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From Gastritis to Gastric Cancer: The Importance of CagPAI of *Helicobacter Pylori* on the Development of Early and Advanced Gastric Adenocarcinoma

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Additional information is available at the end of the chapter

1. Introduction

The genus *Helicobacter* belongs to the ε subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. Members of this genus are all microaerophilic organisms and in most cases are catalase and oxidase positive, and many but not all species are also urease positive [1].

In 1984, Warren and Marshall isolated a bacterium from human stomachs in some patients with gastrointestinal disorders [2]. The organism was initially named "*Campylobacter-like organism*", "*Campylobacter pyloridis*" and "*Campylobacter pylori*", and, after more studies, it is named *Helicobacter pylori* (*H. pylori*) in recognition of the fact that this organism is distinct from members of the genus *Campylobacter* [3].

H. pylori is a spiral-shaped Gram-negative flagellate bacterium that colonizes the human stomach and can establish a long-term infection of the gastric mucosa. Since its isolation, its infection has been associated to the development of various gastrointestinal diseases, such as chronic gastritis, peptic ulcer disease, gastric MALT lymphoma and gastric cancer [1, 4-6]. Nowadays, it has also been related to some extradigestive diseases [7, 8], such as idiopathic thrombocytopenic purpura [9], iron deficiency anemia [10], and hepatobiliary diseases [11, 12], and amongst others.

The prevalence of *H. pylori* infection varies widely by geographical area, age, race, and socioeconomic status [13]. Because it is not possible to ascertain when infection occurs clinically, most of the information on the rates of *H. pylori* in geographically and demographically di-

verse populations comes from seroprevalence studies [14]. There have been numerous reports of a strong correlation between socioeconomic status and prevalence of infection. Generally, low prevalence occurs in industrialized countries and higher prevalence rates have been observed in underdeveloped and developing countries, possibly due to crowded households, habits and lack of sanitary facilities [15-17].

Even though its transmission pathways are not completely clarified [18], the infection appears to be usually acquired during childhood and is characterized as being chronic [19]. Routes of *H. pylori* transmission described include fecal-oral, oral-oral and gastric-oral. *H. pylori* appears to be transmitted most readily within families, possibly from parent to child and among siblings [20]. Evidence also supports child-to-child transmission among those in crowded school or in some living conditions, while transmission among adults is considered rare [21].

After colonization, all patients with *H. pylori* infection develop histological gastritis, which corresponds to classical chronic gastritis and is characterized by the infiltration of neutrophils and other inflammatory cells. However, most patients are asymptomatic for life, while only some will come to develop a digestive disease [22].

Colonization with *H. pylori* virtually leads to infiltration of the gastric mucosa in both antrum and corpus with neutrophilic and mononuclear cells. Gastritis can be classified as an acute or chronic gastritis and it can involve all parts of the stomach or just the fundus, corpus or antrum. The chronic active gastritis is the primary condition related to *H. pylori* colonization, and other *H. pylori*-associated disorders, in particular, resulting from this chronic inflammatory process [1], as atrophic gastritis (Figure 1) and intestinal metaplasia (Figure 2), causing an elevated risk of gastric adenocarcinoma, both intestinal type and diffuse type [23].
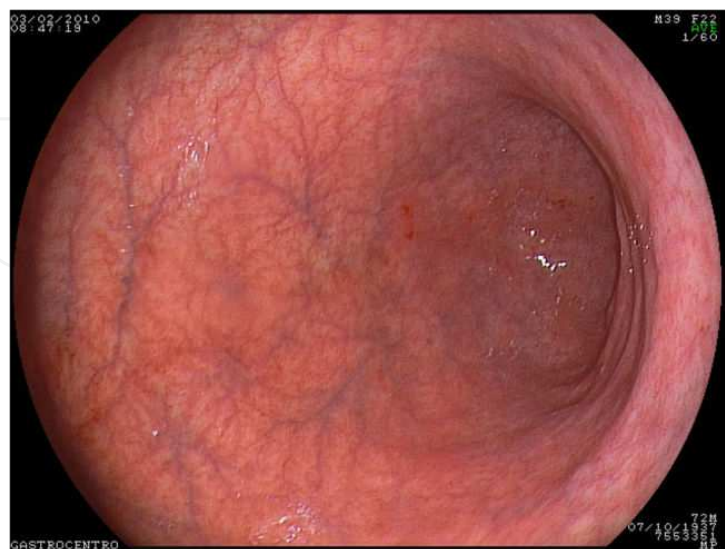

