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Virulence Factors of *Helicobacter pylori* and Their Relationship with the Development of Early and Advanced Distal Intestinal Type Gastric Adenocarcinoma

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1. Introduction

The first isolation of *Helicobacter pylori* (*H. pylori*) in 1980s by Marshall and Warren (Marshall & Warren, 1984) ushered in a new era in gastric microbiology, and, in conjunction with increased interest in the pathogenesis of gastroduodenal diseases, has led to important breakthroughs in medical care (Dunn et al., 1997).

H. pylori is a spiral-shaped gram-negative flagellate bacterium that demonstrates bluntly rounded ends in gastric biopsy specimens (Goodwin et al., 1987). *H. pylori* organisms are 2.5 to 5.0 μm long and 0.5 to 1.0 μm wide, with four to six polar sheathed flagellas, which are essential for bacterial motility (Goodwin & Armstrong, 1990).

H. pylori was the first pathogenic microorganism that had its genome sequenced in two different strains. In 1997, *H. pylori* strain 26695 was firstly isolated from an English patient with chronic gastritis and then sequenced. It had a circular chromosome composed of 1.667.867 base pairs (Tomb et al., 1997). In 1999, strain J99 was isolated from an American patient with duodenal ulcer and then sequenced. This presented a similar, overall genomic organization, gene order and prediction of proteomes when compared to strain 26695 (Alm et al., 1999). In 2006, a chronic atrophic gastritis strain, HPAG1, was isolated and sequenced from a Swedish old patient (Oh et al., 2006). More recently, strain G27 was isolated from an Italian patient and had a similar size to the other three strains (Baltrus et al., 2009). Finally, other strains were sequenced, such as Shi470, B128 and 98-10 (McClain et al., 2009; Thiberge et al., 2010).

The finding of strain-specific genes from the comparison of the sequenced strains is in agreement with the earlier studies which demonstrated the high diversity of the *H. pylori* genome (Akopyanz et al., 1992; Han et al., 2003). Consequently, this high level of genetic diversity can be an important factor in its adaptation to the host stomach and also for the clinical outcome of the infection, an aspect that remains unclear. However, it is thought to involve an interplay between the virulence of the infecting strain, host genetics and environmental factors (Kabir, 2009) and experience with other bacterial pathogens suggests that *H. pylori*-specific factors may exist that influence the microorganism pathogenicity.

According to Yamaoka (2008), many putative virulence genes of *H. pylori* have been reported to determine clinical outcomes, and these are generally classified into three categories. The first one contains strain-specific genes, which are present in only some *H. pylori* strains. Among them, the best studied is the cag pathogenicity island (PAI), which encodes a bacterial type IV secretory apparatus (Censini et al., 1996). The second group is the phase-variable genes which the gene status can be changed during growth or in different conditions. Based on the comparison of the three first sequenced genomes of *H. pylori*, six genes encoding outer-membrane proteins (oipA, sabA, sabB, babB, babC and hopZ) are thought to undergo phase variation (Tomb et al., 1997; Alm et al., 1999; Oh et al., 2006). The last group of genes is with variable structures and genotypes depending on the strain, as example the vacA gene. Besides, the structure of many genes differs between Western strains and East Asian strains, and the structural differences in some genes are reported to influence virulence (Lu et al., 2007; Yamaoka, 2008). So, in this chapter, among virulence factors are described and analysed urease, vacA, cagA, cagT and dupA genes.

H. pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production (Harris et al., 1996). It appears to function as both a colonization factor and a virulence factor (Dun et al., 1997). The urease C gene, or glmM gene (Foxall et al., 1992), a particular region of the urease gene, is specific to *H. pylori* and can be used for primary identification of the microorganism (Moore et al., 1993). The urease gene can also be used to prove the heterogeneity of *H. pylori* strains. For instance, its heterogeneity can be seen by the diversity of patterns obtained by a restriction fragment length polymorphism (RFLP) of urease C and urease B genes, which is able to differentiate many strains presented in diverse pathologies, such as chronic gastritis and peptic ulcer, caused by this bacterium (Roesler et al., 2009).

