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Prevalence of *Helicobacter pylori cagA*, *dupA*, and *vacA* genotypes and their association with the severity of gastropathies in patients with dyspepsia

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ABSTRACT. *Helicobacter pylori* is a gram-negative bacterium associated with the development of severe gastric pathologies, such as atrophy, metaplasia, and gastric adenocarcinoma. This microorganism is considered a class I carcinogen by the International Agency for Research on Cancer. The virulence genes in the strain causing infection influence the clinical outcome and can be used as specific markers for the severity of gastric diseases. We evaluated *H. pylori* infection and associations of *cagA*, *vacA* and *dupA* virulence genes with gastric pathologies. Antral and gastric body biopsies of 117 patients with dyspepsia were analyzed by histological and molecular techniques. Screening for *H. pylori* infection was

performed using the *hpx* gene (16S rRNA). Positive samples were evaluated for *vacA*, *cagA*, and *dupA* virulence genes. The clinical outcomes presented by the patients were stratified according to severity, being considered severe lesions atrophy, metaplasia and adenocarcinoma. Gastritis, duodenitis, esophagitis, ulcer, and xanthelasma were considered non-severe pathologies. The prevalence of infection was 64.1%, with a high frequency of strains positive for *cagA* (80.0%), *dupA* (70.7%), and *vacA* (56.0%). The *cagA* gene was detected in all isolates from patients with severe pathologies, whereas the *vacA* gene was not detected in this group. Simultaneous detection of the three genes was observed in 14.3% and 35.8% of the isolates from individuals with severe and non-severe pathologies, respectively. Furthermore, fewer virulence genes were detected in isolates from patients with severe disease (0.7) than in isolates from non-severe cases (1.4). Patients with severe diseases had a higher mean age and greater number of gastric diseases than patients with non-severe pathologies. The circulating *H. pylori* strains in the Brazilian Midwest exhibit high heterogeneity in the frequencies of virulence genes. Individual virulence factors and their combinations may influence clinical outcomes.

Key words: Gastrointestinal Diseases; Bacteria; Molecular Pathology; Factors Virulence

INTRODUCTION

Helicobacter pylori is a gram-negative bacterium that colonizes the human gastric mucosa. Infections caused by this microorganism are among the most common worldwide, affecting more than half of the global population (Hooi et al., 2017). The prevalence of infection is significantly lower in developed countries than in developing countries; in some regions of Brazil, the prevalence is up to 90% (Pacheco et al., 2013; Toscano et al., 2018).

Although *H. pylori* infection can remain asymptomatic, it is considered the leading cause of gastric disorders, such as chronic gastritis, peptic ulcer, adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma. Based on its clear role in the pathogenesis of gastric cancer, *H. pylori* has been classified by the World Health Organization (WHO) as a class I carcinogen (Kao et al., 2016; McClain et al., 2017). The severity of pathologies associated with bacterial infection depends on the complex and dynamic relationship between the parasite and host, which is determined by several factors, such as host genetic susceptibility, environmental factors, and bacterial virulence factors (Zhang et al., 2016).

H. pylori has a highly variable circular genome of 1,667,867 base pairs (bp) and contains genes that encode virulence factors involved in various processes, such as colonization, chronic persistence, and damage to host cells (Tomb et al., 1997). The microorganism is well-adapted to survive in the inhospitable stomach environment by the synthesis of urease, an important virulence factor that catalyzes the hydrolysis of urea into ammonia and carbon dioxide. This reaction causes a pH increase in the cell membrane and

in areas near the colonization site. In addition, hypervariable regions in genes encoding cell surface structures enable the evasion of host immune responses. Bacterial strains may have different virulence genes that directly influence pathogenicity, such as *cagA*, *vacA*, and *dupA* (Tomb et al., 1997; Pandya et al., 2017; Paredes-Osses et al., 2017; Sallas et al., 2017, 2019).

The *cagA* gene, located in the chromosomal region called the chromosomal pathogenicity island (*cag*PAI), encodes the CagA protein (cytotoxin-associated gene A), a highly immunogenic antigen that stimulates the production of chemotactic factors in the host gastric epithelium, inducing an intense inflammatory response. This gene is considered an important marker of virulence and is associated with an increased risk for the development of gastritis, peptic ulcer, atrophy, and gastric carcinoma (Backert et al., 2010; Zhang et al., 2016).

