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Presence of the Genes *cag*A, *cag*E, *vir*B11 and Allelic Variation of *vac*A of *Helicobacter pylori* Are Associated with the Activity of Gastritis

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Abstract

Non-atrophic active chronic gastritis (ACG) is characterized by the presence of *H. pylori* in the gastric epithelium, known to be one of the first steps that precede progression to gastric adenocarcinoma. Inactive chronic gastritis (ICG) suggests that the patient has *H. pylori* gastritis, but this diagnosis is rarely made in routine histopathology. Clinical manifestations associated with *H. pylori* infection are potentially due to differences in virulence between strains; however, it is unclear if the progression of ACG to ICG depends on the *H. pylori* strain. The aim of this study was to compare the prevalence of the virulence factors of *H. pylori* found in patients with ACG and ICG, and its influence on the development of ICG. A significant association was observed between *H. pylori* detection by histological examination and the activity of gastritis ($p < 0.01$). Long-term use of proton pump inhibitors (PPI) (>1 year) was reported by 28.6% of the ACG group and 42.5% of the ICG, while no evidence of association between long-term use of PPI and decreased inflammation was found in the patients studied. The genes *cag*A, *cag*E and *vir*B11 were statistically associated with ACG ($p = 0.01$, $p < 0.001$ and $p = 0.002$, respectively). In the *vac*As1 allele groups, ACG was associated with the most virulent group ($p = 0.0015$), while ICG was associated with the less virulent group ($p < 0.001$). The rate of co-infection was significantly higher in ICG than in ACG cases ($p = 0.02$). In conclusion, this study points to the role of virulent strains of *H. pylori* in the non-resolution of gastritis.

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Keywords

Active Chronic Gastritis, Inactive Chronic Gastritis, *Helicobacter pylori* Genotypes

1. Introduction

The presence of *Helicobacter pylori* in the gastric epithelium typically leads to an antral-predominant non-atrophic active chronic gastritis (ACG) lesion, known to be one of the first pathologic markers of *H. pylori* infection and one of the steps preceding progression to gastric adenocarcinoma [1]. Inactive chronic gastritis (ICG) is infrequently observed in routine histopathology, and the association with positive *H. pylori* serology and post-eradication status is interpreted as an indicator of pre-existing *H. pylori* gastritis [2]. Goldstain [3] (2002) found substantial rates of *H. pylori* infection in ICG patients.

Differences in the clinical manifestations associated with *H. pylori* infection are potentially due to differences in virulence between *H. pylori* strains [4]. Two factors are well established in *H. pylori* virulence, namely the presence of the *vac*A s1m1 allele [5] and the *cag*A gene, although other virulence factors have been pointed out as potentially relevant to gastric cancer development. In particular, *cag*E and *vir*B11 genes, also belonging to the *cag* pathogenicity island (*cag*-PAI), have been correlated with more severe lesions [6]-[10]. Some authors even consider the *cag*E gene a better marker of *cag*-PAI than the *cag*A gene [11]-[13].

Taking into account the importance of *H. pylori* virulence in gastric lesions, it is unclear if the progression of ACG to ICG depends on the *H. pylori* strain or if the bacterium found in ICG is a casual finding or plays an important role in the pathogenesis of this lesion. There are no published studies that have performed *H. pylori* genetic analyses considering both lesions, ICG and ACG. Therefore, this study aimed to compare the frequency of the virulence factors in *H. pylori* strains detected in patients with ICG versus ACG, to see if the presence or absence of the virulence factors studied was relevant to the development of ICG.

2. Material and Methods

2.1. Clinical Specimens

Samples were collected from 206 patients with dyspeptic symptoms who underwent endoscopy at two hospitals, Hospital Universitário Walter Cantídeo, and Hospital Geral de Fortaleza (HGF), both in Fortaleza, CE. All subjects signed an informed consent form before inclusion and a clinical and socioepidemiologic questionnaire was completed. Patients were excluded in the following cases: if duodenal, gastric or esophageal masses or Barrett esophagus lesions had been seen on endoscopy; previous gastric or duodenal surgery had been performed; intestinal metaplasia or atrophy were present in the gastric biopsy specimen; or if there had been recent use of non-steroidal anti-inflammatory drugs. After these exclusions, 152 patients remained in the study group. *H. pylori* infection was confirmed by two tests, PCR and histopathological analysis. A total of eight biopsy specimens of gastric antrum and body were collected, in which four fragments were for histological examination and four fragments for DNA extraction and PCR assays.

