Prevalence of *Helicobacter pylori* genotypes (*vacA*, *cagA*, *cagE* and *virB11*) in gastric cancer in Brazilian’s patients: An association with histopathological parameters

Valeska Portela Lima *, Isabelle Joyce de Lima Silva-Fernandes, Markênia Kélia Santos Alves, Silvia Helena Barem Rabenhorst

Laboratório de Genética Molecular – LABGEM, Departamento de Patologia e Medicina Legal, Universidade Federal do Ceará-UFC, Brazil

**Article Info**

**Article history:**
Accepted 23 February 2011
Available online 5 April 2011

**Keywords:**
Gastric cancer
*Helicobacter pylori*
caga
vacA
cagE and virB11

**Abstract**

**Purpose:** To investigate the frequency and the association of *vacA* alleles, *cagA*, *cagE* and *virB11* genes of *Helicobacter pylori* from patients with gastric cancer, considering the clinic histopathological parameters.

**Methods:** One hundred and one gastric adenocarcinoma tissues were assessed by PCR to detect *H. pylori* and *vacA* alleles, *cagA*, *cagE* and *virB11*. Results: The distribution of cases according to the presence of the genes studied showed that the group containing *vacA* s1m1, *cagA*, *cagE* and *virB11* *H. pylori* genes was significantly more frequent, followed by the group with at least one marker on the right side and left of the island. They were also present in the early stages and were the most frequent in nearly all histopathological grades. Conclusions: This study verified that *vacAs1m1* and *cag*-*PAI* genes, *cagA*, *cagE* and *virB11* are important *H. pylori* markers for gastric cancer development. Also, this study corroborates the importance of cagE and cagA together as *cag*-PAI marker.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Gastric cancer is the fourth most common cancer and the second leading cause worldwide of cancer deaths [1,2]. About two-thirds of the cases occur in the developing world [3,4]. Adenocarcinoma, the most common gastric cancer type, has been classified by Lauren according to the clinical and histological features into two main types: diffuse and intestinal [5], which differ in their molecular changes and they are considered distinct tumors, having distinct carcinogenic pathways [6,7].

*Helicobacter pylori* is one of the most important environmental causative agents of gastric carcinoma. Over 50% of the population is reported to be infected by *H. pylori* but only 1–2% will develop gastric cancer [8]. The association of *H. pylori* with gastric cancer is strongest for non-cardia cancer, and it holds for both intestinal and diffuse histological types [9]. These observations indicate that besides the host parasite relationship in gastric cancer development, there is a need for studies to improve understanding of *H. pylori* itself. *H. pylori* is one of the most genetically diverse pathogenic bacteria [10]. This feature results in populations of bacteria with regional differences, with particular attributes that make it difficult to establish markers of virulence [10,11]. However, there is evidence of distinct genetic lineages that may have a role in its pathogenesis.

Some virulence factors of *H. pylori* are frequently associated with the most serious clinical outcomes and pathogenic bacteria. These include the *cag* pathogenicity island (*cag*-PAI), which has about 31 genes, and the presence of allelic variation of the *vacA* gene [11,12]. The *vacA* gene produces a 95-kDa homomym secreted protein which induces apoptosis, partly by forming pores in the mitochondrial and cytoplasmic membrane, thereby allowing the efflux of cytchrome *c*, inducing apoptosis and increases the cellular permeability [13]. It also produces immunosuppression by blocking antigen presentation to T cells [14,15] and the maturation of macrophage phagosomes [16]. The signal sequence (s1a, s1b, s1c, s2) and the middle region (m1, m2) of *vacA* vary among *H. pylori* strains, with strains carrying the *vacAs1m1* genotype considered the most pathogenic subtype [17].

Among *cag*-PAI genes, the most studied is *cagA*. This gene is located on the right half of *cag*-PAI. It encodes a protein (CagA) that is injected into the cytoplasm of the host cell and induces cell morphologic alterations, proliferation, adhesion and apoptosis [18–20]. The right half of *cag*-PAI also contains a gene known as *cagE*. This gene encodes a protein responsible for the induction of interleukin (IL)-8, produced by the gastric epithelial cells [21]. It is considered by some authors to be the best marker of *cag*-PAI [22,23]. Additionally, another *cag*-PAI gene, *virB11*, can be an important potential virulent factor, since it is located at a crucial point of the secretory apparatus system and exhibits ATPase activity, assisting in the transport of CagA into the host cell [24,25].
This gene is located on the left half of cag-PAI. Therefore, it can be used as a marker of the integrity of this side of the island.