Other of the major determinants of *H. pylori* virulence is VacA (vacuolating cytotoxin), a toxin that induces cytoplasmic vacuolation in gastric cells (Figura et al., 1989), membrane channel formation, apoptosis and immunomodulation (Atherton et al., 1995; Cover & Blanke, 2005). The vacA gene is present in all strains and comprises of two variable parts. The s-region encodes the signal peptide and is located at the 5´end of the gene which exists as an s1 or s2 allele, while the m-region (middle) occurs as an m1 or m2 allele (van Door et al. 1998). The production of vacuolating cytotoxin is related to the mosaic combination of s and m allelic types and has been associated to specific genotypes with different outcomes (Telford et al., 1994; van Door et al., 1998).

The cytotoxin-associated gene (cag) pathogenicity island (PAI) is approximately a 40-kb cluster of genes in the *H. pylori* chromosome and can be divided into two regions, cag I and cag II (Censini et al., 1996), according to a novel insertion sequence. This gene encodes a type IV secretion system, which delivers CagA 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 (Covacci & Rappuoli, 2000; Rohde et al., 2003). *H. pylori* cagA positive strains are associated with higher grades of inflammation and a significantly increased risk of developing gastric cancer (Parsonnet et al., 1997). 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 (Rohde et al., 2003).

Finally, the duodenal ulcer promoting gene (dupA) was the first putative disease specific marker whose association was described using *H. pylori* strains obtained in both Asian

(Japan and Korea) and Western (Colombia) regions (Lu et al., 2005). dupA gene is suggested to be a vir homologue and encompasses the sequences jhp0917 and jhp0918, as described in strain J99. This gene is associated with interleukin-8 induction (Schmidt et al., 2009) and is possibly part of a yet unidentified type IV secretion system (Lu et al., 2005). The original description of dupA reported that its presence was inversely related to mucosal atrophy and gastric cancer (Zhang et al., 2008, Yamaoka, 2008).

H. pylori is an universal distribution bacterium which affects more than half of the world population, and is considered an important public health problem. Even though its transmission pathways are not completely clarified (Rothenbacher et al., 1998), infection by *H. pylori* is usually acquired during childhood and is characterized as being chronic, with greater prevalence in developing countries in all ages (Kodaira et al., 2002).

Colonization with *H. pylori* is not actually a disease but a condition that affects the relative risk of developing various clinical disorders of the upper gastrointestinal tract (Kusters et al., 2006) and, possibly, extradigestive disorders (Gasbarrini et al., 1999; Figura et al., 2010). In the first case, *H. pylori* infection is responsible for the development of chronic gastritis, peptic ulcers, mucosa associated lymphoid tissue (MALT lymphoma) and gastric adenocarcinoma (Ahmed & Sechi, 2005). Regarding to extradigestive diseases, *H. pylori* infection has been associated with iron-deficiency anemia (Capurso et al., 2001), idiopathic thrombocytopenic purpura (Pellicano et al., 2009; Arnold et al., 2009), cardiovascular diseases (Franceschi et al., 2009), hepatobiliary diseases (Isaeva et al., 2009; Pirouz et al., 2009) and amongst others.

Especially with regards to gastric cancer, the interest in *H. pylori* as a cause of this illness began after the pioneering discoveries of Marshall and Warren. Prior to the discovery of the organism, it was known 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 (Herrera & Parsonnet, 2009). Therefore, on the basis of numerous epidemiological studies, *H. pylori* infection has been shown to be associated with an increased risk of gastric adenocarcinoma development (Parsonnet et al., 1997; Sugiyama & Asaka, 2004). Evidence that the presence of *H. pylori* increases the risk of developing gastric cancer through atrophy and intestinal metaplasia has also been reported (Asaka et al., 1994; Kuipers, 1995), suggesting that *H. pylori* positive patients developed these conditions in a greater proportion than control subjects. Besides, research conducted in animal models (Zhou et al., 2004) demonstrated that the eradication of the bacteria could reduce the incidence of gastric cancer in mice that developed the disease by inoculation of strains of *H. pylori* and administration of low doses of carcinogenic agents. These findings suggest that eradication of these bacteria during precancerous lesions of the disease significantly can reduce the incidence of gastric cancer also in humans (Sugiyama & Asaka, 2004). Consequently, in 1994 the International Agency for Research on Cancer, World Health Organization, concluded that *H. pylori* has a causal link with gastric carcinogenesis and was defined as type I carcinogen, a definite cause of cancer in humans (IARC, 1994).

