Clinical relevance of the *cagA*, *vacA* and *iceA* genotypes of *Helicobacter pylori* in Brazilian clinical isolates

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Abstract

Infection with *Helicobacter pylori* strains harboring determinants of pathogenicity may lead to a strong inflammatory response in gastric mucosa. In this work, we examined the frequency of the *cagA*, *vacA* and *iceA* genotypes in *H. pylori* strains isolated from Brazilian patients and correlated these with the clinical manifestations. *H. pylori* was isolated from 165 patients (30 with non-ulcer dyspepsia cases (NUD); 93 peptic ulcer disease (PUD); 31 gastric ulcers (GU) and 62 duodenal ulcer disease (DU); 18 with erosive gastritis (EG); and 24 gastroesophageal reflux disease (GERD)]. Allelic variants of *cagA*, *vacA* and *iceA* were identified using the polymerase chain reaction. More than one *H. pylori* strain was detected in 28 cases (17%), and these were excluded from the statistical analysis. We were unable to confirm an association between *iceA* status and clinical outcome. There was a strong association between the genotype *cagA*-positive *vacA* s1 and PUD. However, logistic regression analysis showed that *vacA* s1 was the only predictive factor for PUD (OR = 4.19; 95% CI 1.95–8.98). The presence of the less virulent strain *vacA* s2 was related to GERD (OR = 8.59; 95% CI 2.85–25.91). Our results support the hypothesis that virulent strains may protect against the development of GERD.

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1. Introduction

*Helicobacter pylori* is a Gram-negative, microaerophilic, curved bacterium that persistently colonizes the human stomach, where it establishes a long-term infection of gastric and duodenal mucosa [1,2]. *H. pylori* has worldwide distribution, and its prevalence ranges from 25% in developed countries to more than 90% in developing areas, although not all infected individuals develop clinically relevant disease [2]. Colonization with *H. pylori* results in chronic superficial gastritis, which increases the risk for the development of duodenal and gastric ulcers, gastric adenocarcinoma, or non-Hodgkin’s gastric lymphoma [3]. The first strain-specific gene identified in *H. pylori* was *cagA* (cytotoxin-associated gene), considered as a marker for the presence of a pathogenicity island about 40 kb. The presence of an intact *cagA* pathogenicity island is associated with higher interleukin-8 levels and mucosal inflammation [4]. As a result, individuals infected with *cagA*-positive *H. pylori* strains have a higher risk for peptic ulcers and gastric cancer [5,6].

The *vacA* gene encodes a vacuolating toxin excreted by *H. pylori* that can damage epithelial cells [7]. This gene is present in all strains and comprises two variable parts [8]. The s-region encoding the signal peptide is located at the 5’ end of the gene, and exists as an s1 or s2 allele [5]. The m-region (middle) occurs as an m1 or m2 allele. The production of vacuolating cytotoxin is related to the mosaic combination of s and m allelic types. Strains that possess a *vacA* s1 signal allele are associated with peptic ulcer disease [5,6,8,9]. The mosaic combination of the *vacA* gene has been associated to specific genotypes with different outcomes, especially duodenal ulcer disease [5,8,10]. Recently, a novel gene designated *iceA* (induced by contact with epithelium) has been described. There are two main allelic variants of this gene, *iceA1* and *iceA2*, but only *iceA1* RNA is induced following adherence in vitro...
Although iceA1 may be associated with peptic ulcer disease [5,11], other studies have failed to confirm this correlation, and some groups have suggested a reverse relationship [9]. Peek et al. [12] demonstrated that iceA1 expression was significantly related to the host mucosal response, and this led to the hypothesis that the levels of transcription within the host environment may contribute to disease development. In contrast, iceA2 expression may be more influenced by gene structure.

Gastroesophageal reflux disease (GERD) results from exposure of the esophagus to acid [13]. There is no apparent causal relationship between H. pylori colonization and the presence of GERD and its complications [14], although GERD patients are infected with less virulent H. pylori strains, most of which do not have cagA, vacA s1 or iceA1. This finding supports the hypothesis that virulent strains may protect against the development of GERD [6,15].

Since infection with H. pylori harboring of determinants pathogenicity may lead to a strong inflammatory response in the gastric mucosa [4], we have examined the cagA, vacA and iceA genotypes of H. pylori strains isolated from Brazilian patients and correlated these with the clinical manifestations.

2. Materials and methods

2.1. Patients and definition of lesions

H. pylori isolates were obtained from 165 patients (95 males and 70 females; age 45.9 ± 15.8 years) living in southeastern Brazil. There was no significant difference in the age distribution of the different groups. Thirty patients with non-ulcer dyspepsia (NUD), 93 with peptic ulcer disease [PUD; which included gastric ulcers (GU, 31 patients) and duodenal ulcers disease (DU, 62 patients)], 18 with erosive gastritis (EG) and 24 with gastroesophageal reflux disease (GERD) were included in the study. The study was approved by the Ethics Committee of São Francisco University Medical School, and was done in accordance with the Declaration of Helsinki. Each patient signed a written informed consent prior to entering the study.

