Helicobacter pylori genotypes among patients in a university hospital in Egypt: identifying the determinants of disease severity

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ABSTRACT

Objective: To detect the various of H. pylori genotypes, determine the most prevalent one, and to identify the determinants of disease severity.

Methods: Biopsies from 90 patients were collected, however 69 were exclusively analyzed. Recognition of H. pylori was made by rapid urease test, histopathology and polymerase chain reaction. The latter was used to amplify genes encoding for virulence markers such as CagA, vacAs1 and vacAs2.

Results: cagA was identified in 45 isolates (65.2%), vacAs1 in 54 (78.3%, predominantly vacAs1a), vacAs2 in 15 (21.7%), vacAm1 in 37 (53.6%), vacAm2 in 32 (46.4%), vacAi1 in 36 (52.2%), and vacAi2 in 32 (46.4%) of strains. vacAi1 gene was the only independent marker of pathogenicity. cagA/vacAs1(a)i1m1 was the most prevalent genotype.

Conclusion: isolates possess vacAs1(a) as the predominant allelic variant of vacAs types, with a closely equal distributions of m1 and m2 alleles and of i1 and i2 alleles. The majority of the strains from the population studied are of the cagA/vacAs1(a)i1m1 genotype. vacAi1 is the only determinant of disease severity. J Microbiol Infect Dis 2013; 3(3): 109-115

Key words: H. pylori, vacAs, vacAi, cagA, gastroduodenal disease.

Mısır'da bir üniversite hastanesinde hastalar arasında Helicobacter pylori genotipleri: hastalığın ciddiyet belirteçlerinin belirlenmesi

ÖZET

Amaç: Çeşitli H. pylori genotiplerini saptamak, en yaygın olana belirlemek ve hastalığın ciddiyetini gösteren belirteçlerini saptamak

Yöntem: Doksan hastadan alınan biyopsiler toplandı ancak bunlardan sadece 69'u analiz edildi. H. pylori tanınmasının hızlı üreaz testi, histopatoloji ve polimeraz zincir reaksiyonu ile yapıldı. Bu sonucu aynı zamanda CagA, vacAs1 ve vacAs2 gibi virülsans belirteçlerini kodlayan genleri amplifiye etmek için kullanıldı.

Bulgular: cagA 45 suşta (% 65,2), vacAs1 54 suşta (% 78,3, ağırlıklı olarak vacAs1a), vacAs2 15 suşta (% 21,7), vacAm1 37 suşta (% 53,6) olarak, vacAm2 32 suşta (% 46,4), vacAi1 36 suşta (% 52,2) ve vacAi2 32 (% 46,4) tespit edildi. vacAi1 geni patojenite ile ilgili tek markeri. cagA/vacAs1(a) i1m1 geni en yaygın genotip idi.

Sonuç: m1 ve m2 alelleri ve i1 ve i2 alellerinin çok yakın eşit dağılımları ile vacAs tipleri içinde vacAs1(a) genine sahip olan izotlar en basınç aleli varyant idi. Çalışılan suşların büyük çoğunluğu cagA/vacAs1(a)i1m1 genotipinde idi. Hastalığın ciddiyetini gösteren tek tek belirteç vacAi1 idi.

Anahtar kelimeler: H. pylori, vacAs, vacAi, cagA, gastroduodenal hastalık
INTRODUCTION

*H. pylori* is a fastidious, microaerophilic, spiral gram-negative bacterium. It is an important human pathogen causing a variety of gastroduodenal diseases; as chronic gastritis, peptic ulcer and gastric cancer. It is also associated with the development of MALT lymphoma. The ecological niche of *H. pylori* is the human stomach, where it establishes long-term colonization of the gastric mucosa. Lifetime persistence of *H. pylori* within its host is the norm, with rare spontaneous clearance. Transmission can occur by contaminated water and indirect person to person contact e.g., via poorly disinfected endoscopes.

*H. pylori* possess several potential virulence factors. The imbalance between the host defense mechanisms and these factors leads to ailment. The variation in disease outcome is probably due to differences in bacterial virulence genes. The most intensively studied virulence factors are the cytotoxin-in-associated antigen (*cagA*), and the vacuolating cytotoxin (*vacA*).