Gastric cancer continues to be a major global health problem (Malfertheiner et al., 2010) and, despite the decreasing incidence and mortality rates observed worldwide over the last 50 years, it still ranks as a leading cause of cancer-related deaths in many parts of the world (Nardone et al., 2004). As symptoms are often absent or nonspecific in patients with the early stages of the disease, gastric cancer is usually diagnosed in an advanced stage, when curative options are limited. With exceptions in countries that have developed screening

programs for early diagnoses, most patients reach treatment with cancers already in advanced stages (Hohenberger & Gretschel, 2003). Consequently, gastric cancer carries a poor prognosis, with an overall five-year survival rate of less than 20% (Bowles & Benjamin, 2001).

The vast majority of gastric cancers are adenocarcinomas, which can be prevalently divided into two types, the intestinal and the diffuse (Lauren, 1965), which corresponds, respectively, to the well-differentiated type and to the poorly-differentiated type, in the Japanese classification (Sugiyama & Asaka, 2004). In contrast to the diffuse type (**Figure 1**), often associated with familial distribution and developed in the stomach following chronic inflammation, especially in the cardia (Nardone et al., 2004), intestinal type adenocarcinomas (**Figure 2**) are generally thought to be preceded by a sequence of precursor lesions (Correa, 1975). 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 (**Figure 3**) (Correa & Houghton, 2007). In respect to localization in the stomach, premalignant lesions are most frequently localized in the antrum in the transitional zone between the antrum and corpus (de Vries et al., 2007).

Unlike patients with advanced gastric cancer, patients diagnosed in an early stage of the disease present an excellent prognostic, in which a five-year survival rate is more than 90%. This disorder is defined as the adenocarcinoma that is confined to the mucosa or submucosa, irrespective of lymph-node invasion (Everett & Axon, 1998). Many early gastric cancers are believed to go through a life cycle consisting of ulcerations, followed by healing, then reulceration, and some tumours remain at this early stage for years even without treatment (Everett & Axon, 1998). Nevertheless, some early tumours rapidly became advanced and it is one of the principal questions concerning gastric carcinogenesis **(Figure 4).**