Figure 1. Atrophic gastritis(Copyright © Center of Diagnosis of Digestive Diseases, State University of Campinas, SP, Brazil. All rights reserved)
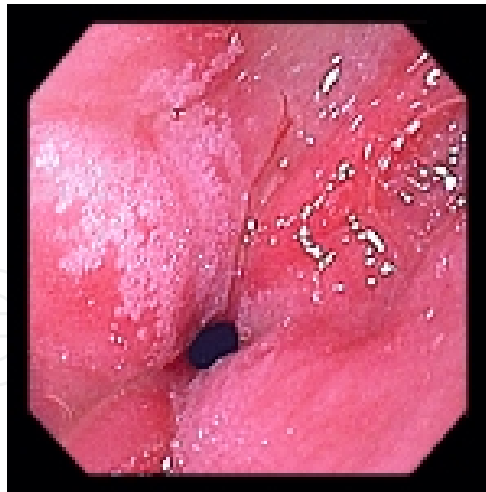
Figure 2. – Intestinal metaplasia, prepyloric antrum(Copyright© Atlanta South Gastroenterology, P.C. All rights reserved)

However, the propensity to develop disease is an aspect that remains unclear, but may depend on host characteristics, particular bacterial factors (virulence of the infecting strains), or to the specific interactions between host and microbe, besides the environmental factors [24].

In fact, the interest in *H. pylori* as a cause of gastric cancer began after the pioneering discoveries of Marshall and Warren [2]. Prior to the isolation of the organism, it was know that gastric adenocarcinomas typically arose in areas of gastritis. When the relationship between *H. pylori* and chronic gastritis was established, investigators began to take interest in the causal role of the bacterium in gastric cancer. Consequently, in 1994, the bacterium was classified as a group I carcinogen by the International Agency for Research on Cancer and is regarded as a primary factor for gastric cancer development [25].

The vast majority of gastric cancers are adenocarcinomas, which can be prevalently divided into two types, the intestinal and the diffuse [26], which corresponds, respectively, to the well-differentiated type and to the poorly-differentiated type, in the Japanese classification [22]. In contrast to the diffuse type often associated with familial distribution and developed in the stomach following chronic inflammation, especially in the cardia [27], intestinal type adenocarcinomas are generally thought to be preceded by a sequence of precursor lesions [28]. The basic components of this process are chronic inflammation of the gastric mucosa, which slowly progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia and dysplasia to gastric cancer [29] that are most frequently localized in the antrum [30].

Patients diagnosed in an early stage of the cancer (Figure 3) present an excellent prognosis, with a five-year survival rate greater than 90%. In cases with advanced lesions, gastric cancer carries a poor prognosis, with an overall five-year survival rate of less than 20% [31].
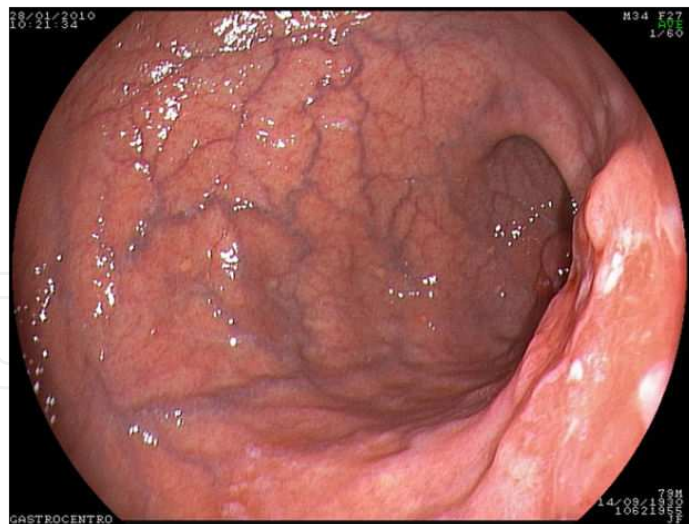
Figure 3. – Early gastric adenocarcinoma(Copyright © Center of Diagnosis of Digestive Diseases, State University of Campinas, SP, Brazil. All rights reserved)