The *vacA* gene encodes vacuolating cytotoxin A (VacA), considered an important virulence factor in the pathogenesis of gastric cancer and peptic ulcer. VacA induces vacuoles in gastric epithelial cells. In addition, it induces the release of cytochrome C from mitochondria, leading to apoptosis, the inhibition of T cell proliferation, and the induction of pro-inflammatory responses (Yamaoka, 2010; Sayehmiri et al., 2015; Pandya et al., 2017).

The duodenal ulcer promoter gene (*dupA*) was the first disease-specific virulence factor identified in *H. pylori*. This gene is located in a region of plasticity in the bacterial genome and is associated with duodenal ulcer formation. The *dupA* gene is also related to neutrophil infiltration in the mucosa and the induction of elevated IL-8 levels by gastric epithelial cells (Lu et al., 2005; Nagashima and Yamaoka, 2015).

Relationships between bacterial virulence factors and gastric diseases have been described in various geographical regions. *H. pylori* strains are highly heterogeneous and this genotypic diversity explains variation in clinical outcomes of infections (Lopes et al., 2014; Paredes-Osses et al., 2017; Sallas et al., 2017; Idowu et al., 2019). The aim of this study was to evaluate the prevalence of *H. pylori* infection and associations of *cagA*, *vacA*, and *dupA* virulence genes with the severity of gastroduodenal diseases in patients with dyspepsia in midwestern Brazil.

MATERIAL AND METHODS

Study population and samples

This study was approved by the Research Ethics Committee of the Hospital das Clínicas of the Universidade Federal de Goiás (approval no. 2.519.032). A total of 117 patients with dyspepsia undergoing upper digestive endoscopy were included. Informed consent was obtained from all patients who agreed to participate in the study. Patients who used proton pump inhibitors within 2 weeks, histamine-2 receptor blockers in the previous week, or immunosuppressants or antibiotics in the last 8 weeks, patients with active gastrointestinal bleeding, pregnant or lactating patients, and those who were unable to undergo endoscopy were excluded.

Gastric specimens were obtained according to the recommendations of the IVth Brazilian Consensus Conference on *Helicobacter pylori* infection (Coelho et al., 2018). From each patient, two fragments of the antrum and gastric body were obtained. The

samples were sent to the clinical pathology laboratory of the university hospital for histopathological analysis and to the Núcleo de Estudo da *Helicobacter pylori*/Universidade Federal de Goiás (NEHP/UFG) for a molecular analysis.

Histopathological analysis

Gastric mucosa biopsies were fixed in 10% buffered formalin, cut, and stained with hematoxylin-eosin and Giemsa. Histological parameters were classified using the criteria described in the Sydney system (Fonseca et al., 2010).

H. pylori detection and genotyping

H. pylori DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was used to detect *H. pylori* by amplifying a 16S ribosomal gene (rRNA) fragment. *H. pylori*-positive samples were used for the detection of the virulence factors *vacA*, *cagA*, and *dupA*.

Each PCR consisted of the following: 33.5 µL of Milli-Q water, 5 µL of PCR Buffer 10× containing $MgCl_2$ (1.5 mM), 2 µL of dNTPs (2.5 mM); 2 µL of each oligonucleotide (10 pmol each); 5 µL of DNA sample (50 ng), and 0.5 µL of Taq DNA Polymerase (2.5 units), for a total volume of 50 µL. For each reaction, positive and negative controls were used. Primer sequences, amplification conditions, and fragment sizes are described in Table 1.

Table 1. Sequences of primers used for the detection of *Helicobacter pylori vacA*, *cagA*, and *dupA*, amplification conditions, and fragment sizes.