Although several studies have described an association between *H. pylori* vacA s1m1 and cagA with gastritis, duodenal ulcers, peptic ulcers and gastric cancer, the relation between these genes with malignancy is still controversial. In Western populations, cagA-positive strains are more commonly associated with peptic ulceration, atrophic gastritis and gastric adenocarcinoma than cagA-negative strains [8,26]. However, this relationship is not observed in many high gastric cancer populations, including in East Asia, where almost all strains are cagA-positive. Also, in Brazil, the studies analyzing vacAS1m1 and cagA association have described conflicting results [27–32].

A relevant point is that some studies which have associated gastric cancer with *H. pylori* were done without considering the *H. pylori* genotypes in the context of the histological subtypes [31,33]. Also, reports of the association of *H. pylori* with histological types of gastric cancers are controversial; some studies have shown an association between *H. pylori* and intestinal type [34,35], while others have observed a balanced distribution between the two histological types [36,37]. Therefore, the aim of this study was to investigate the frequency and the relevance of the association of vacA alleles, cagA, cagE and virB11 genes of *H. pylori* isolated from patients with gastric cancer, considering the clinical histopathological parameters.

2. Materials and methods

2.1. Clinical specimens

The present study was approved by the Ethics Committee of the Hospital Complex of the Federal University of Ceará and all subjects signed the informed consent form before inclusion. Samples from one hundred and one patients with gastric adenocarcinoma who had undergone gastrectomy were collected from two hospitals in Ceará State, Brazil: Walter Cantidéo Hospital at the Federal University of Ceará and Santa Casa de Misericórdia Hospital, both located in Fortaleza, the state capital. The histological classification was made according to the Lauren classification and the tumors were staged according to the TNM criteria by two pathologists of the team.

2.2. DNA extraction

Genomic DNA was extracted from frozen tumor tissue using the cetyltrimethyl ammonium bromide (CTAB) technique, adapted from the method of Foster and Twell [38]. The DNA extraction was done only in fragments that showed more than 80% of tumor cells, and the quality was analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. Also, the amount of DNA was determined using the NanoDropTM 3300 fluorospectrometer.

2.3. Detection of *H. pylori* and the presence of vacA, cagA, cagE and virB11 genes

The *H. pylori* infection was detected by amplification of the *urease* C gene using primers for PCR, as described by Lage et al. [39]. For the *H. pylori*-positive samples, the presence of the vacA and alleles, cagA, cagE and virB11 genes were identified using the primer sets from the published literature. These are shown in Table 1. PCR mixtures, for amplification of cagE and virB11 genes, were prepared in a volume of 20 μL using MasterMix™ (Taq DNA Polymerase, dNTPs and MgCl2) according to the manufacturer's instructions (Promega®), with addition of 0.8% Tween 20; 0.3 μM (virB11), 1 μM (cagE) of each primer and 100 ng of the DNA sample.

The cagA, vacA s1/s2, vacA m1 genes were amplified in a 25 μL volume containing 2.5 μL of 10× PCR buffer (Invitrogen®), Cergy Pontoise, France); 1% Tween 20, 1.5 mM of MgCl2 (Invitrogen®), 200 ng (each) of dNTPs (Invitrogen®), 1 U of Platinum Taq polymerase (Invitrogen®); 0.4 μM (ureC, cagA, vacA s1/s2, vacA m1), 0.3 μM (vacA m2) for each primer and 100 ng of *H. pylori* DNA.