2.2. H. pylori culture and DNA extraction

H. pylori isolates were obtained by inoculating the specimens into selective and non-selective media followed by incubation for 3-5 days at 37°C under microaerophilic conditions, as previously described [16]. The colonies were identified by Gram staining and by oxidase, catalase and urease production. Genomic DNA was extracted with DNAzol® reagent (Gibco BRL, Cincinnati, OH, USA), and the integrity of the DNA was assessed by electrophoresis in 0.8% agarose gels stained with ethidium bromide.

2.3. PCR amplification

The presence of H. pylori was confirmed by polymerase chain reaction (PCR) of the 16S rRNA [17] and glmM [18] genes. The cagA gene was analyzed using the primers D008 and R008, as described by Covacci et al. [19]. For analysis of the vacA m region, primers VA3-F and VA3-R were used, whereas primers VA4-F and VA4-R were used to amplify the m1 and m2 subtypes, respectively. The vacA s region was analyzed using the primers VA1-F and VA1-R, as previously described [8]. For iceA genotype analysis, primers iceA1-F, iceA1-R, iceA2-F and iceA2-R were used. The primers iceA2-F and iceA2-R yielded a fragment of 124, 229 or 334 bp, depending on the existence of repeated sequences of 105 nucleotides [5].

2.4. Statistical analysis

The correlation between H. pylori genotypes and clinical disease was evaluated using either the χ² test with Yates continuity correction or Fisher exact test. Only cases containing single genotypes were included. Student’s t-test was used to analyze the age distribution among different groups of patient. Logistic regression analysis was used to examine the relationship between the cagA, vacA and iceA genotypes of H. pylori and the clinical data. The results are presented as odds ratios (OR) with 95% confidence intervals (CI). A P value of < 0.05 was considered statistically significant.

3. Results

More than one H. pylori strain was detected in 28 (17%) of the 165 patients studied, 9 of whom had NUD, 4 had EG, 5 had GERD, 4 had GU and 6 had DU. None of these patients was included in the analysis of the relationship between clinical disease and the putative virulence factors (vacA, cagA, iceA).

Based on the vacA and iceA genotypes, 137 (83%) specimens were colonized by a single H. pylori strain. In seven (4.2%) cases, multiple vacA genotypes were detected. Sixteen isolates (9.7%) were positive for both iceA1 and iceA2, and 4 (2.4%) of these contained multiple vacA and iceA genotypes. One isolate did not yield a result for the vacA s region and could not be classified.

The vacA genotype was determined in patients with single strain. Most of the isolates had the s1 signal sequence allele (68.8%). Considering all of patients with non-heterogeneous infections, there was a strong association between the vacA s1 strains and the presence of PUD (χ² = 12.95; P = 0.0003) and DU (χ² = 11.55; P = 0.0007), and between vacA s2 and GERD (χ² = 16.11; P = 0.0001, (Table 1)).

The m1 allele was found in 48 (35%) and the m2 in 89 (65%) patients. There was a significant association only
between NUD and the presence of the vacA m2 allele ($\chi^2 = 6.10; P = 0.01$).

Although statistical analysis showed no association between the s1m2 genotype and PUD ($\chi^2 = 2.94; P = 0.08$), a significant association was observed between this genotype and DU ($\chi^2 = 6.07; P = 0.01$). There was an association between vacA s2m2 genotype and patients with GERD ($\chi^2 = 4.56; P = 0.03$).

IceA was detected in 22 (16%) of all the 137 isolates analyzed, and iceA2 was found in 101 isolates (73.7%). There was no relationship between the size of the iceA2 amplicon and the patient’s disease. Fourteen isolates (10.2%) yielded no PCR product for iceA (Table 1). The iceA type was not associated with the vacA genotypes, or with the clinical outcome.

Of the 137 H. pylori isolates, 92 (67.1%) were cagA-positive, and 45 (32.9%) were cagA-negative (Table 1). There was an association between cagA-positive strains and PUD ($\chi^2 = 6.33; P = 0.001$).

The genotype cagA-positive vacA s1 was associated with PUD ($\chi^2 = 7.99; P = 0.004$) and DU ($P = 0.004$), and cagA-positive vacA s2 with GERD ($P = 0.007$). On the other hand, cagA-negative strains was associated with the vacA s1 genotype in PUD ($P = 0.02$) and with vacA s2 in GERD ($P = 0.01$). Statistical analysis also showed an association between the cagA-positive s1m2 genotype and PUD and DU ($P = 0.05$ and $p = 0.02$, respectively). Among patients with GERD, there was no association with cagA-positive vacA s2m2 genotype ($P = 0.006$) and cagA-negative vacA s2m2 combination ($P = 0.01$).