Gene	Primers	Sequence (5′–3′)	Amplification conditions	bp
16S rRNA	*hpx1* *hpx2*	CTGGAGARACTAAGYCCTCC GAGGAATACTCATTGCGAAGGCGA	94°C, 1 min; 59°C, 1 min; 72°C, 1 min (40 cycles)	150
vacA S1	*SA* *SCa*	ATGGAAATACAACAAACACAC CCTGARACCGTTCCTACAGC	94°C, 45 s; 54°C, 45 s; 72°C, 45 s (35 cycles)	176
cagA	*cag1* *cag2*	ATGACTAACGAAACTATTGATC CAGGATTTTTGATCGCTTTATT	94°C, 1 min; 53°C, 1 min; 72°C, 1 min (40 cycles)	232
dupA	*dupA1* *dupA2*	CGTGATCAATATGGATGCTT TCTTTCTAGCTTGAGCGA	94°C, 45 s; 52°C, 45 s; 72°C, 45 s (35 cycles)	197

PCR products were analyzed by 2% agarose gel electrophoresis, containing Blue Green nucleic acid dye (Lac Biotechnology, São Paulo, Brazil), and visualized under an ultraviolet light source. For a size analysis of the amplified fragment, a 100-bp DNA ladder was used.

Diagnosis of gastroduodenal pathologies and severity criteria

The endoscopic and histopathological diagnoses of *H. pylori* infection were made, respectively, by the gastroenterology department and the clinical pathology laboratory of the university hospital. Cases were classified as severe or non-severe according to Paredes-Osses et al. (2017) and Bellolio et al. (2019). Atrophy, metaplasia, and adenocarcinoma

were considered severe pathologies. Gastritis, duodenitis, esophagitis, ulcer, and xanthelasma were considered non-severe pathologies.

Statistical analysis

For statistical comparisons, the chi-square test and Fischer's exact test were used and a P-value of <0.05 was considered statistically significant. Statistical analyses were performed using the BioEstat® 5.3 software.

RESULTS

Prevalence of *H. pylori* infection

Initially, DNA from 117 gastric biopsy specimens was extracted for the amplification of the 16S rRNA gene *hpx* to screen for *H. pylori* infection. Samples that amplified a 150-bp fragment were considered positive (Figure 1A). The prevalence of infection was 64.1% (75/117); among infected individuals, 72.0% (54/75) were women and 28.0% (21/75) were men. The age of the patients ranged from 18 to 83 years, with a mean of 46.6 years.

The 75 *hpx*-positive samples were used for the detection of virulence genes. Samples were considered positive for the *cagA*, *vacA*, and *dupA* genes when they amplified a fragment of 232 bp (Figure 1B), 176 bp (Figure 1C), and 197 bp (Figure 1D), respectively. These genes were confirmed by Sanger sequencing and a bioinformatics analysis.

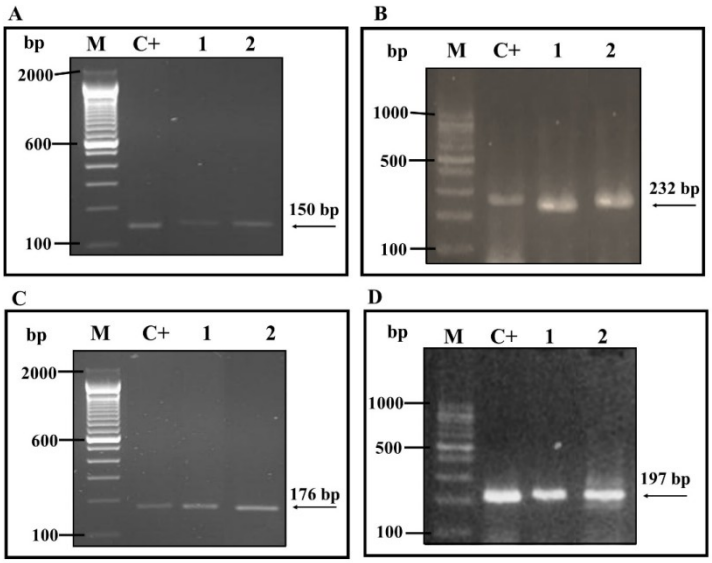

Figure 1. PCR amplification products of the *Helicobacter pylori* ribosomal gene and virulence genes. M: molecular weight marker (100 bp); C+: positive control. (A) Lanes 1 and 2: *hpx*-gene positive samples. (B) Lanes 1 and 2: positive samples for the *cagA* gene. (C) Lanes 1 and 2: v*acA* gene-positive samples. (D) Lanes 1 and 2: positive samples for the *dupA* gene.