With respect specifically to the bacterium strains, *H. pylori* has a high level of genetic diversity that represents an important factor in its adaptation to the host stomach and also for the clinical outcome of the infection [32,33]. Virulence factors of *Helicobacter pylori* are essential players in modulating the immune response involved in the initiation of carcinogenesis in the stomach. One category of the genes that are responsible for produce virulence factors is the strain-specific ones, which are present in only some *H. pylori* strains [34]. Among then, the best studied is the *cytotoxin-associated gene pathogenicity island* (cagPAI).

The cagPAI is a 40 kb region of chromosomal DNA encoding approximately 31 genes that forms a type IV secretion system and can be divided into two regions, cag I and cag II, according to a novel insertion sequence [35]. This secretion system forms a pilus that delivers CagA, an oncoprotein, into the cytosol of gastric epithelial cells through a rigid needle structure covered by CagY, a VirB10-homologous protein and CagT, a Virb7-homologous protein, at the base [36-38].

Upon delivery into host cells by the *cag* secretion system, the product of the terminal gene in the island, CagA, undergoes Src-dependent tyrosine phosphorylation and activates an eukaryotic phosphatase (SHP-2), leading to dephosphorylation of host cell proteins and cellular morphologic changes [39,40]. CagA has also been shown to dysregulate β-catenin signaling [41,42] and apical-junctional complexes [43], events that have been linked to increased cell motility and oncogenic transformation in a variety of models [44,45]. In addition, some studies have been reported that the cagPAI appears to be involved in the induction of gastric interleukin-8 (IL-8) production, a potent neutrophil-activating chemokine [46].

Consequently, the presence of the cagA gene has been associated with higher grades of inflammation, which may lead to the development of the most severe gastrointestinal diseases, such as peptic ulcer [47-49] and gastric cancer [50-54]. In Western countries, it has been reported that individuals infected with cagA-positive strains of *H. pylori* are at a higher risk

of peptic ulcer or gastric cancer than those infected with cagA-negative strains [34,55]. However, in East Asia, most strains of *H. pylori* have the cagA gene irrespective of the disease [56].

Furthermore, cagA is a polymorphic gene that presents different numbers of repeat sequences located in its 3' region. Each repeat region of the CagA protein contains Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, including a tyrosine phosphorylation site. The first repeat region is commonly named as EPIYA-A and EPIYA-B segments and the second repeat region is named EPIYA-C or EPIYA-D segments, for Western and East Asian strains, respectively [57]. Despite the know variability in the N-terminal cagA gene and other cagPAI genes, there has been limited information concerning clinical relevance of genetic variants outside the EPIYAs [58].

Together with cagA gene, cagT gene is found at the base of the outgrowing pilus of the type IV secretion system and is supposed to be responsible for binding to a cellular receptor to induce interleukine-8 secretion and eject CagA [37]. The cagT gene has also been associated with higher degrees of inflammation, being encountered in *H. pylori* strains from patients with early and advanced gastric adenocarcinoma [54].

Thus, considering each gene, there are two types of clinical *H. pylori* isolate, CagA-producing (cagA positive) strains and CagA-nonproducing (cagA negative) strains, and CagT-producing (cagT positive) strains and CagT-nonproducing (cagT negative) strains, genotypes that were considered in this study.

As gastric cancer still ranks as a leading cause of cancer-related deaths in many parts of the world, the aims of the present study were to investigate the presence of cagA and cagT genes in Brazilian patients with early and advanced distal type intestinal adenocarcinoma, in order to determine the general incidence of this gene in gastric cancer and to compare the results obtained between the two stages of the disease, trying to understand if the cagA and the cagT genes and/or the genotype cagAcagT could be important to the development of one or other stage of distal intestinal type gastric adenocarcinoma in Brazil.