The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. The size of the amplification product was used to confirm the identity of the PCR product. The sample was considered *H. pylori* positive when an ureC fragment of 294 bp was amplified. For confirmation of the specificity of reaction, PCR products from *urease* C gene were cloned with TOPO TA Cloning Kit (Invitrogen®) and sequenced using the ABI PRISM® BigDye Terminator v.3.0 Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 3100 DNA Sequencer (Applied Biosystems). vacA, cagA, cagE and virB11 genes were considered positive when a specific fragment were detected (Table 1). DNAse-free water was used as a negative control. DNA preservation has also been confirmed by amplification of different genes in other approaches under study in the laboratory. Random samples were reanalyzed for confirmation of the results.

2.4. Statistical analyses

These were carried out using the statistical programs SPSS® version 15.0 (Chicago, IL, USA). Statistically significant differences

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Annealing</th>
<th>Size of PCR product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ureC</td>
<td>F - 5'-AAGCTTCTAGGCGTGTAGGCGT-3'; R - 5'-AAGCTTACTTCTCTAAGCTAAGC-3';</td>
<td>55 °C</td>
<td>294 bp</td>
<td>[39]</td>
</tr>
<tr>
<td>vacA s1/s2</td>
<td>F - 5'-ATGAAATACACACACACACAC-3'; R - 5'-CTGCTTGAATGGCACAACAC-3';</td>
<td>55 °C</td>
<td>259/286 bp</td>
<td>[17]</td>
</tr>
<tr>
<td>m1</td>
<td>F - 5'-GGTAAAGCGGTCCGGGCGG-3'; R - 5'-CCATCTGCATTGAGAAGAC-3';</td>
<td>55 °C</td>
<td>290 bp</td>
<td></td>
</tr>
<tr>
<td>m2</td>
<td>F - 5'-GGAGGCCCCAGAAGAATTGC-3'; R - 5'-CATAATCAGGCGTGCCGAC-3';</td>
<td>52 °C</td>
<td>192 bp</td>
<td></td>
</tr>
<tr>
<td>cagA</td>
<td>F - 5'-ATAGCCTGCTAATTACAACCATGACCGGCA-3'; R - 5'-TTGAAATCCAACACAAAACATAACGGCCT-3';</td>
<td>56 °C</td>
<td>297 bp</td>
<td>[40]</td>
</tr>
<tr>
<td>cagE</td>
<td>F - 5'-TGGAAAATCTTACGAGCTAGAAGGAC-3'; R - 5'-GCCTAAGGCTATTCACATACCACAT-3';</td>
<td>50 °C</td>
<td>509 bp</td>
<td>[23]</td>
</tr>
<tr>
<td>virB11</td>
<td>F - 5'-TATAATTCTTCAAGGCGACTGCT-3'; R - 5'-GATATAACTCGTGTGTAACCCGTC-3';</td>
<td>49 °C</td>
<td>491 bp</td>
<td>[23]</td>
</tr>
</tbody>
</table>
3. Results

Among the 101 analyzed cases, 68 (67.3%) were males and 33 (32.7%) females. The average age was 62.7, ranging from 23 to 90 years old. *H. pylori* infection was detected in 94 out of 101 (93%) gastric adenocarcinomas. The males were more frequent (68%) in the *H. pylori* (+) cases. The rate of *H. pylori* infection in the antrum was statistically higher (*p < 0.001*) than the cardia and body (53.2%, 26.6% and 17%, respectively). Among *H. pylori* positive cases, the intestinal and diffuse histological subtype had similar frequency, although the intestinal type was slightly more frequent (58.5% and 41.5%, respectively).

3.1. Relationship between *H. pylori* infection and vacA, cagA, cagE and virB11 genes with gastric adenocarcinoma

The frequency of *H. pylori* genes is shown on Table 2. The frequency of vacAs1m1 associated with cagA was 56.4%. Considering the integrity of cag-PAI and the association among the cag-PAI genes, we found a positive correlation between cagA and virB11 (*r = 0.229*, *p = 0.027*), and between cagE and virB11 (*r = 0.728**; *p < 0.001*). In 6.4% (6/94) of the cases only the cagA and virB11 strains were present, while only cagE and virB11 were present in 11.7% (11/94) of the cases.