The *cagA* gene is one of several genes in a pathogenicity island known as cagPAI, in the *H. pylori* genome. The *cagA* gene encodes for a protein that causes a number of cellular changes. It has been reported that most cagA-positive strains carry all of the other genes of the cagPAI, but all cagA-negative strains lack the cagPAI. Based on these findings, there is a general consensus that the presence of cagA is the marker for the presence of the cagPAI. In comparison with cagPAI-negative strains, infection with cagPAI-positive strains of *H. pylori* may significantly increase the risk of developing severe gastric mucosal inflammation, duodenal ulceration and gastric cancer.

*VacA*, encoded by the *vacA* gene induces the formation of pores and anion channels in epithelial cell membranes with vacuoles formation. It has also been described as a permease that promotes urea diffusion across epithelia providing an additional source of nutrients to sustain *H. pylori* growth in vivo. This cytotoxin can induce apoptosis in epithelial cells and a specific inhibition of immune response. *vacA* gene is present in all *H. pylori*, but is not similarly expressed. The gene contains a number of polymorphisms: signal (s), intermediate (i) and middle (m) regions. Each of these polymorphic regions has two main types/alleles that divide them further into type/allele 1 and type/allele 2. Within the ‘s1’ type, several subtypes are distinguished (s1a, s1b and s1c). The ‘s1’ type has more ability to form membrane channels than ‘s2’ type. The m1 type shows toxicity to a broader range of cells than the m2 type. The i1 type has stronger vacuolating activity than the i2 type. More recently, a new intermediate variant (i3) has been reported. The variations in the three regions of the vacA gene, are known to cause different vacuolating activities, and consequently disease progression.

Although *H. pylori* have a global distribution, several reports have evidenced geographical differences in the prevalence of cagA status and vacA alleles among *H. pylori* isolates. To our knowledge, there is only one study done in Egypt, focusing on *H. pylori* virulence factors. However, that study addressed only cagA in relation to clinical presentation. The objectives of this work were to detect the various *H. pylori* genotypes, determine the most prevalent one(s), and identify the determinants of disease severity.

**Patients and methods**

Inclusion criteria: Ninety patients who underwent standard gastroscopy procedures were included in this study. Exclusive criteria: Patients who had received antibiotics, proton-pump inhibitors or NSAIDs, throughout the last 2 months were excluded. The symptoms of patients reported were abdominal pain and discomfort, changes in bowel habits, weight loss, loss of appetite, nausea and/or vomiting, melena, and bloating. Of these patients, 50 had gastritis, 15 had gastric ulcer, 15 had duodenal ulcer, and 10 had gastric carcinoma.

Six gastric biopsies; 3 antral and 3 corpal were collected from each patient (Pentax Video–Endoscopy EG/3485). The study has been approved by the Institutional Review Board. Written consent was obtained from all patients before biopsy collection. A set of one antral and one corpal biopsies were inserted into rapid urease test tube. The second set was dispatched in 10% buffered formalin, and processed for histopathologic examination using hematoxyline-eosin, and Giemsa stains. The PCR set was placed in 0.9% normal saline.

For PCR, DNA was extracted from biopsies by QIAamp DNA Mini Kit (Qiagen, Germany). Extracted DNA was stored at -20°C until used for identification of ureA gene, and detection of virulence markers; cagA2 and vacA alleles [vacAs, vacAm, and vacAl], and subtypes. Primers used are shown in Table 1. Amplification was carried out in a final volume of 25 μL containing 12.5 μL master mix (Qiagen, Germany), 0.2 μM of each primer and 10 ng of DNA. Amplification cycles were performed with Cyclogene Thermal Cycler (Techne, England), as mentioned elsewhere. For each batch of
A patient with *H. pylori* infection was defined as the patient diagnosed by the clinician, and having a positive result for any of the three diagnostic tests. Descriptive statistical analysis for categorical variables was expressed as numbers and percentages. The association between *H. pylori* virulence markers, and the association between markers (separate or in combination) and the clinical outcomes, were analyzed using Chi-square and Fisher's exact tests when expected values were less than 5. Moreover, we identified the virulence markers which can be considered as predictors of pathogenicity, using backward stepwise logistic regression analyses. For that purpose, cases of gastric and duodenal ulcers (PUD), and malignancy, were analyzed separately in relation to gastritis. Odds Ratio and 95% confidence interval were calculated. P <0.05 was considered significant. Statistical analysis was conducted by using SPSS version.\textsuperscript{11}

### RESULTS

The age, gender and clinical picture of the 90 patients studied, are shown in Table 2. Eighty-two (91%) patients were found positive for *H. pylori*. Sixty-five (79%) were positive by the rapid urease test, 68 (83%) were positive on histopathologic examination, and all (100%) were positive by PCR.