2. Clinical samples, methods and results

2.1. Clinical Samples

The present study was carried out utilizing clinical samples of *H. pylori* obtained from 2005 January to 2009 January from the Laboratory of Pathology of the Center of Diagnosis of Digestive Diseases, Faculty of Medical Sciences, State University of Campinas (UNICAMP), São Paulo, Brazil. Eighty nine paraffin wax-embedded specimens of gastric tissue were analysed from a total of 89 patients, 31 from patients diagnosed with early distal type intestinal gastric adenocarcinoma (group one) and 58 from patients with advanced distal type intestinal gastric adenocarcinoma (group two). The mean ages of group one was 61.0 years, with 20 male cases (64.5%) and 11 female cases (35.5%). For group two the mean ages was 64.5 years, with 40 male cases (69.0%) and 18 female cases (31.0%). All the gastric tissue samples were obtained from endoscopic biopsy and had positive results for *H. pylori* by histological

analysis. *H. pylori* positive strains for cagA and cagT genes were used as a control for all the reactions performed in this study. All the stages of this study were approved by the Ethics Committee of the Faculty of Medical Sciences, State University of Campinas (UNICAMP), São Paulo, Brazil.

2.2. Methods

2.2.1. DNA extraction

Paraffin wax-embedded tissue DNA extraction was performed by carrying out the pre-extraction treatment of fixed tissues, using xylene and ethanol washes for paraffin removal. Subsequently, successive steps using proteinase K, phenol, chloroform and isoamyl alcohol were carried out, in order to isolate and purify the DNA [59]. Quantification of the extracted DNA and polymerase chain reaction (PCR) for human betaglobin gene [60] were carried out in order to guarantee the quality of this research.

2.2.2. PCR for urease C gene, cagA gene and cagT gene

Primers pairs for all the genes are described in Table 1, as well as the length of the fragments amplified for each reaction, urease C [61], cagA [62], and cagT [63]. PCR for urease C gene was performed with the aim to identify the bacterium DNA in samples.

After amplification, each PCR product was submitted to eletrophoresis on a 2.0% agarose gel stained by ethidium bromide with a 0.5X tris-acetate-EDTA buffer. A 100-bp ladder was used as standard.

Then, for each specific reaction, after being tested positive for urease C gene, products obtained were classified in cagA positive or negative and cagT positive or negative.

Gene	Strand	Primer sequence (5´-3´)	Length (bp)
betaglobin	+	ACA CAA CTG TGT TCA CTA GC	110
	-	CAA CTT CAT CCA CGT TTC ACC	
urease C	+	AAG CTT TTA GGG GTG TTA GGG GTT T	294
	-	AAG CTT ACT TTC TAA CAC TAA CGC	
cagA	+	GAT AAC AGG CAA GCT TTT GAG G	349
	-	CTG CAA AAG ATT GTT TGG CAG A	
cagT	+	CCA TGT TTA TAC GCC TGT GT	301
	-	CAT CAC CAC ACC CTT TTG AT	

Table 1. Sequence of synthetic oligonucleotide primers used to characterize *H. pylori* strains.

2.2.3. Statistical Analysis

After the amplification reactions, the results were analysed by a chi-square test at the Statistical Service of the Faculty of Medical Sciences, State University of Campinas (UNICAMP), with the *Statistical Analysis for Windows ®* (*SAS*) 9.1.3 (SAS Institute Inc, 2002-2003, Cary, NC, USA). Results were then related to the diseases in study, observing possible differences among H. pylori strains encountered in early and in advanced distal type intestinal gastric adenocarcinoma. Values of p<0.05 were considered to be statistically significant. The results as a whole, after comparison between the two groups, were depicted. Odd ratios with a confidence interval of 95% were also observed. Tables with absolute frequencies (n) and percentages (%) were made in order to determine genotypes combinations. Finally, Exact Fisher´s Test was used to compare the genotypes combinations between early and advanced gastric adenocarcinoma groups. Values of p<0.05 were considered to be statistically significant.

2.3. Results

PCR for the urease C gene of *H. pylori* was positive in all 89 samples, identifying the bacterium DNA (Table 2). Analyzing the results obtained for cagA gene, from patients with early gastric cancer, 61.3% (19 cases) were positive and from patients with advanced gastric cancer, 82.8% (48 cases) were positive, with a p = 0.025 (OR = 3.032; 95% CI = 1.123-8.185) (Table 2; Figure 4). In the group as a whole, there were 67 positive cases (75.3%) for the cagA gene.