Most of the *H. pylori* strains cagA(+) were associated with cagE(+) (62.3%). To investigate the relationship between *H. pylori* infection and the presence of vacA alleles, cagA, cagE and virB11 genes in gastric tumorigenesis, the cases were divided into three groups according to vacA alleles and each one was then subdivided into four subgroups according to the integrity of cag-PAI considering the studied genes, the cases which no presented any cag-PAI genes were classified as cag-PAI (–). These groups, their frequencies and histological types are shown in Table 3.

No statistical difference was found between intestinal and diffuse histological types and the *H. pylori* groups. The most frequent subgroup in all adenocarcinomas cases was A1 (vacA s1m1, cagA, cagE and virB11), which represented 36.2% (34/94) of *H. pylori* infected gastric cancer cases. There was a statistically significant difference in the frequency of cases found in the A1 group and the other groups: A2 (*p = 0.002*), A3 (*p = 0.001*), A4 (*p = 0.026*), B3 (*p = 0.038*). Also, comparing the A1 against the B group (all B sub-groups together, since the number of cases were insufficient for individual statistical analysis), the A1 group was statistically more frequent (*p = 0.001*) than the B group. Considering the *H. pylori* A groups with at least one marker in the right and left side of cag-PAI (A1 and A2 groups), a significant number of the cases were included (51%; 48/94), with a statistically significant difference when compared to the A3 (*p < 0.001*), A4 (*p = 0.003*), B3 (*p = 0.005*) and B4 (*p = 0.010*) groups.

In almost all groups, the predominant anatomical site was the gastric antrum. In the *H. pylori* groups (other than group A) in which it was possible to do statistical analysis, a significant difference was found between group A3 and the presence of the tumors in the cardia region (8/15; *p = 0.015*). Interestingly, of all gastric cancers located in the cardia, 70.8% (17/24) belonged to the intestinal subtype.

Fig. 1 shows the distribution of the *H. pylori* genotype groups according to the tumor stages. Due to a small number of cases, the subgroups of groups B and C were not considered. From this figure, it is possible to observe that the A1 group was present in almost all stages and also was the most frequent except the III tumor stage. Although groups A4, B and C were present in almost all stages, they were less frequent in the majority of the grades. We did not carefully analyze other risk factors, like alcohol consumption and smoking, due of the difficulties of measuring the levels of exposure. However, we observed that these factors were frequent in this group, since 5/6 cases had at least one such associated risk factor.

Figs. 2 and 3 show the distribution of the *H. pylori* genotype groups according to tumor stage and histological subtypes. As in Fig. 1, all cases of B and C subgroups are shown together. In both histological subtypes it is possible to observe that the A1 group was present from earlier stages with high frequency. Although in the diffuse tumors the frequencies of the A1 and A2 groups increased with tumor stage, in the intestinal subtype there was a decrease in the frequency of the A1 subgroup in grades III and IV. Even when

<table>
<thead>
<tr>
<th>Table 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of <em>H. pylori</em> genes.</td>
</tr>
<tr>
<td><em>H. pylori</em> genes</td>
</tr>
<tr>
<td>vacA</td>
</tr>
<tr>
<td>s1m1</td>
</tr>
<tr>
<td>s1m2</td>
</tr>
<tr>
<td>s2m1</td>
</tr>
<tr>
<td>s2m2</td>
</tr>
<tr>
<td>cagA</td>
</tr>
<tr>
<td>cagE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. pylori</em> infection cases in gastric cancer divided according to <em>H. pylori</em> genotypes and histological types.</td>
</tr>
<tr>
<td>Groups</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Group A: vacAs1m1</td>
</tr>
<tr>
<td>A1: cagA(+) and cagE(+) and virB11(+)</td>
</tr>
<tr>
<td>A2: cagA(+) and virB11(+) or cagE(+) and virB11(+)</td>
</tr>
<tr>
<td>A3: cagA(+) or cagE(+) or virB11(+) or cagA(+) and cagE(+)</td>
</tr>
<tr>
<td>A4: cag-PAI (–)</td>
</tr>
<tr>
<td>Group B: vacAs1m2 e vacAs2m1</td>
</tr>
<tr>
<td>B1: cagA(+) and cagE(+) and virB11(+)</td>
</tr>
<tr>
<td>B2: cagA(+) and virB11(+) or cagE(+) and virB11(+)</td>
</tr>
<tr>
<td>B3: cagA(+) or cagE(+) or virB11(+) or cagA(+) and cagE(+)</td>
</tr>
<tr>
<td>B4: cag-PAI (–)</td>
</tr>
</tbody>
</table>
A1 and A2 were considered together, the same tendency can be observed. The distribution of the *H. pylori* groups by gender shows that women were more frequent than men in the A1 (56.2% × 43.8%) and A2 (26% × 16.7%) groups. However, this sex ratio was reversed in the A3 (25% × 13%) and A4 (14.5% × 4.8%) groups, but without statistical significance.