Samples for PCR were immediately frozen at −80˚C. Samples for histopathological examination were preserved in 10% formaldehyde and submitted to routine processing. The levels of lymphoplasmacytic inflammation (chronic gastritis) and polymorphonuclear neutrophil inflammation (activity) were assessed using the updated Sydney gastritis classification system, with chronic gastritis being scored as normal, mild, moderate or marked. This study was approved by the Ethics Committee of the Federal University of Ceará (047.06.09) and by the Committee for Ethics and Research of HGF (070714/10).

2.2. DNA Extraction

Genomic DNA was extracted from two frozen fragments according to the lesion location, one obtained from the antrum and the other from the body, using cetyltrimethyl ammonium bromide (CTAB), adapted from the method of Foster and Twell [14] with some modifications. DNA extraction quality was analyzed by 1% agarose gel electrophoresis with ethidium bromide staining, and the DNA amount was determined using the NanoDropTM 3300 fluorospectrometer.

2.3. *H. pylori* and *cag*A, *cag*E, *vir*B11 and *vac*A Arrangement Gene Detection

The *H. pylori* infection was detected by PCR amplification of the *urease* C gene using primers described by Lage [15]. For the *H. pylori*-positive samples, the presence of the *vac*A alleles, *cag*A, *cag*E and *vir*B11 genes were identified using primers described in the literature (**Table 1**). PCR mixtures, for amplification of *ure*C, *cag*E and *vir*B11 genes, were performed using Green Master Mix® 2× (Promega®, Madison, USA), according to the manufacturer's instructions, with addition of 0.8% Tween 20 mix for *cag*E, and water with 0.1% of BSA for *vir*B11. The concentration of each primer was 0.48 μM for *ure*C and 0.4 μM for both *vir*B11 and *cag*E. DNA sample was used at a concentration of 100 ng. For the detection of *cag*A, *vac*A s1/s2, *vac*A m1 and *vac*Am2 genes, the PCR mixture consisted of 10× PCR buffer Invitrogen®, a final concentration of 1× taq buffer and 2U of enzyme (Invitrogen Platinum® Taq DNA Polymerase), BSA, 0.2 mM dNTP, 1.5 mM MgCl$_2$, 0.4 μM primer and 100 ng DNA.

Amplified products were electrophoresed in a 6% polyacrylamide gel (**Figure 1** and **Figure 2**), silver staining, for the genes *ure*C, *cag*A and *vac*A, and in 1% agarose gel (**Figure 3**) with ethidium bromide staining, for the genes *vir*B11 and *cag*E. The size of the amplification product (**Table 1**) was used to confirm the identity of the PCR product.
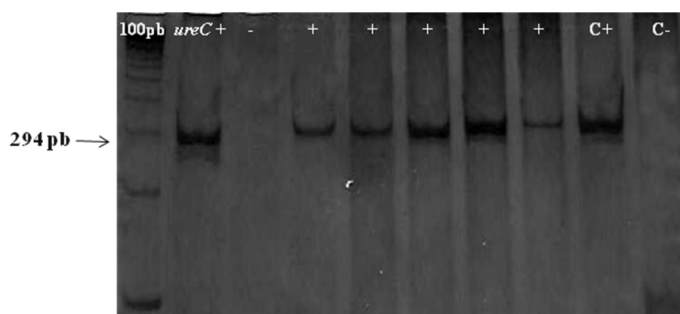

Figure 1. Gel 6% polyacrylamide stained with silver nitrate, showing the detection of specific fragment of 294 pb (arrow) from the amplification reaction of the *ure*C gene.
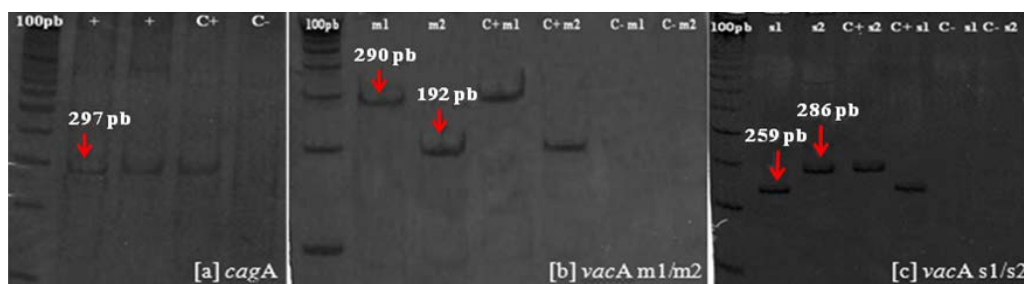

Figure 2. Polyacrylamide gels to 6% by detecting specific fragments of 297 pb for *cag*A gene (a), and 290 bp for 192 pb alleles of *vac*A m1 and m2 (b) and 259 pb and 286 pb allele of *vac*A s1 and s2, respectively (c).

