CHARACTERIZATION OF VIRULENCE GENES *cagA* AND *vacA* IN *HELICOBACTER PYLORI* AND THEIR PREVALENCE IN GASTROINTESTINAL DISORDERS

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ABSTRACT

Prevalence of *H. pylori* infection was determined using cultures of gastric biopsy samples of patients attended at the academic hospital of the Federal University of Paraná, Curitiba, Paraná, Brazil. Molecular methods were used to characterize the *cagA* and *vacA* genes from bacterial isolates associated with different diseases presented by patients. Out of a total of 81, forty-two gastric biopsy samples tested were positive for *H. pylori*, with a prevalence of 51.9%. No significant difference was found with regard to the gender (p=0.793) and age (p=0.183) of the patients. Genotype s1m1 *vacA* gene was found in 67% of the cases of peptic ulcer investigated (p=1.0), despite the limited number of patients with this disease (n=3). A correlation between the presence of less virulent strains (s2m2) and reflux esophagitis was found in the majority of the cases (45%), but without statistical significance. An association between the prevalence of *cagA* gene, found in 92% of isolates, and peptic ulcer was not observed (p=1.0), suggesting that this gene cannot be considered a specific marker of severity in our environment. The results reinforce the importance of conducting regional studies and the need to characterize *H. pylori* virulence genes associated with different diseases.

Key words: *Helicobacter pylori*; virulence genes; *cagA*; *vacA*.

INTRODUCTION

Helicobacter pylori is a gram-negative, microaerophilic, spiral rod-shaped bacillus that, since it was discovered in 1983, has been associated with the etiopathogenesis of several diseases of the digestive system (11). This bacterium colonizes the gastric mucosa of more than half of the world's population (8, 9) and is recognized as the main cause of histological

gastritis, the majority of peptic ulcers, in addition to being involved in the development of adenocarcinomas of the stomach and primary gastric mucosa-associated lymphoid tissue lymphoma (26).

Although most individuals do not develop adverse clinical consequences from *H. pylori* colonization, between 10 and 15% will experience some form of symptomatology (13) and the clinical result of the infection will be determined by complex interactions between host and bacterial factors. While the host factors remain unknown, the identification of specific bacterial factors is continuously advancing (16).

Over the past few years, research on pathogenicity markers has become gradually more important and intense in an attempt to detect bacterial strains associated with each of these diseases. The cytotoxin-associated gene A (*cagA* gene) was the first virulence factor detected in *H. pylori* strains. This gene encodes a protein that is associated with an increase in intensity of gastric inflammation and, consequently, with severe clinical outcomes, inducing an intense inflammatory process, with dense neutrophil infiltration, which can cause severe damage to the gastric mucosa (17).

The *vacA* gene (*vacuolating cytotoxin gene A*) - another important virulence factor - encodes the production of a vacuolating cytotoxin. The activity of this cytotoxin causes cytoskeleton changes, apoptosis and vacuolation of host cells (19). Although the *vacA* gene is present in almost all *H. pylori* strains, only half produce cell vacuolation *in vitro*, suggesting genetic diversity (2).

The *vacA* gene has two variable regions, the *s* region - which encodes the signal peptide and exists as two alleles, s1 or s2 - and the m region - which is located in the middle region of the gene and has the m1 or m2 alleles. The mosaic combination of the alleles of the s region with the alleles of the m region allelic types determines cytotoxin production and activity and is responsible for the degree of virulence of the bacterium (3, 27). Bacterial strains classified as type s1m1 are associated with the production of high levels of vacuolating cytotoxin, type s1m2 strains produce moderate levels, while type s2m2 show little or no vacuolating cytotoxin production (19).

In addition to the genetic diversity among *H. pylori* strains, epidemiologic studies have demonstrated that the microrganism is also not evenly distributed among population groups and varies greatly with age, race, social economic conditions and geographical region (7). Differences in infection rates may be observed within the same country, particularly in Brazil with its continental dimensions and socioeconomic inequalities.

Since *H. pylori* is considered a risk factor for the development of numerous pathologies, including gastric cancer, it is relevant not only to determine the prevalence of *H. pylori* in our region, but also to associate the presence of the *cagA* and *vacA* virulence genes with the different diseases manifested by the patients investigated in this study.