A high prevalence of virulence genes was found in circulating strains in central western Brazil. The most frequent gene was *cagA*, present in 80.0% (60/75) of samples, followed by *dupA* in 70.7% of samples (53/75) and *vacA* in 56.0% of samples (42/75) (Figure 2).

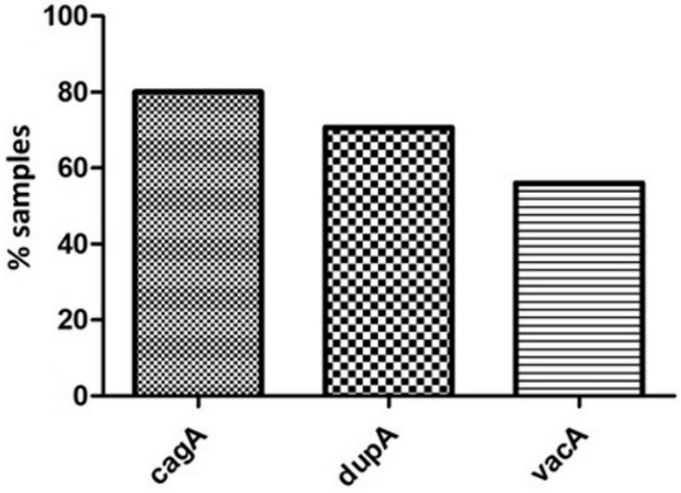

Figure 2. Prevalence of *Helicobacter pylori* genotypes in patients with dyspepsia

Associations between clinical outcomes and virulence genes

Most patients in our study had more than one endoscopic diagnosis. In patients infected with *H. pylori,* the most frequent non-severe gastric pathology was gastritis (54/75; 72.0%), followed by esophagitis (11/75; 14.7%) and duodenitis (11/75; 14.7%). The most frequent severe pathologies were gastric atrophy (4/75; 5.3%), intestinal metaplasia (2/75; 2.7%), and gastric adenocarcinoma (2/75; 2.7%). In addition, among infected patients, 20.0% (15/75) did not present any gastric pathology (Table 2).

In the group of patients with non-severe pathologies, the most frequent clinical outcome was gastritis, and *dupA*, *cagA*, and *vacA* were present in 73.0, 70.0, and 69.0%, respectively, of isolates from these patients. In patients with severe pathologies, atrophy was the most common clinical outcome, and unlike in the severe group, the *cagA* gene was most frequent, followed by *dupA* and *vacA*. Despite the differences in the frequencies of virulence genes between groups, their presence was not associated with any specific clinical outcome (Table 2).

Table 2. Clinical profile of patients infected with *Helicobacter pylori* and genotypes of isolates.

Clinical outcomes	*hpx* (+)		*cagA* (+)		*vacA* (+)		*dupA* (+)	
	n (75)	f (%)	n (60)	f (%)	n (42)	f (%)	n (53)	f (%)
Gastric adenocarcinoma	2	2.7	2	3.3	1	2.4	0	0
Atrophy	4	5.3	4	6.7	2	4.8	3	5.7
Duodenitis	11	14.7	7	11.7	6	14.3	8	15.1
Esophagitis	11	14.7	8	13.3	4	9.5	6	11.3
Gastritis	54	72.0	42	70.0	29	69.0	39	73.6
Metaplasia	2	2.7	2	3.3	1	2.4	2	3.8
Normal	15	20.0	12	20.0	9	21.4	10	18.9
Ulcer	1	1.3	1	1.7	0	0	1	1.9
Xanthelasma	1	1.3	0	0	0	0	0	0

n: number / f: frequency

Number of virulence genes and the severity of gastric disorders

H. pylori-positive samples were used for the detection of the virulence genes *vacA*, *cagA*, and *dupA*. The results showed that isolates from 3/75 patients (4.0%) did not harbor these genes, 14/75 (18.7%) had only one gene, 32/75 (42.7%) contained two of the genes, and 26/75 (34.7%) harbored all three genes (Figure 3A). The association between the number of virulence genes and clinical outcomes was verified. Interestingly, we observed that the mean number of virulence genes was significantly higher in strains from patients with non-severe pathologies than from severe pathologies ($p = 0.0199$) (Figure 3B).

