and gastric adenocarcinoma respectively to be cagA positive in contrast to only 28% of gastritis cases. The vacA s1 allele was the commonest in PUD and gastric adenocarcinoma cases (85.7% and 75% respectively), while the vacA s2 allele was the commonest in gastritis cases (70.7%). In conclusion, we suggest the possibility of a genotype-phenotype association of H. pylori disease. Determination of cagA status and vacA genotypes may contribute to the potential clinical identification of patients at different levels of risk. We recommend further studies involving other virulence genes.

INTRODUCTION

Helicobacter pylori, the spiral-shaped, microaerophilic bacterium that colonizes the stomach in approximately half of the world’s human population, has long been associated with diseases such as chronic gastritis, peptic ulcer disease (PUD), gastric cancer, and mucosa-associated lymphoid tissue lymphoma. Once established, it may reside in the gastric mucosa for years, possibly for the life of the host, because the immunological defence mechanisms of the host fail to eliminate it. Although most H. pylori infections are clinically silent, the organism is associated with substantial morbidity and mortality. It is uncertain why the bacteria are able to produce severe diseases in some hosts and be innocuous in others.

Different virulence factors have been proposed for H. pylori such as urease, flagella, heat-shock protein, lipopolysaccharide and adhesins. The vacuolating cytotoxin (vacA) and the cytotoxin-associated protein (cagA), encoded by cagA and vacA genes, respectively, are other virulence determinants of H. pylori which have been linked with gastroduodenal diseases.

The cytotoxin associated gene (cagA) is a marker for a pathogenicity island of 40 kb. Several genes of this cag island encode proteins that enhance the inflammatory responses such as interleukin (IL)-8 production in gastric epithelial cells.

The vacuolating cytotoxin gene is present in virtually all H. pylori strains but only about 50% of them can produce detectable amounts of this cytotoxin. VacA, so far mainly regarded as a cytotoxin for the gastric epithelial cell layer, resulting in cell vacuolation, apparently has profound effects in modulating the immune response. The gene contains at least two variable regions, the signal (s) region, which encodes the signal peptide, and the middle (m) region. The s region has been divided into two subtypes, s1 and s2, and the m region has been divided into 2 subtypes m1 and m2. The amount of cytotoxin is highest with the s1/m1 allele, followed by the s1/m2 allele, while little or no cytotoxin activity is found when s2/m2 is present.

There is increasing evidence that the genetic variability of H. pylori may have a clinical importance. A significant association between the presence of ulcers or gastric carcinoma and the presence of vacA type s1.
and cagA gene has been reported (9-12). Previous studies have also shown that the vacA subtypes show regional and racial differences (13, 14).

The aim of this study was to detect and type the cagA status and the vacA genotypes directly from biopsy DNA, and to further define the relationship between H. pylori genotypes and gastroduodenal pathology.

MATERIAL AND METHODS

The material of this study included biopsy specimens obtained from 105 patients with dyspeptic symptoms who underwent an upper gastrointestinal endoscopy. All the patients attended the Gastroenterology Department of Alexandria Main University Hospital, and were subjected to full history taking and clinical assessment. Patients receiving antibiotics, non-steroidal anti-inflammatory drugs (NSAIDS) and antisecretory drugs (H2 blockers and protein pump inhibitors) 4 weeks prior to endoscopy were excluded from the study.

Antral gastric biopsy samples were taken from each dyspeptic patient. Biopsy samples for molecular analysis were kept frozen in 15% tryptone soy broth (Oxoid Ltd., Basingstoke, England) and stored at −70°C until analyzed. Other biopsy specimens were immersed in 10% formalin and submitted for histology. Endoscopic and histological diagnoses were recorded for all patients.

Genomic DNA extraction. Biopsy specimens were centrifuged at 10,000 × g for 5 min. The DNA was extracted from the pellets by use of the QIAamp DNA kit (QIAGEN, Hilden, Germany) according to the manufacturer’s recommendations and DNA stored at −20°C until analysis. DNA extraction negative controls were performed in 10% formalin and submitted for histology. Endoscopic and histological diagnoses were recorded for all patients.