PATIENTS, MATERIAL AND METHODS

Casuistics and samples

This study included patients attended at the hospital of the Federal University of Paraná (Hospital de Clínicas, HC-UFPR) located in the city of Curitiba – Paraná, Brazil, which had been diagnosed with gastritis (GT), peptic ulcer (PU) or reflux esophagitis (RE) based on the results of endoscopic and anatomopathological examinations. The present study was submitted to the Ethics Committee of HC-UFPR for approval (protocol CEP 982.021/2005-01). The participating patients signed a free informed consent form prior to sample collection.

The study was conducted with a total of 81 patients, 18 of which were male and 63 female, with age varying from 20 to 80 (mean ± 47) years. Gastroduodenal biopsies were obtained by upper digestive endoscopy, with one biopsy being taken from the gastric corpus and another from the gastric antrum of each patient.

Bacterial identification

The samples were macerated and plated onto Petri dishes

containing a culture medium consisting of brain heart infusion agar (Difco, MI, USA) with 10% defribinated sheep blood (Newprov, Curitiba, Brazil), amphotericin B (10mg/L), vancomicin (10mg/L), nalidixic acid (20 mg/L), and triphenyltetrazolium chloride (Merck, Darmstadt, Germany) (40 mg/L) (22). The dishes were incubated at 37ºC under microaerophilic conditions, for a period of 7 days (18).

The colonies that formed in the selective medium were Gram-stained and subjected to complementary biochemical tests. The microrganism was identified as *H. pylori* if it had developed small, round smooth colonies with a golden appearance (22) with the typical cell morphology of a gram-negative spiral rod-shaped bacillus after Gram-staining. Additionally, it was tested positive for catalase, oxidase and urease (18).

After identification, part of the growth was removed with a sterile swab and transferred to a microcentrifuge tube containing 500 µL chemically pure water. The suspension was centrifuged at 6000 x g for 5 minutes and the supernatant fluids discarded. The pellet was stored in a freezer at - 80ºC until DNA extraction (2).

DNA extraction

In this stage, 300 µL of an extraction buffer solution (Tris-HCl 50 mM pH 8.0; EDTA and Tween 20 0.5%) was added to the cryotubes containing the *H. pylori* pellets and subsequently mixed by inversion. After that, Proteinase K (Sigma Chemical CO., St. Louis, MO) was added to a final concentration of 0.5 mg/mL. The samples were then incubated at 37ºC overnight and the bacterial DNA extracted using the phenol/chloroform method (23).

Amplification of virulence genes by Polymerase Chain Reaction (PCR)

The *cagA* and *vacA* virulence genes (alleles s1, s2, m1, m2) of the clinical isolates were amplified by the polymerase chain reaction in a final volume of 50 µL containing 2.5 µL genomic DNA, 20 ρmol of each primer, 200 µM of each dNTP

(Invitrogen Life Technologies, Carlsbad, EUA), 1.5% $MgCl_2$, 2.5 units of the *Taq* DNA Polimerase enzyme (Invitrogen Life Technologies, Carlsbad, EUA) and a reaction buffer for the enzyme. The *cagA* gene was analyzed using the D008/R008 primers (14). For the amplification of *vacA* m region, VA3-R/VA3-F primers were used for type m1, VA4-R/VA4-F primers for type m2 and the s region was amplified with VA1-R/VA1-F primers (3). Positive and negative controls were used in all experiments and the PCR amplified products were analyzed by 2% agarose gel electrophoresis.

Statistical Analysis

The results were statistically analyzed using the Chi-square or Fisher's exact test. The level of significance was set at $p < 0.05$.

RESULTS

Of the 81 patients included in this search, 42 (51.9%; CI95%: 42.0% - 62.7%)   presented positive culture for *H. pylori*, with three of the patients (7.1%) presenting growth only in the biopsy of the gastric corpus, six patients (14.3%) in the biopsy of the antrum, while 33 patients (78.6%) were positive for *H. pylori* growth in both biopsy cultures.

In general, no statistically significant differences were observed for the prevalence of *H. pylori* as a function of gender (*p*=0.793). The prevalence of *H. pylori* in five age subgroups 20-29; 30-39; 40-49; 50-59; and 60-80 years - depicted in Table 1 - show that prevalence increased with age up to 60 years and then slightly decreased, but without reaching statistical significance (*p*=0.183).