As regards to cagT gene of *H. pylori*, from patients with early gastric adenocarcinoma, 54.8% (17 cases) were positive and, in patients with advanced gastric cancer, 65.5% (38 cases) were positive, with a p = 0.323 (OR = 1.565; 95% CI = 0.642-3.813). (Table 3; Figure 5) In the group as a whole, there were 55 positive cases (61.8%) for the cagT gene.

Classification	Early gastric cancer	Advanced gastric cancer	Total	p value
urease C	31 (100.0%)	58 (100.0%)	89 (100.0%)	
cagA positive	19 (61.3%)	48 (82.8%)	89 (100.0%)	0.025*
cagA negative	12 (38.7%)	10 (17.2%)		
cagT positive	17 (54.8%)	38 (65.5%)	89 (100.0%)	0.323
cagT negative	14 (45.2%)	20 (34.5%)		
Total	31 (100.0%)	58 (100.0%)	89 (100.0%)	

Table 2. General results obtained after PCRs for urease C, cagA and cagT genes of *H. pylori*. * cagA gene (p=0.025) OR=3.032, 95% CI = 1.123-8.185)
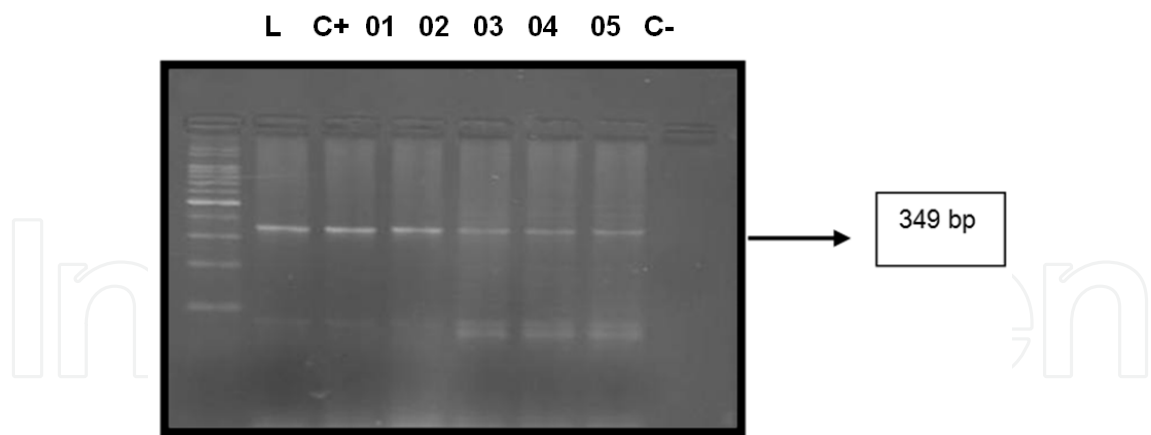
Figure 4. Amplification of cagA gene of *H. pylori* (349 bp). L, ladder 100 bp; C+, positive control; 01-05, positive samples for cagA gene; C-, negative control.
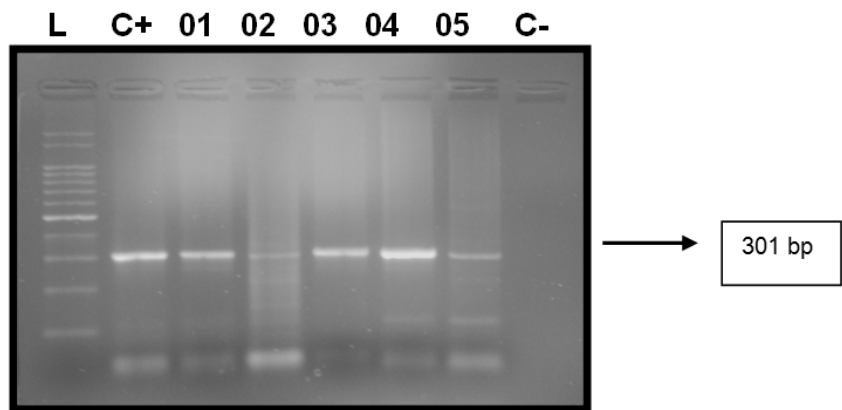

Figure 5. Amplification of cagT gene of *H. pylori* (301 bp). L, ladder 100 bp; C+, positive control; 01-05, positive samples for cagT gene; C-, negative control.