DNA amplification and gel electrophoresis: Amplification was performed in a final volume of 50 µl of PCR mixture containing 0.8 uM of each primer, 0.2 mM of each deoxynucleotide (d ATP, d GTP, d TTP, and d CTP), 75 mM Tris base (pH 9.0), 20 mM ammonium sulphate, 0.01% Tween 20, 3 mM MgCl2, 1 unit of thermostable DNA polymerase and 50-100 ng of DNA template. The reaction was carried out in a Techne probe thermocycler. Negative and positive controls were run in parallel for each amplification. All primers were synthesized by a DNA synthesizer (QIAGEN OPERON). The following sets of primers were used:

- ure C (136 bp) specific for urease gene, (5’- AAGCATTAGGGGTGTTAGGGTTT-3’ and 5’-CGCAATGTCTTCAAATCTTTG-3’) which is indicative of H. pylori infection (15).
- cagA (128 bp), (5’-ATAATGCTAAATTAGACAACTTGAGAGCGA-3’ and 5’-AGAAACAAAGCAATACGATCTTTC-3’) (5)
- DNA amplification by PCR was carried out by denaturation at 94°C for 5 minutes in the first cycle, followed by annealing for 30 seconds at 60°C for ure C and 55°C for CagA, extension for 2 minutes at 72°C, and denaturation for 30 seconds at 94°C, for a total of 40 PCR cycles. The extension of the last cycle was increased to 5 minutes to ensure complete extension of the amplified fragment.
- For vacA, primers vac1F (5’-GAAATACAACAAACACACCGC-3’) and vac1R (5’-GGCTTGGTTTGGACCCCCAG-3’) were used to amplify the signal sequence region. Amplification fragments of 201 and 228 bp were expected from genotype s1 and s2, respectively. The middle region of the vacA gene was analyzed with primers vac3F (5’-GGTCAAATGCGGCTATG-3’) and vac3R (5’-GGCTTGGTTTGGACCCCCAG-3’) for m1 and vac4F (5’-CCAGGAAACATTGCCGCG-3’) and vac4R (5’-CATAACTAGCGCCTGAC-3’) for m2, which amplified 388-bp fragments for m1 and 346-bp fragments for m2 (9).
- PCR amplification was performed under the following conditions: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation.
at 95°C for 50 seconds, annealing and extension for 160 seconds, and final extension at 72°C for 2 min. Annealing temperatures were set at 55°C for vac1F-vac1R, at 60°C for vac3F-vac3R and vac4F-vac4R (16).

The PCR products were resolved by 2% agarose gel electrophoresis and were visualized after ethidium bromide (0.5 µg/ml) staining using an ultraviolet transilluminator.

**Histopathology.** Gastric biopsy specimens were fixed in 10% formalin and embedded in paraffin. The sections (4 to 5 µm thick) were cut and stained with hematoxylin and eosin (17). The histological findings from the sections stained with hematoxylin and eosin were scored according to the updated Sydney system of classification and grading of gastritis (18).

**Statistical analysis:** Minitab 11.2 was used for statistical analysis. Chi-square test was applied to test whether differences between some values were significant. P values < 0.01 were considered statistically significant.

**RESULTS**

Antral biopsy specimens were obtained from 105 patients with dyspeptic symptoms. The age of the patients ranged from 19 to 69 years with a mean of 37 years. Of the 105 patients, 56 (53%) were males and 49 (47%) were females (resulting in an overall male to female ratio of approximately 1.1:1).

Endoscopic and histopathological examinations revealed gastritis in 75 (71%) patients (Chronic gastritis (CG) in 15 (14.3%), chronic active gastritis (CAG) in 48 (45.7%), chronic atrophic gastritis (CAAG) in 11 (10.5%), and chronic atrophic gastritis with intestinal metaplasia (CAAGI) in 3 (2.9%) patients). Other findings were peptic ulcer disease (PUD) in 21 (20%) patients (9 (8.6%) with gastric ulcers (GU) of the antrum and 12 (11.4%) with duodenal ulcers (DU)), and gastric adenocarcinoma in 4 (3.8%) patients. No abnormal findings were detected in 3 (2.9%) patients who were diagnosed as nonulcer dyspepsia (NUD). In all patients with gastric ulcers, moderate to severe antral predominant chronic gastritis was diagnosed histologically. Patients with duodenal ulcers presented with duodenitis and, in some cases, with chronic antral gastritis.