It is interesting to note that the C4 genotype group was significantly more frequent (*p* < 0.001) in the younger patients (up to 44 years old, six cases) than the oldest ones and five of them were in the diffuse type, but no family history was described. Additionally, the A3 group was associated with the older patients, those above 65 years old (*p* = 0.018).

### 4. Discussion

The association of gastric cancer with *H. pylori* has been well established [41]. Some aspects, like host genetic profile, lifestyle can explain the gastric carcinogenesis, but the bacterial genotype seems to have a relevant role [13]. *H. pylori* strains have substantial phenotypic and genotypic diversity, which may engender individual host inflammatory responses that influence clinical outcome [42–44]. The well established virulence factors, vacA alleles (s1m1) and cagA, although having an impact on bacterial pathogenesis, cannot explain all gastric cancer development. Therefore, other potential virulence factors need to be investigated. In this respect, cagE and virB11 genes are interesting since they belong to the cag-Pal and have an important role [21–25] and along with cagA could be markers of the pathogenic strain for gastric cancer development.

As expected, a close association of gastric cancer with *H. pylori* infection was observed in this study. Similar frequencies observed in this study (93%) have been found in other Brazilian studies from the southeast region, and in studies from Turkey and Hawaii [28,36,45–48]. As in other studies, the most frequent mosaic of the vacA gene found in our study was vacA s1m1 (75.5%). This finding is consistent with the pathogenic aspect of this gene, since the production of VacA toxin is related to the allelic structure, where the s1m1 and s1m2 genotype strains are high and moderate, respectively, producers of VacA protein, whereas s2m2 genotype strains do not produce VacA. Also, s1/m1 is toxic to a wider range of epithelial cells than s1/m2 [49].

A relatively high frequency (64.9%) of *H. pylori*-infected gastric cancers were infected by the cagA (+) variety. The frequency of cagA is diverse according to region and associated disease. In Eastern countries, almost all *H. pylori* strains are cagA positive (88.9% and 88.4%); and therefore the presence of this gene is not related to gastrointestinal diseases [50,51]. On the other hand, in Western countries the frequency of the cagA variety is between 26.4% and
90.4%, and it seems that the presence of this gene is associated with the development of more severe gastric diseases, including gastric cancer [47,52,53]. In Brazilian studies, a great variation is also reported (54.7–100%) of association with gastric cancer cases [54,55]. There are suggestions that the regional divergence could be due of polymorphism in the cagA gene, but this subject is not fully elucidated.

An association between H. pylori strains carrying vacA s1m1 and cagA genes has been related with clinical outcome in some studies, but the pathogenicity of these strains is still controversial. Also, there is a little knowledge about the interaction between these two genes and the gastric cancer development. Recently, Yokoyama et al. [56] proposed a mechanism based in the opposite effects of these genes on the nuclear factor of activated T cells (NFAT). They demonstrated that CagA activates NFAT, which in turn activates many genes, including p21, a cell cycle inhibitor. Conversely, they demonstrated that VacA counteracts this activation. Therefore, the nuclear activation of NFAT by CagA is counteracted by the presence of VacA s1m1, reducing the p21 induction and thus stimulating deregulated cell growth. Since H. pylori-infected CagA also deregulates SHP-2 and other cellular target molecules that promote cell proliferation, this process could lead to transformation in gastric epithelial cells and, therefore, explain the association of these genes with the pathogenic process and the high frequency of the associations found in this study.