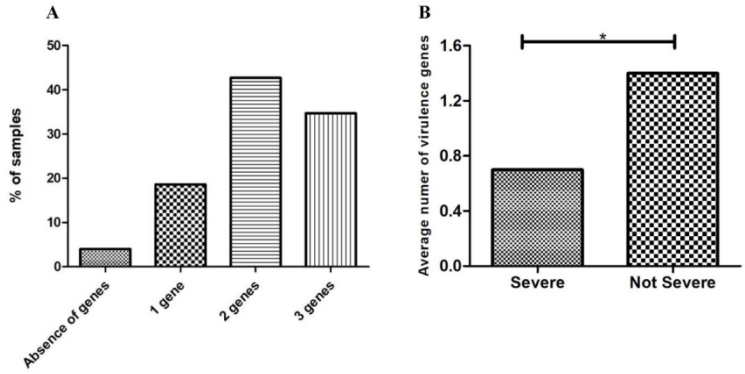

Figure 3. (A) Percentage of *Helicobacter pylori* isolates with various virulence genes. (B) Relationship between the number of virulence genes in isolates and severity of gastric pathologies. *P < 0.005.

In addition, we observed that patients with severe pathologies were 10 years older, on average, than patients with non-severe pathologies ($p = 0.0047$) and had approximately twice the number of gastric diseases ($p < 0.001$) (Table 3).

Table 3. Associations between the severity of gastropathies and age and the number of gastric diseases in dyspeptic patients.

Variables	Severe (n=7) Average	SD	Not Severe (n=53) Average	SD	P-*value*
Age	56.7	14.3	46.0	15.2	**0.0047**
Number of clinical outcomes	1.9	0.6	1.0	0.7	**<0.0001**

SD: Standard deviation

Gene profile of infective strains in patients

The genotype profiles of strains isolated from individuals with severe and non-severe pathologies were evaluated. In the first group, *cagA* was present in 14.3% (1/7), *cagA* and *vacA* were present in 28.6% (2/7), *cagA* and *dupA* were present in 42.8% (3/7), and all three genes were present in 14.3% (1/7) of isolates. The *vacA* and *dupA* genes, were not found in any strain isolated from patients with severe pathologies (Figure 4A).

In the group of patients with non-severe pathologies, the *cagA*, *vacA,* and *dupA* genes were present in 5.7% (3/53); 5.7% (3/53), and 9.4% (5/53) of isolates, respectively. The combinations of *cagA* and *dupA*, *cagA* and *vacA*, and *vacA* and *dupA* were identified in 24.5%, 11.3%, and 3.8% of isolates. The simultaneous detection of the three genes was observed in 35.8% of isolates, while 3.8% did not show any virulence genes (Figure 4B).

In the group of patients without gastric pathologies, the *vacA* gene was not found in any of the isolates. Additionally, *cagA* was found in 6.7% of isolates. The same frequency was obtained when analyzing the *dupA* gene alone. The combinations of *cagA* and *dupA*, *cagA* and *vacA*, and *vacA* and *dupA* were present in 20.0%, 13.3%, and 6.7% of isolates, respectively. All three genes were found in 40.0% of isolates in this group. Additionally, 6.6% of individuals were infected with strains that had none of the virulence genes (Figure 4C).