**Helicobacter pylori** DNA was detected by PCR assay in 100 (95.2%) of gastric biopsy specimens. Three cases with NUD and 2 cases with CAAG were negative.

Table 1 shows the distribution of cagA and vacA allelic types according to endoscopic and histopathological findings in gastric biopsies of *Helicobacter pylori* positive patients.

Of the 100 *H. pylori* PCR-positive biopsy specimens, 43 (43%) were cagA positive. The vacA gene was detected in all (100%) of them.

The prevalence of the different vacA subtypes detected is displayed in table 2.
Table 1: Distribution of vacA and cagA allelic types according to endoscopic and histopathological findings in gastric biopsies of 100 Helicobacter pylori positive dyspeptic patients.

<table>
<thead>
<tr>
<th>Endoscopic and histopathological findings</th>
<th>No. (%) with vacA and cagA results</th>
<th>s1/m1</th>
<th>s1/m2</th>
<th>s2/m2</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cagA</td>
<td>cagA</td>
<td>cagA</td>
<td>cagA</td>
<td>cagA</td>
</tr>
<tr>
<td>Full Dyspepsia:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUD:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GU (n=9)</td>
<td>3(33.3)</td>
<td>0</td>
<td>4(44.4)</td>
<td>1(11.11)</td>
<td>0</td>
</tr>
<tr>
<td>DU (n=12)</td>
<td>4(33.3)</td>
<td>0</td>
<td>5(41.66)</td>
<td>1(8.33)</td>
<td>0</td>
</tr>
<tr>
<td>Nonatrophic gastritis:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG (n=15)</td>
<td>2(13.33)</td>
<td>1(6.66)</td>
<td>1(6.66)</td>
<td>6(12.5)</td>
<td>0</td>
</tr>
<tr>
<td>CAG (n=48)</td>
<td>3(6.25)</td>
<td>2(4.17)</td>
<td>5(10.4)</td>
<td>4(8.33)</td>
<td>0</td>
</tr>
<tr>
<td>Atrophic gastritis:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAAG (n=9)</td>
<td>1(11.11)</td>
<td>0</td>
<td>0</td>
<td>1(11.11)</td>
<td>2(22.22)</td>
</tr>
<tr>
<td>CAAG1 (n=3)</td>
<td>0</td>
<td>0</td>
<td>1(33.33)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malignant lesions:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gastric adenocarcinoma (n=4)</td>
<td>0</td>
<td>0</td>
<td>2(50)</td>
<td>1(25)</td>
<td>0</td>
</tr>
<tr>
<td>Total (n=100)</td>
<td>13(13)</td>
<td>3</td>
<td>18</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2: H. pylori vacA genotypes in 100 gastric biopsy samples

<table>
<thead>
<tr>
<th>VacA genotype</th>
<th>No. (%) of gastric biopsy samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1/m1</td>
<td>16(16)</td>
</tr>
<tr>
<td>s1/m2</td>
<td>27(27)</td>
</tr>
<tr>
<td>s2/m2</td>
<td>54(54)</td>
</tr>
<tr>
<td>Multiple vacA genotypes</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Total vacA positive</td>
<td>100(100%)</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Helicobacter pylori infection is usually acquired during early childhood and causes persistent infection and inflammation of the human gastric mucosa. The prevalence of H. pylori in adults ranges from approximately 25% in developed countries to more than 80% in the developing world [19].

In the present study, Helicobacter pylori DNA was detected by PCR assay in 100 (95.2%) out of the 105 gastric biopsy specimens from dyspeptic patients. In a study carried out on patients with gastritis and peptic ulcer disease from many Middle Eastern countries, including Egypt, the reported prevalence rate ranged from 71 to 92% [20]. Thus, the prevalence in our study slightly exceeded the higher end of the range in the other study which could be partially explained by the high detection rate and sensitivity of PCR procedure performed directly on gastric biopsies compared to other procedures [21].