After the individual analysis, a table with absolute frequencies (n) and percentages (%) was made in order to compare the results individually for each gene and the genotypes combinations (Table 3; Table 4, respectively). There were 75.28% of positive cases for cagA gene and 61.80% of positive cases for cagT gene (table 3). As regards to cagAcagT, the most frequent genotype was cagA+cagT+, that was presented in 52.81% of the samples (table 4). The second most frequent genotype was cagA+cagT-, presented in 22.47% of the samples (table 4).

Subsequently, the combinations of genotypes were made and then compared between the two groups of patients, with early and advanced gastric adenocarcinoma. Although the most frequent genotype (cagA+cagT+) was the same in both groups of patiens, there was found a statistically significant difference between the groups, with cagA+cagT+ in advanced gastric cancer group (62.07%, 36 cases) and with cagA-cagT+ in early gastric cancer group (19.35%, 6 cases), p=0.027 (table 5).

Genes	Frequency (n)	Percentage (%)
cagA negative	22	24.72
cagA positive	67	75.28
cagT negative	34	38.20
cagT positive	55	61.80

Table 3. Descriptive analysis of categorical variables for total samples

cagA cagT combination	Frequency (n)	Percentage (%)
cagA cagT		
negative negative	14	15.73
negative positive	8	8.99
positive negative	20	22.47
positive positive	47	52.81

Table 4. Descriptive analysis of categorical variables for total samples according to genotype (cagAcagT)

cagAcagT	Advanced gastric cancer	Early Gastric cancer	Total
negative negative	8 13.79	6 19.35	14
negative positive	2 3.45	6 **19.35**	8
positive negative	12 20.69	8 25.81	20
positive positive	36 **62.07**	11 35.48	47
Total	58	31	89

Table 5. - Genotypes comparison between the two groups of patients, advanced and early gastric cancerp = 0.027

3. Conclusion

Gastritis is the most common illness associated to the stomach and can be considered as the beginning of different complication that may led to peptic ulcers disease and gastric adeno-carcinoma. Specifically concerning to gastric cancer, the understanding of the development of this disease has advanced considerably in recent decades, especially as regards to the role of *H. pylori* (the principal etiologic agent) in the progression of chronic gastritis in precancer-

ous lesions and cancer, changes that occur in the gastric mucosa in the development of the intestinal type gastric adenocarcinoma [30].

The risk of development of this disease is also related to genetic characteristics of the host and environmental factors, which, associated with specific bacterium strain characteristics, influence the severity of the chronic inflammatory response [64]. Thus, although infection with *H. pylori* almost always results in chronic active gastritis, many infected patients do not develop any complication, even those not showing clinical symptoms of infection [65]. This leads to the conclusion that some strains are more virulent than others [1], expressing, in different ways, specific bacterial products.

Other question that remains unclear, and that can be associated with these bacterium virulence factors, is why some tumors remain in the early stage and others are in advanced stage almost always in a short period of time? Are the different strains of *H. pylori* important to these changes? And, if the bacterium is eradicated, when this eradication is important? Is there a point of the development of the cancer when the eradication is not more important or when the bacterium presence does not make a difference?

In the present study we tried to understand some of these questions considering only one aspect of the development of the cancer, which were *H. pylori* strains presented in Brazilian patients with early and advanced distal type intestinal gastric adenocarcinoma. Considering the mentioned issues and a large number of studies that demonstrate that the cagPAI as one of the most important virulence factors of *H. pylori*, associated with higher grades of mucosa inflammation, we studied two genes that is in the cagPAI, the cagA and the cagT genes.