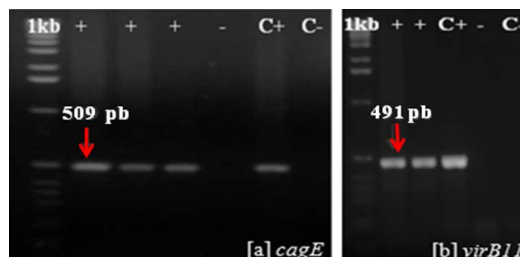

Figure 3. Agarose gels 2% detecting fragments of 509 bp generated from the amplification of the *cag*E gene (a) and 491 pb to *vir*B11 (b). The molecular weight marker used in the gels of 2% agarose was 1 kb to *cag*E, *vir*B11 (Invitrogen).

Table 1. PCR primers used for genotyping *Helicobacter pylori.*

Gene	Primer sequence	Reference	Annealing (°C)	Size (bp) of the PCR product
*Ure*C	F5-AAGCTTTTAGGGGTGTTAGGGGTTT-3	[15]	57	294
	R5-AAGCTTACTTTCTAACACTAACGC-3			
	s1s2		54	259 - 286
	F5-ATGGAAATACAACAAACACAC-3			
	R5-CTGCTTGAATGCGCCAAAC-3			
	m1		55	290
*vac*A	F5-GGTCAAAATGCGGTCATGG-3	[16]		
	R5-CCATTGGTACCTGTAGAAAC-3			
	m2		51	192
	F5-GGAGCCCCAGGAAACATTG-3			
	R5-CATAACTAGCGCCTTGCAC-3			
*cag*A	F5-ATAATGCTAAATTAGACAACTTGAGCGA-3	[17]	56	297
	R5-TTAGAATAATCAACAAACATAACGCCAT-3			
cagE	F5-TTGAAAACTTCAAGGATAGGATAGAGC-3	[11]	50	509
	R5-GCCTAGCGTAATATCACCATTACCC-3			
*vir*B11	F5-TTAAATCCTCTAAGGCATGCTAC-3	[11]	49	491
	R5-GATATAAGTCGTTTTACCGCTTC-3			

F—Forward; R—Reverse.

2.4. Statistical Analysis

The statistical analyses were conducted using the EPINFO® 6.0 and SPSS® 15.0 versions of the statistical software programs (SPSS, Chicago, IL, USA). Statistically significant differences were evaluated by the Chi-square test (χ^2) and Fisher's exact test. A p-value lower than 0.05 was considered as statistically significant.

3. Results

Among the samples analyzed, 40 cases of ICG (26.3%) and 112 of ACG (73.7%) were identified. Clinical and epidemiological data, such as the predominant location of the injury evaluation, were compared between patients with ICG or ACG, as shown in **Table 2**. No statistically significant differences were found between the groups.

In relation to the intensity of the inflammatory process, 80% (32/40) of the ICG group showed mild gastritis and 20% had moderate gastritis. No intense inflammation was found among the ICG patients. When the CGA group was evaluated, 50.9% (57/112) had mild, 37.7% (40/112) moderate and 11.4% (15/112) intense gastritis. Long-term use of proton pump inhibitors (PPI) (>1 year) was reported by 28.6% (32/112) of the patients diagnosed with ACG and 42.5% (17/40) of the ICG group. Although a higher number of cases of ICG were associated with treatment, no evidence of association between long-term use of PPI and decrease in polymorphonuclear infiltrate was found in the sample studied (p = 0.15).

H. pylori infection was positive in 98.2% (110/112) of patients with ACG and in 92.5% (37/40) with ICG, according to PCR detection. Histological examination showed a significantly lower frequency of *H. pylori* infection in the ICG group than in the ACG group (**Table 3**). A significant association between *H. pylori* detection by histological examination and the presence of polymorfonuclear infiltrate in the gastric mucosa was also observed (p < 0.01).