Despite the importance of cagA and vacA s1m1 genes for bacterial virulence, they do not appear sufficient for gastric cancer development. So other H. pylori cag-PAI genes must be involve due of the importance for the functionality of the IV system secretion. Additionally, these genes could work, along with cagA, as markers for cag-PAI integrity. Studies of cag-PAI integrity associated with diseases are controversial because there are no patterns for the selected genes [22,23,57,58]. In the present study, we did not analyze the composition of cag-PAI was in depth. We chose the genes based for their location on the island as well as on the putative role of their proteins.

The importance of cagE can be observed by its high frequency in gastric cancer from India (100%), Turkey (81.8%) and Thailand (93.8%) [51,59,60]. In the present study, 53.2% the cases had H. pylori that were cagE positive, which is lower than reported in these other studies. Also, cagE has been suggested as a better marker for the integrity of cag-PAI than cagA [23,33]. Interestingly, in our study, although cagA was more frequent than cagE, most of the time it was associated with cagE. Therefore, our data corroborate the suggestion that cagA is a better single marker of the pathogenicity island. However, the association with cagE improved the accuracy, suggesting the use of both as markers for cag-PAI presence and also for the pathological significance of these genes.

In addition to cagE, virB11 was found in a significant (60.6%) number of the gastric cancer cases analyzed. So far, there are no studies linking the presence of gastric cancer to virB11. Nevertheless, Tomasini et al. [61] and Sozzi et al. [23] studied Italian dyspeptic patients and found 90% and 94.7% presence, respectively, of the virB11 gene.

To verify the most frequent H. pylori genotype in gastric cancer and their contribution to the development of gastric cancer, we divided the cases according to vacA alleles and cag-PAI integrity. According this distribution, we found that the vacA s1m1 plus all cag-PAI genes studied (A1) was the most frequent genotype (36.2%), which was considered the most pathogenic genotype in this study. Additionally, considering the cag-PAI integrity using cagA and/or cagE as right side markers (A1 + A2), 51% of the cases were included. Since gastric cancer is expected to result from more virulent strains, the combination genotypes presented in this study suggests that cagE and virB11 genes were involved in gastric carcinogenesis. Also, virB11 could be used as left marker for cag-PAI. Also, the significant frequency of more virulent strains in all tumor stages, include the early stage, indicates the importance of these H. pylori strains to gastric carcinogenesis. The low frequency of early stage cases found in this study is explained by the fact that the samples were taken from patients treated by the public health system, in which low-income individuals who seek hospitals when the disease is already in its advanced stages predominate.

In this study, the frequency of H. pylori A1 and A2 strains did not differ between the intestinal and diffuse histological types, corroborating the suggestion of H. pylori’s involvement in both tumor subtypes [36,37,62,63]. Interestingly, we observed an increase in frequency of these groups with malignancy in the diffuse tumors, while in the intestinal tumors the highest frequencies were in the early stage, suggesting that H. pylori is involved in gastric carcinogenesis independently of the histological subtype, although having different carcinogenic pathways.

The gastric cancer cases located in the antrum were predominant in patients infected with more pathogenic strains of group A, corroborating with some studies that have reported that H. pylori strains were associated with the gastric antrum [64]. Noteworthy, same less virulent strain was related to gastric cardia (A3 and B4) and body (C2 and C4). This fact can be related to associate risks, such as alcohol consumption (at least 40 mL a day) and smoking (at least 2 box a day) in patients infected by less virulent strains. In fact, 53.8% of patients with less virulent strains were smokers and drinkers.

In conclusion, this study allowed verifying that cag-PAI genes in association with vacA s1m1, are potentially important H. pylori strain markers for gastric cancer development, since this strain was the most frequent and was present from the initial tumorigenic process. The H. pylori strains seem to be important for gastric cancer development in both histological subtypes, but probably act through different carcinogenic pathways. The presence of less virulent strains in gastric cancer could be because of the association with host genetic predisposition; other risk factors like smoking and drinking or some other bacterial factors that were not analyzed in this study. Also, this study corroborates the importance of cagA as a marker for cag-PAI, but the association with cagE seems a better marker in this respect and also suggests virB11 as a left side cag-PAI marker.

References