#### Table 2. Characters of the patients studied

<table>
<thead>
<tr>
<th>Patients characters (n=90)</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>51 (56.67%)</td>
</tr>
<tr>
<td>Females</td>
<td>39 (43.33%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>16-69 (mean 40.5)</td>
</tr>
<tr>
<td>Abdominal pain and discomfort</td>
<td>70 (77.78%)</td>
</tr>
<tr>
<td>Changes in bowel habits</td>
<td>54 (60%)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>23 (25.56%)</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>11 (12.22%)</td>
</tr>
<tr>
<td>Nausea and/or vomiting</td>
<td>7 (7.78%)</td>
</tr>
<tr>
<td>Melena</td>
<td>4 (4.44%)</td>
</tr>
<tr>
<td>Bloating</td>
<td>4 (4.44%)</td>
</tr>
</tbody>
</table>

Fifty-three *H. pylori* strains were cagA-positive. The vacAs gene was amplified in all isolates. Fifty-three isolates had vacAs1, and the remaining 29 had vacAs2 alleles. vacAm1 alone was identified in 37 isolates, vacAm2 alone in 32 isolates, while both vacAm1 and vacAm2 were identified in 13 isolates.
vacAi1 was found in 42 and vacAi2 in 39 isolates. Because each *H. pylori* isolate possesses a single copy of various vacA types, the detection of more than one type (s, i, or m) in a DNA sample indicates colonization by two or more isolates with dissimilar vacA genotypes. Accordingly, only 69 isolates were subjected to further studies. The incidence of virulence markers, their association with disease outcome and the statistical analyses are shown in Table 3.

Table 3. Virulence markers and their association with disease outcome

<table>
<thead>
<tr>
<th>Virulence marker (n=69) (%)</th>
<th>Gastritis (n=29) n (%)</th>
<th>Gastric ulcer (n=15) n (%)</th>
<th>Duodenal ulcer (n=15) n (%)</th>
<th>Gastric carcinoma (n=10) n (%)</th>
<th>Total (69)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cagA</em> (65.2)</td>
<td>15 (51.7)</td>
<td>10 (66.7)</td>
<td>11 (73.3)</td>
<td>9 (90.0)</td>
<td>45</td>
<td>0.140</td>
</tr>
<tr>
<td><em>vacAs1</em> (78.3)</td>
<td>19 (65.5)</td>
<td>12 (80.0)</td>
<td>13 (86.7)</td>
<td>10 (100)</td>
<td>54</td>
<td>0.102</td>
</tr>
<tr>
<td><em>vacAs1a</em> (72.5)</td>
<td>17 (58.6)</td>
<td>12 (80)</td>
<td>12 (80)</td>
<td>9 (90)</td>
<td>50</td>
<td>0.159</td>
</tr>
<tr>
<td><em>vacAs1b</em> (7.2)</td>
<td>2 (6.9)</td>
<td>1 (6.7)</td>
<td>1 (6.7)</td>
<td>1 (10)</td>
<td>5</td>
<td>0.988</td>
</tr>
<tr>
<td><em>vacAs2</em> (21.7)</td>
<td>10 (34.5)</td>
<td>3 (20.0)</td>
<td>2 (13.3)</td>
<td></td>
<td>15</td>
<td>0.102</td>
</tr>
<tr>
<td><em>vacAm1</em> (53.6)</td>
<td>5 (17.2)</td>
<td>11 (73.3)</td>
<td>12 (80)</td>
<td>9 (90)</td>
<td>37</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><em>vacAm2</em> (46.4)</td>
<td>24 (82.8)</td>
<td>4 (26.7)</td>
<td>3 (20)</td>
<td>1 (10)</td>
<td>32</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td><em>vacAi1</em> (52.2)</td>
<td>3 (10.3)</td>
<td>12 (80)</td>
<td>12 (80)</td>
<td>9 (90)</td>
<td>36</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><em>vacAi2</em> (46.4)</td>
<td>25 (86.2)</td>
<td>3 (20)</td>
<td>3 (20)</td>
<td>1 (10)</td>
<td>32</td>
<td>&lt;0.001**</td>
</tr>
</tbody>
</table>