Table 2. Clinical epidemiological data of surveyed population: comparison between ACG and ICG.

	ACG	ICG
Gender:		
Male	44.6% (50/112)	40% (16/40)
Female	54.6% (62/112)	60% (24/40)
Age:		
0 - 14	-	-
15 - 44	39.3% (44/112)	30% (12/40)
45 - 54	21.4% (24/112)	25% (10/40)
55 - 64	22.3% (25/112)	22.5% (09/40)
>65	17% (19/112)	22.5% (09/40)
Location:		
Body	17% (19/112)	20% (08/40)
Antrum	50% (56/112)	47.5% (19/40)
Pangastritis	33% (37/112)	32.5% (13/40)

ACG—Active chronic gastritis; ICG—Inactive chronic gastritis.

Table 3. Comparison of PCR and histological methods used for diagnosis of *Helicobacter pylori* infection.

Methods	ACG (n = 112)%		ICG (n = 40)%	
	H. pylori+	*H. pylori−*	*H. pylori+*	*H. pylori−*
Histologic	99 (88.4)	13 (11.6)	07 (17.5)	33 (82.5)
PCR	110 (98.2)	02 (1.8)	37 (92.5)	03 (7.5)
p	0.007[*]		<0.001[*]	

ACG—Active chronic gastritis; ICG—Inactive chronic gastritis; [*]Significant if $p < 0.05$.

Genotyping results for the *H. pylori* virulence genes studied are summarized in **Table 4**. There was a significantly higher frequency of *H. pylori* with *cag*A, *cag*E and *vir*B11 genes in the ACG group. The allelic combination of *vac*A s1m1 was the most frequent in both groups, and none of the allelic combinations showed a statistically significant association with gastritis classification. The analysis of *vac*A allelic variability revealed the presence of a co-infection, characterized by the detection of strains with both *vac*A alleles. This was significantly more frequent among the ICG cases (86.5%) than the ACG cases (64.5%) (p = 0.02). Regardless of the presence of co-infection, it was observed that strains with a high virulence gene profile, concerning *cag*-PAI genes, were more frequent in ACG.

Based on the importance of the *vac*A s1 allele, the *H. pylori* strains were divided into two groups (I and II), according to allele presence. Within these groups, the strains were also grouped into four subgroups (A, B, C or D), in an attempt to assess the integrity of the *cag*-PAI island, as shown in **Table 5**. The strains with a high virulence gene profile (IA group) were strongly associated with ACG cases (p = 0.0015), while the ID group was associated with ICG (p < 0.001). Strains with the *vac*A s2 phenotype were not associated with any of the two groups analyzed.

The virulence of *H. pylori* strains was categorized according to the presence of *vac*A alleles and the *cag*-PAI genes. Strains with *vac*As1 allele and all *cag*-PAI genes studied (IA) were considered the most virulent followed by the ones which had at least one right marker (*cag*A/*cag*E) and one left marker (*virb*11).

Table 4. Frequencies distribution of the *H. pylori* genes according to active chronic gastritis and Inactive chronic gastritis in the population studied.

	ACG % (n)	ICG % (n)	p
cag-PAI genes:			
*cag*A	64.5% (71/110)	40.5% (15/37)	0.01[*]
*cag*E	56.4% (62/110)	19% (07/37)	<0.001[*]
*vir*B11	58.2% (64/110)	29.7% (11/37)	0.002[*]
*vac*A allelic combination:			
*vac*A s1m1	66.3% (73/110)	75.7% (28/37)	0.39
*vac*A s1m2	19.1% (21/110)	10.8% (04/37)	0.36
*vac*A s2m1	7.3% (08/110)	8.1% (03/37)	0.86
*vac*A s2m2	7.3% (08/110)	5.4% (02/37)	0.98

ACG—Active chronic gastritis; ICG—Inactive chronic gastritis; [*]Significant if p < 0.05.

Table 5. Distribution of strains of *H. pylori* according to virulence genes in consonance to activity of gastritis (chronic active gastritis and chronic inactive gastritis) in the population studied.