Fig. 1. Diffuse type adenocarcinoma
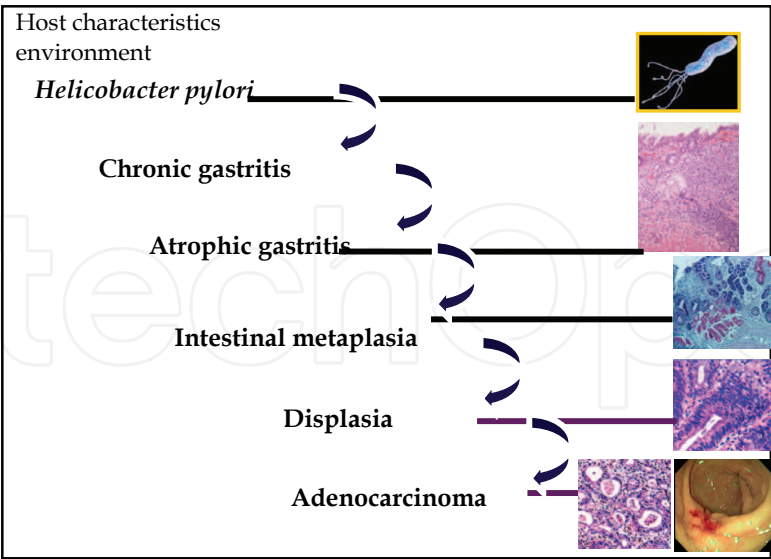

Fig. 2. Intestinal type adenocarcinoma

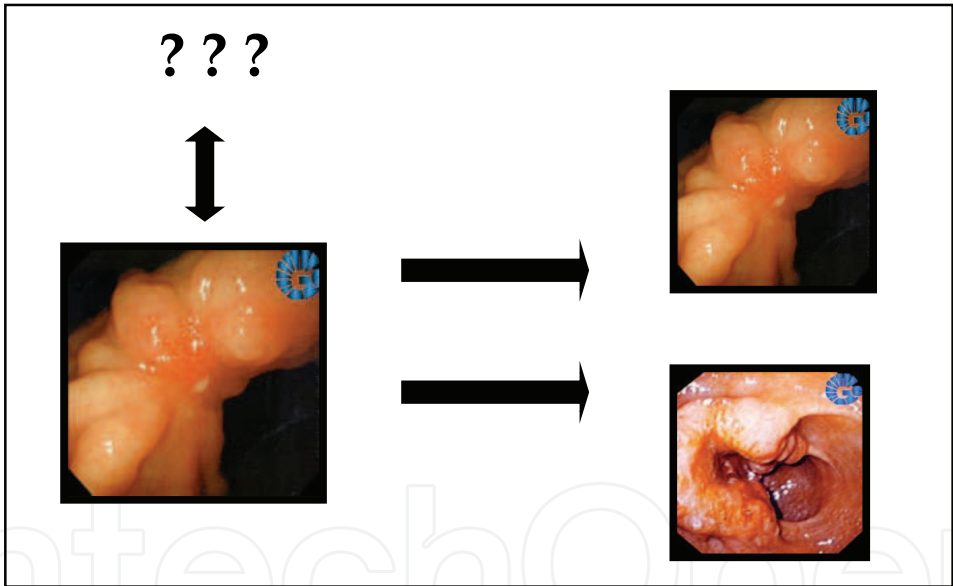Fig. 3. Sequence of precursor lesions of the intestinal type adenocarcinoma development

Fig. 4. Early gastric cancer: healing, maintenance or progression.

Have *H. pylori* virulence factors influenced this alteration? Why any tumour remains at an early stage for years even without treatment, or why they are able to heal by themselves, we do not know. Obviously, gastric carcinogenesis involves the interaction of the etiologic agent (*H. pylori*), the host characteristics and the external environment (Kabir, 2009), and, in this relationship, it should be considered the different strains of *H. pylori* and their virulence factors, important keys in the role of gastric carcinogenesis. Consequently, with the aim to try and understand better the mechanism of gastric carcinogenesis, especially concerning about the act of *H. pylori* , in which present studies have identified and analysed possible and essential differences among *H. pylori* strains derived from patients with early and advanced distal intestinal type gastric adenocarcinoma. Molecular biology techniques were

used in order to amplify genes of this bacterium that act as virulence factors, comparing the results obtained in the two severities of illness.

2. Clinical samples, methods and results

2.1 Clinical samples

Clinical isolates of *H. pylori* used in this study were obtained from the Laboratory of Pathology of the Centre 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 of these were diagnosed with early distal type intestinal gastric adenocarcinoma (group one) and 58 with advanced distal type intestinal gastric adenocarcinoma (group two). The mean ages of group one were 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. Samples from gastric tissues obtained by endoscopic biopsy of patients with chronic gastritis and peptic ulcers were used as a control for all the reactions performed in this study. Primer pairs were chosen in order to amplify final products with similar length (base pairs) and, with this aim, only for the cagA gene, we used the nested PCR. Finally, all the stages of this study were approved by the Ethics Committee of the Faculty of Medical Sciences, State University of Campinas (UNICAMP).