Of the 100 H. pylori PCR-positive biopsy specimens, 43 (43%) were cagA positive. This is considered to be a low prevalence compared to other parts of the world [10, 11, 14, 22-26]. In an international collaborative study comparing the prevalence of different H. pylori alleles in several countries, the H. pylori isolates from most countries had a prevalence of cagA positivity greater than 67%, whereas the cagA positivity in Egyptian strains was only 36% [13]. Similarly, a low prevalence of 25.5% was reported in a study involving Israeli children [27].
In our study, the vacA gene was detected in all (100%) H. pylori PCR-positive biopsy specimens. In other studies, it was detected in more than 90% of specimens (10, 13, 22, 25, 28). We found the vacA s2/m2 genotype to be the most prevalent (54%) followed by s1/m2 (27%), then s1/m1 (16%) and 3% of specimens showed multiple genotypes. These findings are markedly different from those reported in other parts of the world, which show a marked predominance of the s1 allele whether in gastric biopsy samples or in clinical isolates (10, 13, 14, 22, 25, 29). However, in the international collaborative study mentioned earlier, in most countries, the vacA s2 allele was present in less than 30%, but the prevalence in biopsy specimens from Egypt was approximately 50%, which approaches our results (13). In further agreement with our findings, one study found the vacA s2/m2 genotype to be present in 65% of isolates (27), and another study reported that African Arabs were predominantly infected with the s2 type (30).

An association between the presence of the vacA s1 allele and cagA positivity of more than 85% has been reported from many countries (9, 13). However, in our study, this association was only 31% (table 1), which raises the possibility that the low cagA positivity may be related to the low prevalence of the vacA s1 allele in our samples. Other workers similarly found this association to be only 50% in Egyptian as well as in Israeli strains (13, 27). The observation that in two neighboring countries, despite the obvious host differences, the H. pylori strain prevalence is so similar, indicates a primary geographic influence, which may be important in the adaptation of H. pylori to the environment and climatic conditions. It is probable that evolution has selected the H. pylori strains that are best able to colonise the population of each country through extensive inter-strain gene transfer and recombination (31). The considerable geographical variation in the distribution of allelic types of H. pylori has been emphasized in many studies (13, 20, 27, 30).

There have been attempts to correlate between different H. pylori genotypes and disease severity, histopathological and endoscopic findings (29, 32-34). In the present study, cagA positive results were detected to a significantly higher degree in patients with PUD (90.5%), followed by gastric adenocarcinoma (75%), in contrast to only 28% of gastritis cases (p=0.0). The vacA s1 allele was the commonest in PUD and gastric adenocarcinoma cases (85.7% and 75% respectively), while the vacA s2 allele was the commonest in gastritis cases (70.7%). The vacA s2/m2 genotype which is the most prevalent among our cases was the most detected (71%) in the gastritis cases, but the least detected (5%) in PUD. The vacA s1/m2 genotype, on the other hand was the most detected in malignant lesions and PUD (75% and 52% respectively). All those findings were statistically significant (p=0.0). In agreement, a significant correlation between the s1/m2 genotype and PUD was confirmed by other workers (29, 30). Another study considered patients who are infected by vacA s1m1 genotype H. pylori strains to be at high risk for developing peptic ulcers (34).

In our study, 45% of all H. pylori positive specimens had the cagA negative status with vacA s2/m2 genotype, all of which came from gastritis cases, 38% from nonatrophic and 7% from atrophic gastritis. These were considered to be the least pathogenic strains by some authors who suggested that the s2 allele of vacA is carried by the more benign strains of H. pylori isolated from patients with gastritis only (10, 35). The second most common to be detected was the cagA positive status with the vacA s1/m2 genotype found in 18% of all H. pylori positive cases, half of these had PUD. This was followed by the cagA positive vacA s1/m1 genotype (13%) and again more than half of these had PUD, which suggests the role of association of cagA positivity and the vacA s1 allele in the pathogenesis of PUD. Overall, our data are consistent with other reports emphasizing this association (10, 22, 33, 36, 37).