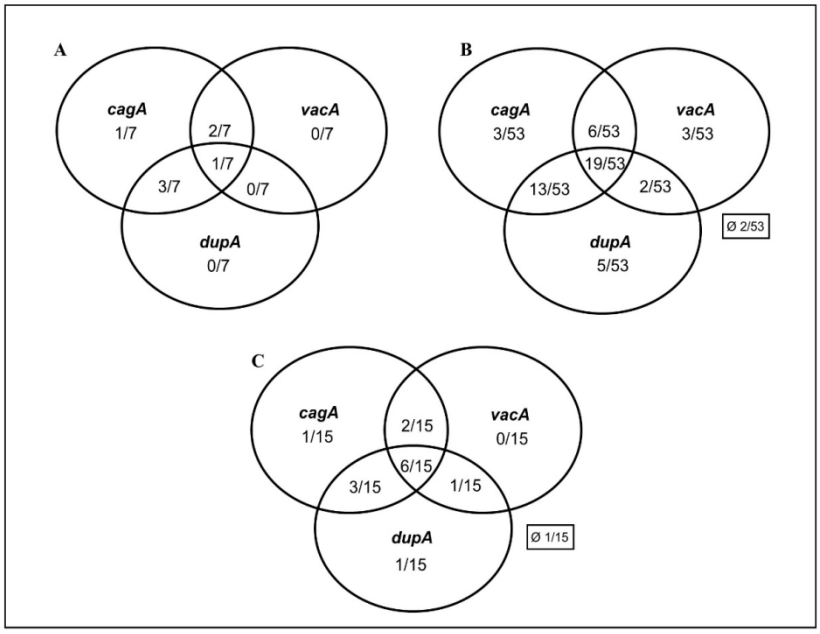

Figure 4. Association between severity of gastropathies and the presence of *cagA*, *vacA* and *dupA Helicobacter pylori* virulence genes. (A) Individuals with severe pathologies. (B) Individuals with non-severe pathologies. (C) Individuals without gastric disorders. Φ: absence of all genes.

These results showed that the most frequent gene combination in patients classified as severe was *cagA* and *dupA*. In the group of non-severe patients and in the group of individuals without gastric pathologies, strains harboring all three genes were most frequent (Figure 4).

DISCUSSION

The reported prevalences of *H. pylori cagA*-positive strains among patients with dyspepsia in India (72.7%), Iran (70.0%), Greece (73.0%), and Bulgaria (84.7%) (Khodaii

et al., 2011; Yordanov et al., 2012; Pandya et al., 2017) are similar to the high prevalence found in our study (80.0%).

When the CagA oncoprotein is present in host cells, the risks of developing a peptic ulcer and gastric cancer appear to be higher than those for infections with *cagA*-negative strains (Kim et al., 2013; Sallas et al., 2017). In our study, 77.3% of patients with non-severe pathologies were infected with *cagA*-positive strains; however, this gene was present in all strains of patients with severe pathologies. These data suggest that the *cagA* gene is an important marker of severity and corroborate the studies by Boukhris et al. (2013), who found a significant association ($p = 0.04$) between the *cagA* genotype and gastric cancer in the Moroccan population. In addition, in our work patients with severe pathologies were older, with the potential for a longer time for disease progression (Table 3).

VacA is a toxin secreted by *H. pylori* with the ability to induce vacuolization in epithelial cells. We detected a low frequency of *vacA* (i.e., 56%), which can be explained by the amplification of only one allelic subtype of the gene. These results corroborate those obtained by Idowu et al. (2019), who reported a *vacA* frequency of 66.2% when only the s1 allele was evaluated. However, this frequency is considered low when compared to those for northern, northeastern, and southeastern regions of Brazil, where several studies have evaluated more than one allelic region, showing frequencies of 84.6%, 86.1%, and 69.4%, respectively (Cavalcante et al., 2012; Rasmussen et al., 2012; Silva Júnior et al., 2013; Vinagre et al., 2015).

Studies of other countries, such as Saudi Arabia, Morocco, and China, have found a vacA gene frequency of greater than 90% in patients with dyspepsia (Pinto-Ribeiro et al., 2010; El Khadir et al., 2017; Akeel et al., 2019). The high frequencies found in these studies may also reflect the analysis of several allelic subtypes of the gene.

In the present study, none of the patients with severe pathologies were infected with strains of *H. pylori* that contained only the *vacA* gene; however, the combination of *vacA* and *cagA* was detected in 28.7% of isolates. A study conducted in northeastern Brazil reported that a high prevalence of the combination of *H. pylori cagA* and *vacA* s1m1 genes was associated with a more severe gastric condition (Cavalcante et al., 2012). Other studies conducted in Venezuela, Cuba, and Colombia have also demonstrated an association between the combination of *cagA* and *vacA* and the risk of developing gastric diseases (Ortiz-Princz et al., 2010; Takahashi et al., 2013; Trujillo et al., 2014; Sallas et al., 2017). In the Moroccan population, *cagA*-positive strains were also the most prevalent type and were most highly associated with the *vacA* allelic subtype, s2m2 (Boukhris et al., 2012).