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 (Goelz et al., 1985). Quantification of the extracted DNA and polymerase chain reaction (PCR) for human betaglobin gene (Saiki et al., 1988) were carried out in order to guarantee the quality of this research.

2.2.2 PCR for urease C, vacA (regions s and m), cagA, cagT and dupA (regions jhp0917 and jhp0918) genes

Primer pairs for all the genes are described in **Table 1**, as well as the length of the fragments amplified for each reaction: urease C (Lage et al., 1995), vacA s1/s2 (Atherton et al., 1997), vacA m1/m2 (Atherton et al., 1999; Thomazini et al., 2006), cagA (Faundez et al., 2002), cagT (Mattar et al., 2007) and dupA jhp0917 and jhp0918 (Lu et al., 2005). 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% agarose gel stained by ethidium bromide with a 0.5 X 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 vacA s1m1, s2m1, s1m2 or s2m2, cagA positive or negative, cagT positive or negative, dupA jhp0917 positive or negative and dupA jhp0918 positive or negative; dupA gene was considered positive when its two regions were positive simultaneously.

Gene	Strand	Primer sequence (5´ - 3´)	Length (bp)
Betaglobin	+	ACACAACTGTGTTCACTAGC	110
	-	CAACTTCATCCACGTTTCACC	
Urease C	+	AAGCTTTTAGGGGGTGTTAGGGGTTT	294
	-	AAGCTTACTTTCTAACACTAACGC	
vacA s (s1/s2)	+	ATGGAAATACAACAAACACAC	S1,259; s2: 286
	-	CTGCTTGAATGCGCCAAAC	
vacA m1	+	GGTCAAAATGCGGTCATGG	290
	-	CCATTGGTACCTGTAGAAAC	
vacA m2	+	ATGCTTTAATATCGTTGAGA	198
	-	GAA CAT GTT TTA GTG AAA GC	
cagA (1st PCR)	+	GATAACAGGCAAGCTTTTGAGAGGGA	393
	-	CCATGAATTTTTGATCCGTTCGG	
cagA (nested)	+	GATAACAGGCAAGCTTTTGAGG	349
	-	CTGCAAAAGATTGTTTGGCAGA	
cagT	+	CCATGTTTATACGCCTGTGT	301
	-	CATCACCACACCCTTTTGAT	
dupA (jhp0917)	+	TGGTTTCTACTGACAGAGCGC	307
	-	AACACGCTGACAGGACAATCTCCC	
dupA (jhp0918)	+	CCTATATCGCTAACGCGCGCTC	276
	-	AAGCTGAAGCGTTTGTAACG	

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

2.2.3 Statistical analysis

After all amplification reactions, the results were analysed by a chi-square test at the Statistical Service of the Faculty of Medical Sciences at UNICAMP. 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. Then, Exact Fischer´s Test was used in order to compare the genotypes combinations between early and advanced gastric cancer. 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; Figure 5**). For the vacA gene, with regard to region s, of 31 samples from patients with early gastric cancer, 71.0% (22 cases) were s1 and 29.0% (9 cases) s2. Fifty eight samples from patients with advanced gastric cancer, 70.7% (41 cases) were s1 and 29.3% (17 cases) s2 (p = 0.978) (**Table 2; Figure 6**). In conclusion, 63 (70.8%) samples were diagnosed with vacA subtype s1 and 26 (29.2%) samples were with subtype vacA s2. Related to the vacA gene subunit m, no positive results were observed for the vacA gene subunit m2. From all 89 samples, 31 patients with early gastric cancer and 58 patients with

advanced gastric cancer were m1 positive (**Table 2; Figure 7**). Following this analysis, samples were classified into s1m1 or s2m1. For patients with early gastric cancer, 71.0% (22 cases) were s1m1 and 29.0% (9 cases) were s2m1. From 58 samples of patients with advanced gastric cancer, 70.7% (41 cases) were s1m1 and 29.3% (17 cases) s2m1 (table 3). Results were not statistically significant (p = 0.978). In conclusion, 63 (70.8%) samples were vacA s1m1 and 26 (29.2%) were vacA s2m1 (**Table 2**).