One study proposed that patients with this type of H. pylori strain but without PUD might be at higher risk of developing PUD (37). According to Ribeiro et al. (38), vacA s1 was the only predictive factor for PUD. Another study found the extent of DNA damage in the gastric mucosa to be related to infection by cagA positive/vacA s1m1 and iceA1 strains which could serve as biomarkers for the risk of extensive DNA damage and possibly gastric cancer (39). In contrast, other studies failed to detect a correlation between cagA status or vacA genotypes and clinical outcome (14, 27). One of
these, explained this lack of correlation by the possibility that the virulence of *H. pylori* infection depends on host-pathogen interaction and other environmental factors and not on the *H. pylori* genotype alone (27).

In this study, we were unable to detect a significant correlation between the *cagA* status or *vacA* alleles and the histopathological findings in the different grades of gastritis (CG, CAG, CAAG and CAAGI) unlike others (40). It may be that with larger study groups, statistically significant results could be achieved.

To conclude, we agree with the concept of geographical variation in the distribution of allelic types of *H. pylori* as genotypes detected in our study were to some extent similar to those reported in neighboring countries, but differed from those reported in other parts of the world. We detected a significant relationship between *H. pylori* genotypes and gastroduodenal pathology and hence emphasise the possibility of a genotype-phenotype association of *H. pylori* disease. Determination of *vacA* genotypes and *cagA* gene may thus contribute to the potential clinical identification of patients at different levels of risk. We recommend further molecular epidemiological studies involving other virulence genes in order to elucidate the circulating genotypes and to better understand the genetic diversity of this pathogen. Determining the genetic sequences of strains ought to be considered in future studies.

**REFERENCES**


الملخص العربي

ارتباط الأصناف الجينية كاج أ و فاك أ للبكتريا الحلوانية ( هلوكوباكتر بيلوري)

بأمراض المعدة والإثني عشر.

مها مصطفى الغرباوي، مجدي محمد حلمي، أيمن محمد إمام

على الرغم من أن الغالبية العظمى من سكان الدول النامية مصابون بعدوي البكتريا الحلوانية إلا أن ذلك لا يوازي معدل الإصابة بالمضاعفات في المعدة والأمعاء.

كان الهدف من هذا البحث هو تصنيف البكتريا الحلوانية على أساس وجود أو عدم وجود الجين المصاحب للتوكسين الخلوي (كاج أ) و كذلك الأنواع الجينية للتوكسين الخلوي التجويفي (فاك أ) كما استهدف البحث دراسة العلاقة بين الأصناف الجينية للبكتريا الحلوانية و أمراض المعدة والإثني عشر المصاحبة لها.

وقد تم أخذ عينات باثولوجية بواسطة منظار المعدة من 105 من المرضى الذين يعانون من أعراض تشتت اجراء منظار علوي للمعدة و الأمعاء. استخدمت العينات لعمل تفاعل السلسلة المتبلمرة لتشخيص البكتريا الحلوانية و الكاج أ و الفاك أ و أصنافه س و W وكذلك لأجراء الفحص الهستوبيولوجي.

و قد وجد الحامض النووي للبكتريا الحلوانية في 100 (95.7%) من العينات كما وجد أن 43 (43%) منها تحتوي علي كاج أ و جميعها (100%) تحتوي علي فاك أ. وكان الصنف الجيني فاك أ س/2 م/2 هو الأكثر شيوعا (64%) و يليه الصنف س/1 م/2 (27%) ثم س/1 م/1 (16%) كما أظهرت 3% من العينات أصناف متعددة. كما وجدنا أن 90% من حالات فرحه المعده و الإثني عشر و 75% من حالات سرطان المعده تحتوي علي كاج أ و في المقابل احتوت 8% فقط من حالات التهاب المعده علي كاج أ. كان الصنف فاك أ س/1 هو الأكثر شيوعا في حالات الفرح و سرطان المعده بنسبة 50.7% و 75% على التوالي، أما الصنف فاك أ س 2 فقد كان الأكثر شيوعا في حالات التهاب المعده بنسبة 67.7%.

و تختلف من هذا البحث إمكانية وجود علاقة بين الصنف الجيني للبكتريا الحلوانية و المرض الناتج عنها، و تشير النتائج المذكورة إلى أهمية ذلك في اكتشاف المرضي المعرضين لكل مرض منهم كما نقترح أن تبحث الدراسات المستقبلية في جينات الضراو أخر للمورها الحلوانية.