* significant towards increased pathogenicity
** significant towards less pathogenicity

*Figure 1. Amplification products of *cagA* and *vacA* alleles and subtypes, and genotypes of 6 strains (24-29), isolated from gastritis.*

**Primers used are:** 1) *cagA* gene: cagA1 and cagA2 ; 2) *vacAs1a* gene: SS1-F and VA1-R ; 3) *vacAs1b*: SS3-F and VA1-R; 4) *vacAm1* gene: VA3-F and VA3-R; 5) *vacAm2* gene: VA4-F and VA4-R; 6) *vacAi1* gene: Vac-F1 and C1R; 7) *vacAi2* gene: Vac-F1 and C2R; 8) *vacAs2* (not shown in figure: SS2-F and VA1-R).

**Genotypes of isolated strains:** 24: cagA/vacAs1(a)/i2/m2, 25: vacAs1(a)/i2/m1/m2 (excluded from analysis) 26: vacAs1(b)/i2/m1/m2 (excluded from analysis), 27: cagA/vacAs1(a)/i1/m1, 28: vacAs2/i2/m2, 29: cagA/vacAs1(a)/i2/m2.
The virulence markers which appeared to be statistically associated with disease outcomes, as shown in Table 3, were introduced into a backward stepwise logistic regression study, vacA1 was found to be the only independent marker of pathogenicity. The strongest association was with malignancy [OR 77.99 (CI 7.7-848.5), p<0.001]. For PUD the OR was 34.7 (7.79-154.3), p<0.001.

In this work, all possible associations existed among allelic variants of *H. pylori*, except for vacAs2, as seen in Table 4 and Figure 1. The majority of our isolates, were cagA/vacAs1(a)/i1/m1 genotype. The distribution of various genotypes and the statistical significance of such distribution are shown in Table 4.

Table 4. Genotypes of *H. pylori* isolates

<table>
<thead>
<tr>
<th>vacA status</th>
<th>cagA positive</th>
<th>cagA negative</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>s1a/i1/m1</td>
<td>29†</td>
<td>3</td>
<td>32</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>s1b/i1/m1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1*</td>
</tr>
<tr>
<td>s1/i2/m1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0.35</td>
</tr>
<tr>
<td>s1/m2</td>
<td>13</td>
<td>3</td>
<td>16</td>
<td>0.11</td>
</tr>
<tr>
<td>s2/i2/m2</td>
<td>0</td>
<td>15</td>
<td>15</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>24</td>
<td>68</td>
<td></td>
</tr>
</tbody>
</table>

† the most prevalent genotype: cagA/s1(a)/i1/m1

**A significant association between the presence of cagA and being of the vacAs1/i1/m1 genotype.

* cagA+ strains were more likely to be vacAs1a, than vacA1b.

**Statistically significant relationship between vacA-s2/i2/m2 genotype and absence of cagA.

**DISCUSSION**

More than half of the population worldwide, are persistently infected with *H. pylori.* In developing countries, 70-90% of the population harbors this organism; and the majority acquire the infection before the age of 10 years. In developed countries, the prevalence is less, due to better socioeconomic circumstances.

In the current work, eighty-two (91%) patients were found to have *H. pylori*. This high rate may be due to the fact that our hospital is a tertiary-care facility with a large number of patients being diagnosed, and referred through primary and secondary care facilities.

Despite the worldwide existence of *H. pylori*, a noteworthy genetic dissimilarity is evident among different geographic areas. The incidence of cagA-positive strains differs in various parts of the world, and there is a clear regional variation in the distribution of vacA alleles and subtypes.

Among our isolates, a high incidence of cagA-positive strains was detected, as in Table 3, which is comparable to the results obtained in of a recent study conducted in Minofya Egypt. East Asian, U.S. African, and Western European, Latin American and Lebanese strains have been shown to have cagA positivity of nearly 100% 14, 80% 15 to 70%16,17 and 37.5%18, respectively. As to the Middle East, a wide range of cagA distribution has been identified. The lowest has been from Jordan, (26.4%), and the highest from Kuwait (87%). Those from Saudi Arabia, Iraq, Iran, and Turkey (52%, 71%, 76%, and 78% respectively) fall in between.19

When the prevalence of cagA has been linked to the clinical presentations, a direct relation has been found. While it was the lowest in gastritis, it was the highest in gastric carcinoma, as shown in Table 3. The same was recognized in Minofya, Egypt and another study from Iran. On the other hand, studies from Europe and North America reported a significant correlation between the possession of cagA and the risk of developing atrophic gastritis, peptic ulcer diseases and gastric cancer.