Studies have demonstrated that *H. pylori* strains with the allelic subtype s2m2 and s2m1 do not induce vacuolization in gastric epithelial cells (Foegeding et al., 2016; Yahiro et al., 2016). According to Molina-Castro et al. (2019), the presence of strains with the combination of *cagA* and *vacA* s1m1 increased the risks of atrophic gastritis and duodenal ulcer by 3.2 and 2.5 times, respectively. In this study, the *vacA* gene alone or in combination with *cagA* showed no association with severe diseases. This result can be explained by the limited analysis of allelic subtypes of the *vacA* gene.

It has been suggested that the detection of *dupA* is not sufficient to assess its role in gastric pathologies. In addition to its presence, it is necessary to evaluate the size of the gene product, as shorter forms of the protein are inactive (Trujillo et al., 2014). Analyses of the whole *dupA* gene cluster may provide a better understanding of its role in the pathogenesis of *H. pylori* infection (Jung et al., 2012).

The *dupA* gene appears to act as a protective factor against gastric cancer and may also be associated with an increased risk of duodenal ulcer (Shiota et al., 2010). The presence and activity of the *dupA* gene as well as its association with gastric diseases is poorly studied, especially in Brazil. Our results demonstrated a high frequency of *dupA,* although no association with the severity of gastric diseases was found.

All three genes were detected simultaneously in 34.7% of isolates in the present study, corroborating the results of Sallas et al. (2017), who detected a high frequency of the combination of *cagA*, *vacA*, *dupA,* and *oipA*. In this previous study, the combination of *cagA* and *dupA* was associated with a normal gastric mucosa. In contrast, in the present study, the most frequent combination in isolates from patients without gastric alterations was *cagA*, *vacA,* and *dupA* (40.0%). In the study by Pereira et al. (2014) with gastric samples from adults and children infected with *H. pylori*, the presence of the *dupA* gene was associated with *cagA*-positive strains, in addition, there was also an association between the *dupA* gene and the s1/m1 genotypes of the *vacA* gene.

The study provides the first demonstration that infectious strains in patients with severe diseases have fewer virulence genes when compared to isolates from patients with non-severe pathologies. There is evidence suggesting that an interaction between CagA and VacA can influence the severity of gastrointestinal disease. Studies have demonstrated an antagonistic interaction between CagA and VacA with respect to different biochemical signaling pathways and cellular morphologies. In addition, CagA can block the apoptotic activity of VacA (Bridge and Merrell, 2013). The pathogenic mechanism of DupA is still not well understood and interactions between this protein and other virulence factors have not been established. Therefore, we suggest that severe manifestations of gastrointestinal diseases result from interactions among virulence factors (Šterbenc et al., 2019).

According to Yamaoka (2010), *H. pylori* is a highly heterogeneous bacterium and its virulence varies geographically. Furthermore, geographical differences in the incidence of gastric cancer can be partially explained by the presence of different types of virulence factors such as CagA, VacA and OipA. In our study, the presence of the *oipA* gene in the infecting strain was not evaluated, some studies demonstrate that the functional status of this gene may be associated with the activity of the *cagA* and *vacA* genes (Sallas et al., 2010; Farzi et al., 2018). Additional studies of different genotypes of these virulence factors as well as proteomic and metabolomic analyses are necessary to better elucidate the mechanisms underlying different clinical outcomes.

CONCLUSIONS

Taken together, our results revealed a higher prevalence of the *cagA* gene, followed by *dupA* and *vacA*, demonstrating heterogeneity in the presence of these genotypes in local circulating strains. Simultaneous detection of the three genes was more prevalent in patients with non-severe pathologies, while the combination of *cagA* and *dupA* genes was more often detected in patients with severe pathologies. Our hypothesis is that combinations between the different virulence genes of *H. pylori* may be a trigger for the development of gastroduodenal diseases. Our findings reinforce the importance of understanding the correlation between *H. pylori* virulence and host susceptibility.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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