	ACG (n)	ICG (n)	p
Allele *s*1 *vac*A (I):			
IA.*cag*A+/*cag*E+/*vir*B11+	43	04	0.0015[*]
IB.*cag*A+ or c*ag*E+/*vir*B11+	21	08	0.73
IC.*cag*A+ or *cag*E+ or *vir*B11+ *cag*A+/*cag*E+/*vir*B11−	14	04	0.98
ID.*cag*A−/*cag*E−/*vir*B11−	16	16	<0.001[*]
Allele *s*2 *vac*A (II):			
IIA.*cag*A+/*cag*E+/*vir*B11+	03	01	1
IIB.*cag*A+ or c*ag*E+/*vir*B11+	06	00	0.15
IIC.*cag*A+ or *cag*E+ or *vir*B11+ *cag*A+/*cag*E+/*vir*B11−	01	01	0.44
IID.*cag*A−/*cag*E−/*vir*B11−	06	03	0.69

ACG—Active chronic gastritis; ICG—Inactive chronic gastritis; [*]Significant if p < 0.05.

4. Discussion

In this study, high rates of *H. pylori* infection determined by PCR detection were found in both gastritis groups, ACG and ICG. The rate of *H. pylori* detection found was higher than that reported in other studies [18] [19], probably because this study was conducted in a population with a low socioeconomic status and deficient sanitary conditions [20]. Histological staining showed statistically lower rates of *H. pylori* detection when compared with PCR, mainly in the ICG group in which false negatives made up 75% of cases. The low frequency of *H. pylori* in the histological specimens could be explained by two studies that showed that stress conditions, such as use of PPI and antibiotics, may inhibit bacterial growth cause changes in morphology from the spiral to coccoid form, making the bacteria difficult to identify in routine staining for *H. pylori* [3] [21]. Most studies determining the presence of *H. pylori* in ICG have used histological analysis for bacterial identification, explaining the low detection rates found in these studies. In addition, PCR is a technique with higher sensitivity and specificity compared histological assays [22] [23].

Untreated *H. pylori*-associated gastritis is usually antral-predominant and ACG [1]. PPI intake and *H. pylori*

eradication therapy rapidly result in a reduction or absence of polymorphonuclear infiltrate in the antrum and a slower long-term decrease in lymphoplasmacytic inflammation in this region [24] [25]. Although the literature shows an association between inactive chronic gastritis and previous *H. pylori* eradication regime and PPI intake [2] [3], in the present study no significant association was found between chronic uptake of PPI and inactive gastritis. A higher number of cases of ICG were associated with treatment. It is important to note that in other published studies, the association between PPI uptake and ICG could only be demonstrated in some of the cases studied, and therefore, other causative factors may be involved in the development of ICG.

Genotyping results for *H. pylori* virulence factors showed a statistically significant association between the *cag*-PAI genes and the presence of active gastritis. Several genes from *H. pylori cag*-PAI, such as *cag*A, *cag*E and *vir*B11, can induce the expression of cytokines in the gastric epithelium, especially IL-1B and IL-8 [8] [26] [27]. As IL-8 is an important neutrophil chemotactic and activating factor [28], the *cag*-PAI genes seem to have an important role in the development of polymorphonuclear infiltrate, which characterizes ACG. This is supported by the results obtained when the genotypes were grouped in an attempt to assess the integrity of the *cag*-PAI. Strains with a complete island were associated with activity, while the absence of the island showed an association with lack of polymorphonuclear infiltrate. *vac*A alleles were not associated with the development of activity. Nevertheless, it seemed to be related to the intensity of the inflammation response. It has been shown that the *vac*A toxin can act as an immunomodulator by interfering with the IL-2 signaling pathway in T-cells [29] or interfering with antigen presentation mediated by MHC class II cells [30]. In the present study, regardless of the high prevalence of *vac*A *s*1 strains in the population analyzed, *i.e.*, 85.7% of all samples, no association was found between *vac*A genotypes and the development of gastritis activity or the intensity of gastric inflammation in the samples studied.

5. Conclusion

In conclusion, this study showed that the genes *cag*A, *cag*E and *vir*B11 and *vac*As1 allele were statistically associated with ACG. These data point to the fact that the non-resolution of gastritis may be due to the bacterial genotype. There have been no reported studies comparing *H. pylori* strains in ACG and IGG considering *cag*-PAI genes. Additionally, the present study corroborates others in demonstrating that PCR is better than histological analysis for *H. pylori* detection. In contrast to reports in the literature taking into account the use of PPI, no association was found between the use of this medication and the decrease in polymorphonuclear infiltrate.

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