Examination of the different alleles of the *vacA* gene showed that three samples (7.1%) were found to have more than one allelic form simultaneously, besides being present in different pathologies in these patients (one had GT, one had GT and RE, and another had GT, RE and PU; all of them presented the s1m1, s1m2, s2m1 e s2m2 alleles). For that

reason, the samples were not included in the statistical analyses of this study, thus reducing the number of bacterial isolates to 39. The distribution of *vacA* and *cagA* genotypes found in patients suffering from gastritis, peptic ulcer and reflux esophagitis is shown in Table 2.

The associations between the *vacA* genotypes and clinical manifestations exhibited by the patients, such as those between reflux esophagitis and the s2m2 allele ($p=0.718$), and peptic ulcer and the s1m1 allele ($p=1.0$), were not statistically significant.

The *cagA* gene was found to be present in the majority of the samples analyzed (92%) and a comparison between the presence of the gene and the different pathologies studies did not show any significant association ($p=1.0$).

Table 1. Characteristics of the population studied by gender and age.

Variable	Total of Patients	Hp+ Group[*]	Prevalence	p-value
Gender				
Male	18	10	56%	
Female	63	32	51%	0.793
Age (years)				
20 – 29	8	2	25%	
30 – 39	7	3	43%	
40 – 49	22	12	54%	
50 – 59	25	15	60%	
60 – 80	19	10	53%	0.183

[*]Hp+ Group: patients with a positive culture for *H. pylori*

Table 1. Distribution of *vacA* and *cagA* genotypes according to the main pathologies of the patients.

Genotype	Total	Pathologies		
		GT[*] (n=25)	PU[**] (n=3)	RE[***] (n=11)
cagA				
cagA +	36 (92)	22 (88)	3 (100)	11 (100)
cagA -	3 (8)	3 (12)	0 (0)	0 (0)
vacA				
s1	23 (59)	15 (60)	2 (67)	6 (55)
s2	16 (41)	10 (40)	1 (33)	5 (45)
m1	21 (54)	15 (60)	2 (67)	4 (36)
m2	18 (46)	10 (40)	1 (33)	7 (64)
s1m1	20 (51)	14 (56)	2 (67)	4 (36)
s1m2	3 (8)	1 (4)	0 (0)	2 (18)
s2m2	15 (38)	9 (36)	1 (33)	5 (45)
s2m1	1 (3)	1 (4)	0 (0)	0 (0)

Results expressed by frequency (percent)
[*]gastritis (GT) ; [**]peptic ulcer (PU) ; [***]reflux esophagitis (RE)

DISCUSSION

H. pylori is responsible for one of the most commonly ocurring bacterial infections in the world and its prevalence is well-documented in several countries. However, in Brazil there is only a limited number of epidemiological reports. An epidemiological study conducted by Pilonetto in 2001 (21), revealed that seroprevalence of *H. pylori* infection in the city of Curitiba was 52.9%. The similar rates (51.9%) of our study confirm the high rate of infection in our environment.

It was also observed that, in spite of the lack of statistical significance (*p*=0.183), *H. pylori* prevalence increased with age. This can be attributed to the cohort effect, in which the higher incidence observed in adult and elderly individuals reflects the greater risk of acquiring the infection these people were exposed to in their childhood (5). The results also demonstrate a slight drop in the *H. pylori* prevalence rate in the age group 60-80 years. Some authors report that a decrease in the prevalence of this microrganism in the gastric mucosa of elderly individuals may occur due to a reduction of the *H. pylori*-specific immune response, or that a reduction of the number of microrganisms in individuals that have been infected for several decades may occur as a result of gastric atrophy (25). No significant difference (*p*=0.793) in the prevalence of *H. pylori* infection between male and female patients was found in this study, a finding which is confirmed by studies conducted by Brown (7).