We analyzed the vacAs, vacAm, and vacAi regions separately. A higher rate of vacAs1 than vacAs2 allele was encountered, which goes in consonance with reports elsewhere. Similar to data from Northern and Eastern Europe, the majority of our vacAs1 alleles was of the vacAs1a subtype, whereas in Spain, Portugal, Central America, and Brazil 89% of strains are of the vacAs1b subtype. The distribution of vacAs1a and vacAs1b varies also between different areas in the same country, as in Pakistan and Chili.

Contradictory to studies demonstrating a higher risk for development of peptic ulcer disease upon gastric infection with *H. pylori* strains containing type vacAs1 allele/subtypes, we found that this correlation is non-significant, as shown in table 3. A similar finding has also been concluded from eastern Asia, Iraq and Iran.

Nearly equal distribution of vacAm1 and vacAm2 alleles was encountered in this study. The same has been found in Jordan. Studies from Turkey, Iraq, Iran and Saudi Arabia characterized m2 as the main allele. In a recent study in China, most of the Chinese, and Uruguayan strains were m2, whereas most of the U.S. African strains were m1. Previous studies from eastern Asia revealed
that the m region had country-specific differences.\textsuperscript{14} Showing no role in predicting \textit{H. pylori} virulence by vacAm, has been concluded in this work, as was previously reported.\textsuperscript{7} On the other hand, exhibiting a major, primary role in that context was formerly identified.\textsuperscript{28}

Contrary to studies showing marked differences in the occurrence of the i1 and i2 alleles in \textit{H. pylori} strains studied within various populations\textsuperscript{6}, our isolates had nearly equal rates. Meanwhile, we found that vacA\textsuperscript{1} allele was the only independent marker of intensity of illness. Since it has been first reported by Rhead, et al. in 2007 its value as a strong and independent predictor of \textit{H. pylori} pathogenicity, has been focused upon.\textsuperscript{6} Authors suggested the vacAI-region to be a better determinant of disease severity than vacAs- and m-regions. Review of literature has revealed vacAI to be linked to more severe forms of \textit{H. pylori} -induced disease \textsuperscript{5} In contrast, no association was found between vacAI genotypes and clinical outcomes in East and Southeast Asian countries.\textsuperscript{29} In fact, more studies are needed to determine whether this new region is a major contributor to disease state or not.

Dissimilar to studies with Western and Iranian strains, which noted that vacAs1-m2 strains varied in their i-region genotypes; while vacAs1-m1 strains were exclusively i1 \textsuperscript{5,6} all possible combination of vacAs1 with m and i alleles were recognized in this work. This goes in accord with others.\textsuperscript{11} Of note, we found vacAs2 allele exclusively with i2 and m2 alleles, a finding that have been previously identified.\textsuperscript{5,6} Nevertheless, the presence of (s2-m1) genotypes was reported, but with very rare prevalence (0-3%).\textsuperscript{30}

All above mentioned geographic differences raise the possibility that other bacterial virulence factors may influence disease phenotypes. Moreover, other genes with varying degrees of expression, which have not yet been characterized, may exist. Such genes as well as their expression may be governed by multiple host geographic origin, environmental, ethnic and racial make-up along with socioeconomic conditions. Further studies are required to determine the factors involved.

When cagA/vacA were correlated, the majority of our isolates, were of cagA/vacAs1(a)/i1/m1 genotype. vacAs1(a)/i1/m1 genotype has been suggested to be the most virulent form of the vacuolating toxin.\textsuperscript{3} Additionally, we and others\textsuperscript{6}, documented a significant association between cagA status and vacAs1(a)/i1/m1 genotype in favor of increased disease severity, as in Table 4. It has been reported\textsuperscript{31}, that vacA possibly works as an immune modulator altering the immune response to the immunogenic cagA. The cytoskeleton of gastric epithelial cells is disorganized by cagA, and vacA, which leads to amplified cell dispersion and growth. When cagA and vacA are combined, they can diminish the effect of each protein alone, probably leading to much more survival of infected host cells. This, conceivably, occurs by preventing vacA induced apoptosis or by inhibiting the vacA induced autophagy pathway by cagA.

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