Logistic regression showed that vacA s1 was the only predictive factor for PUD (OR = 4.19; 95% CI 1.95–8.98) and DU (OR = 4.57; 95% CI 1.92–10.87). And the less virulent strain vacA s2 was related to GERD (OR = 8.59; 95% CI 2.85–25.91).

### 4. Discussion

The clinical relevance of putative virulence-associated genes of *H. pylori* is still a matter of controversy. The present study investigated the relationship between some virulence factors (vacA, cagA, and iceA) of *H. pylori* and clinical disease.

Evidence for the presence of multiple strains, based on vacA and iceA genotyping was found in 17% of the cases. The prevalence of multiple *H. pylori* strains may be still underestimated, especially in areas with a high prevalence of *H. pylori* infection [5,6,20].

Particular vacA genotypes have been considered markers for the pathogenesis of individual *H. pylori* strains since production of cytotoxin in vitro, epithelial damage in vivo, and the development of peptic ulcer disease are related to specific vacA genotypes [5,10]. Our results suggest a higher prevalence of the vacA s1 allele in patients with PUD and DU, in agreement with other reports [5,6,8,9]. The elevated toxicities of s1 strains may contribute to the development of ulcers. Indeed, vacA s1 strains secrete larger amounts of cytotoxin than vacA s2 strains in vitro, the latter supposedly being less virulent [8,10]. In relation to the nucleotide sequence of the vacA middle region, our data did not indicate any vacA m genotype as a significant virulence factor, as previously described [6].

The relationship between vacA s1m1 and peptic ulcer remains controversial even though this genotype has
been correlated with clinical outcome [5,6,8,9]. Although our data indicated that the vacA s1m2 genotype was associated with DU, logistic regression analysis suggested that this genotype could be due to a higher prevalence of the vacA s1 allele among patients with DU, as noted before [5].

The iceA contains two main allelic variants, iceA1 and iceA2, of which iceA1 is associated with PUD [5,11]. Recently, iceA1 expression was found to be significantly related to the host mucosal response [12]. However, the iceA1 genotype was not associated with peptic ulceration [9,20]. In contrast to results obtained from Dutch, Japanese and Korean patients [5,9,20], iceA2 was the most frequent genotype detected in our population, as previously described [20]. These results may reflect important geographic differences between *H. pylori* strains and patients. The present study in Brazilian patients did not identify any iceA genotype as a significant virulence factor. This finding agrees with previous reports and suggests that this gene should not be used as a marker for predicting the clinical outcome of *H. pylori* infection [20].

With regard to cagA, this gene was detected in 67% of our patients. Although PCR-based methods for cagA detection may result in false negative results, in silico analysis from sequences available in GenBank indicated that the sequences for the primers set used in this study were highly conserved, as noted before [21].

Our data are supported by previous reports and suggest that persons colonized with cagA-positive *H. pylori* strains are at increased risk of developing peptic ulceration [5,6]. Our results also indicate that both cagA-positive and vacA s1 are associated with PUD and DU. This agrees with other reports, and suggests a possible role for these factors in the pathogenesis of *H. pylori*-related peptic ulceration [5,6,10,19]. The present study also demonstrated an association between the cagA-positive vacA s1m2 genotype and the presence of PUD and DU. However, a logistic regression analysis pointed to vacA s1 as a predictor factor for underlying disease. This finding indicates that the presence of cagA is not independently associated with PUD or DU, as shown by similar data from patients with a peptic ulcer in the Netherlands [6]. Thus, the vacA m allele, cagA, and iceA are not useful for discriminating or predicting a specific disease risk in our patient population.

Patients with cagA-positive strains have a threefold lower risk of complications in GERD when compared with patients carrying cagA-negative strains [22]. Additionally, infection by more virulent strains may protect against GERD, so these patients may have a lower frequency of the vacA s1 genotype and a higher frequency of the vacA s2 and m2 genotypes [6,15]. Our data support this hypothesis. When the putative virulence factors were combined, a higher relationship was detected only between cagA-positive vacA s2m2 and cagA-negative vacA s2m2 genotypes and GERD. However, a logistic regression analysis showed a relationship between the presence of a less virulent strain (vacA s2) and this particular disease.

Despite the limitations of the present study, the analysis of virulence genes revealed a specific association between *H. pylori* strains and clinical outcome. We were unable to confirm an association between iceA genotypes and clinical status, suggesting that this gene is not useful for the universal discrimination or prediction of specific disease risk. Our data did reveal an association between vacA s1 and peptic ulceration in Brazilian patients. This study also supports the hypothesis that virulent strains may protect against the development of GERD. The identification of bacterial virulence factors predicting a pathological outcome would be beneficial not only for the understanding the pathogenesis of peptic disease but also for targeting treatment to those in greatest need of therapy.

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