The diagnosis of *H. pylori* infection by invasive means, as is the case of cultures, allowed isolating the microrganism from biopsies of both the gastric body and antrum of most of the patients (78.6%). However, in a minority, *H. pylori* growth was detected only in the corpus (7.1%) or antrum (14.3%) biopsies. In order to study the presence of *H. pylori* in the stomach, biopsies may be taken of the mucosa of either the body or the antrum, but it is not clear in which of these two anatomical sites should be used. Some researchers suggest that the examination of biopsies of both the antrum and the corpus would be a better option for the diagnosis of the infection (12). The findings of our study suggest that the probability of positive diagnosis may be greater when cultures of both anatomic sites are examined.

PCR using allele-specific primers was performed to allow the identification of vacA types s1 and s2 of *H. pylori* isolates, the majority of these isolates (59%) was classified as type s1 and the rest (41%) as type s2. Regarding the geographical distribution of the *vacA* alleles, the frequency with which type s1 was detected in this study was similar to that found in France, Canada, Italy and the United States. Higher rates were reported in studies conducted in South America (71.4%) (29) and the central portion of Brazil (72.7%) (2).

Some authors propose that the prevalence of the s1 allele in peptic ulcer disease would be greater compared to that of the s2 allele, since the former would induce secretion of greater amounts of active cytotoxins and should, for that reason, be considered more virulent (4). In this study, only three patients presented with peptic ulcer, two of them carried the s1 allele.

Analysis of the alleles of the m region showed that 54% of the samples were identified as m1 and 46% as m2. The alleles of the middle region (m1 and m2) are associated with the levels of cytotoxin produced, with the allele m1 being associated with the production of higher amounts of the toxin (3). In general, the m alleles are distributed more evenly than the s alleles, with half of the samples carrying m1 and the other half m2 (28). Although this was confirmed by our study, this finding contrasts with the results reported by Brazilian researchers (24), who observed the presence of the m1 and m2 alleles in 35 and 65% of the samples analyzed, respectively.

There is controversy in literature about the association between the s1m1 genotype and the development of peptic ulcer, although some authors suggest a greater frequency of the allele among individuals with the disease (10). Even though only three patients presented with this symptomatology, two of them carried the s1m1 allele (*p*=1.0).

It is believed that patients infected with more virulent *H.*

pylori strains may develop "protection" against reflux esophagitis, i.e. they would be inversely associated with the onset of the disease (20). Several hypotheses have been formulated in an attempt to explain this phenomenon. One of these hypotheses postulates that this protective effect would be due to the reduction in acid secretion induced by infections caused by more virulent strains. Other hypotheses suggests that, in addition to the virulence factors of the bacterium, host genetic factors should contribute to this process (15). In the present study, the less virulent strains (s2m2) of the *vacA* were present in greater percentage (45%) in the patients with reflux esophagitis, however without statistical significance (*p*=0.718).

Like the *vacA* gene, the prevalence of *cagA*-positive samples varies between geographical regions. The frequency of the patients included in this study that carried the *cagA* gene was 92%. In large parts of Western Europe and the United States, the prevalence of *cagA*-positive samples varies between 59 and 75%; Italy and Portugal exhibit rates greater than 80%, while in Mexico, practically the whole population carries the gene (15).

Several studies have demonstrated that infections caused by *cagA*-positive *H. pylori* strains are associated with greater intensity of intestinal inflammation, activity, atrophy and metaplasia (1). According to Parsonnet and collaborators, patients infected by these strains would have a three times greater risk of developing gastric cancer (15). On the other hand, Gatti and collaborators (14) did not find any association between *cagA*-positive samples and chronic gastritis, in spite of the fact that most of the samples analyzed in their study were *cagA* positive, suggesting that other bacterial factors may be involved in the pathogenesis of this disease, in addition to the host characteristics.

An association between the prevalence of the *cagA* gene and severe diseases such as peptic ulcer suggested by some authors (6), was not observed (*p*=1.0). However, the high prevalence of this gene in other diseases, such as gastritis and reflux esophagitis, suggests that the gene may not be

considered a specific marker of severity in our environment.

Thus, the data generated in this study demonstrate that the genetic profiles determined for the *H. pylori* isolates in this specific region of the country are different from those reported by other Brazilian authors, thereby emphasizing the importance of regional studies. Furthermore, the correlation between the clinical findings and the bacterial genotypes investigated may contribute to a better understanding of the infections associated with this microrganism.

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