Analyzing the 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 (p = 0.025), with an odds ratio of 3.032, 95% confidence interval (1.123-8.185) **(Table 2; Figure 8)**. In the group as a whole, there were 67 positive cases (75.3%) for the cagA gene. On the other hand, analyzing the cagT gene of *H. pylori*, from patients with early gastric adenocarcinoma 54.8% (17 cases) were positive and in patients with advanced gastric adenocarcinoma, 65.5% (38 cases) were positive (p = 0.323) (**Table 2; Figure 9**). In the group there were 55 positive cases (61.8%) for the cagT gene.

Results of dupA jhp0917 in patients with early gastric cancer, 35.5% (11 cases) presented positive results and in patients with advanced gastric cancer, 39.7% (23 cases) presented positive (p = 0.700) (**Table 2; Figure 10**). In the group as a whole, there were 34 positive cases (38.2%) and 55 negative cases (61.8%) for jhp0917. Tests of dupA jhp0918 in patients with early gastric cancer, 51.6% (16 cases) had positive results and In patients with advanced gastric cancer, 55.2% (32 cases) were positive (p = 0.748) (**Table 2; Figure 11**). The p value obtained was 0.748, which was not statistically significant. In the group there were 48 cases (53.9%) that were positive for dupA jhp0918 gene and 41 cases (46.1%) that were negative.

Classification	Early gastric cancer	Advanced gastric cancer	Total	p value
urease C	31 (100.0%)	58 (100.0%)	89 (100.0%)	
s1	22 (71.0%)	41 (70.7%)	63 (70.8%)	
s2	9 (29.0%)	17 (29.3%)	26 (29.2%)	p = 0.978
m1	31 (100.0%)	58 (100.0%)	89 (100.0%)	
m2	0 (0.0%)	0 (0.0%)	0 (0.0%)	
s1m1	22 (71.0%)	41 (70.7%)	63 (70.8%)	
s2m1	9 (29.0%)	17 (29.3%)	26 (29.2%)	p = 0.978
cagA positive	19 (61.3%)	48 (82.8%)	67 (75.3%)	
cagA negative	12 (38.7%)	10 (17.2%)	22 (24.7%)	p = 0.025*
cagT positive	17 (54.8%)	38 (65.5%)	55 (61.8%)	
cagT negative	14 (45.2%)	20 (34.5%)	34 (38.2%)	p = 0.323
dupA jhp0917 positive	11 (35.5%)	23 (39.7%)	34 (38.2%)	
dupA jhp0917 negative	20 (64.5%)	35 (60.3%)	55 (61.8%)	P = 0.700
dupA jhp0918 positive	16 (51.6%)	32 (55.2%)	48 (53.9%)	
dupA jhp0918 negative	15 (48.4%)	26 (44.8%)	41 (46.1%)	P = 0.748
Total	31 (100.0%)	58 (100.0%)	89 (100.0%)	

* cagA gene (p = 0.025), OR = 3.032 (95% CI, 1.123-8.185)