As regards to cagA gene, in an isolated analysis, our results were similar to those of in which strains cagA positive were related to the development of gastric cancer. cagA positive strains tend to be more virulent and induce higher levels of expression of cytokines such as interleukin 1b and 8 [66]. Some studies have shown that patients with strains that express CagA are three times more likely to develop gastric cancer (52,57,67] than those infected with cagA negative strains [56,68]. Besides, other study demonstrated that strains that express CagA are three times more likely to develop advanced gastric cancer than the early stage [54]. In the present study the same characteristics were observed and patients infected with cagA positive strains demonstrated a high risk of advanced gastric cancer development (p=0.025; OR=3.023, 95%CI). Besides, studies conducted in Western countries [69,70] and in Asian countries [71] reported that the most patients with gastric cancer are infected with *H. pylori* cagA positive strains.

One limitation for the comparisons of our results with others were that our study classified the two stages of the gastric cancer, the early and the advanced ones, and the most part of the studies consider only the gastric cancer disease, without classifying it in early or advanced stages. Besides, it was not our aim the study of the polymorphism of this gene, which are related to the EPIYA motifs, which can be important if we considerer the cagA gene in an isolated analysis, even though there has been limited information concerning clinical relevance of genetic variants outside the EPIYAs.

With our results, we can conclude that cagA gene is important for the development both of early and advanced gastric cancer, because it was presented in the most part of the strains in the two stages of the disease. Nevertheless, we can not conclude if it is important for the change of one stage in other (early in advanced stage) and we have also to consider that we do not really know if the bacterium presence is important or crucial after the beginning of the development of the disease. Some studies have demonstrated that the eradication of *H. pylori* when the precancerous lesions are present can be very important to prevent the cancer development, but, after this development beginning, we do not know if the regression of the early gastric cancer lesions can occur with the bacterium eradication.

Like the cagA gene, the cagT also belongs to the cagPAI and it is assumed that is related to the type IV secretion system, responsible for binding to cell receptors and inducing the release of interleukin-8 and also by ejecting the CagA protein [37]. The cagT gene has been linked to the development of peptic ulcer, and strains with the absence of this gene were generally related to chronic gastritis [63]. In this study, values were not found to be statistically significant between the two studied groups (p=0.323). However, the cagT gene was found, in the group as a whole, in 61.8% of the samples, which reflects an important result, showing that this gene, like cagA, or acting together with cagA, may be related to the gastric cancer development.

As regards to the comparison of the genotype cagAcagT, we reported again as a limitation for our study the fact that there are no studies that consider the early and the advanced stages of gastric cancer separately, so there were no relevant studies with which to compare the results from different stages of the same disease, gastric cancer. Further, we considered it unnecessary to make a comparison between these results and possible results from healthy individuals, because our aim was exactly to compare the strains presented in two stages of the same disease and not between the strains in the disease and in healthy volunteers.

For both groups, advanced and early, the most frequent genotype was cagA+cagT+ (62.07% and 35.48%, respectively). However, when comparison between the two groups was made, we found a statistically significance concerning the cagA+cagT+ and cagA-cagT+ strains (p=0.027). It can be considered very important because even though the genes cagA+ and cagT+ status were more frequent in both groups, the genotype cagA-cagT+ occurred with a higher frequency in the early gastric cancer group (advanced = 3.45%; early = 19.35%). Moreover, the genotype cagA+cagT+ occurred with a higher frequency in the advanced cancer group, a result that can be considered important too (advanced = 62.07%; early = 35.48%). We can suggest, with these results, that cagA gene positivity, independently of its polymorphisms, can be considered an essential virulence factor for the development of most severe gastric diseases, as gastric cancer.

Finally, as told before, the understanding of gastric carcinogenesis has advanced considerably over the past decades, especially with regards to insights into the role of *H. pylori* infection and the progression of chronic gastritis from premalignant stages to gastric cancer. Thereby, with the results obtained in the present study, cagA and cagT genes can be considered important *H. pylori* virulence factors implicated on the gastric cancer development.

Obviously, more studies are necessary to elucidate the *H. pylori* mechanisms of gastric tissue injury and we can also suggest that the identification of strains positive for cagA and cagT genes, besides others that are just considered virulence factors, can become very important and an useful tool to identify subjects most at risk for cancer, especially in places when this disease presents a higher incidence.

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