Table 2. General results obtained after reactions for urease C, vacA (s/m), cagA, cagT and dupA jhp0917 and jhp0918 genes of *Helicobacter pylori*
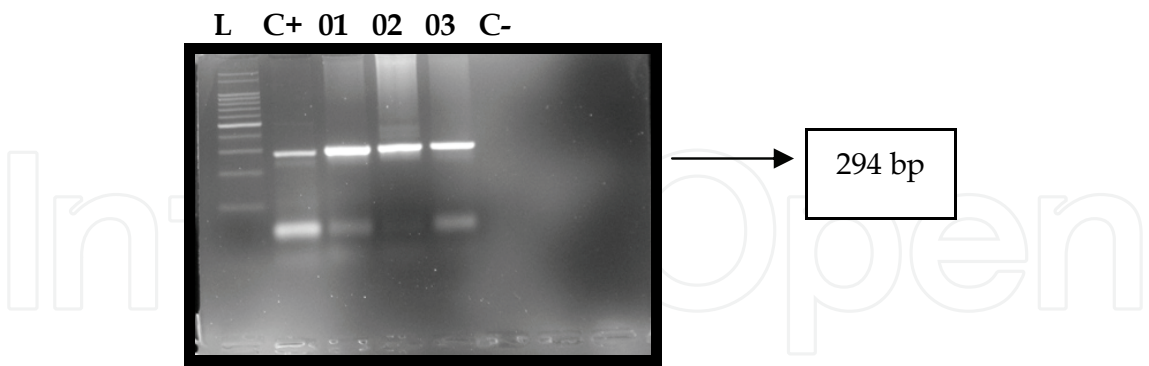
Fig. 5. Amplification of the urease C gene of *H. pylori* (294 bp); L, ladder 100 bp; C+, positive control; 01 to 03, positive samples; C-, negative control.
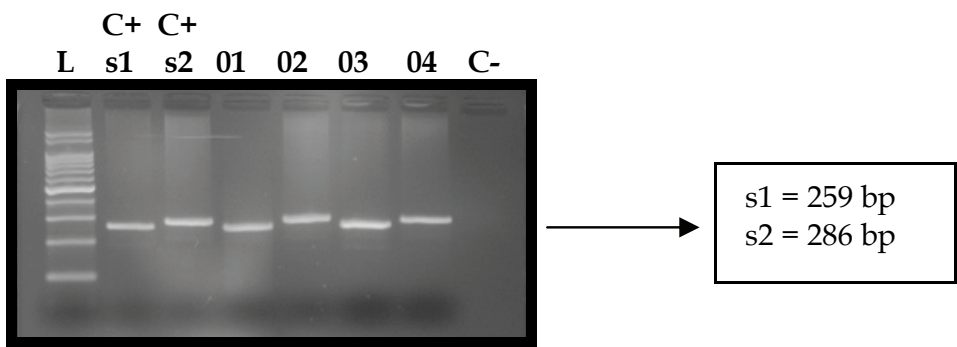

Fig. 6. Amplification of the vacA region s gene of *H. pylori* (s1, 259 bp; s2, 286 bp); L, ladder 100 bp; C+s1, positive control for vacA s1; C+s2, positive control for vacA s2; 01 and 03, positive samples for s1; 02 and 04, positive samples for s2; C-, negative control.
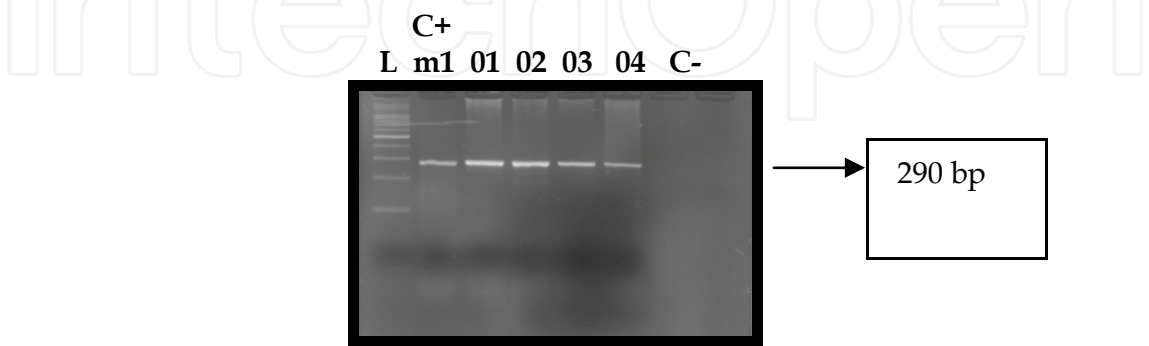

Fig. 7. Amplification of the vacA m1 region of *H. pylori* (290 bp). L, ladder 100 bp; C+ m1, positive control for vacA m1; 01 to 04, positive samples; C-, negative control.
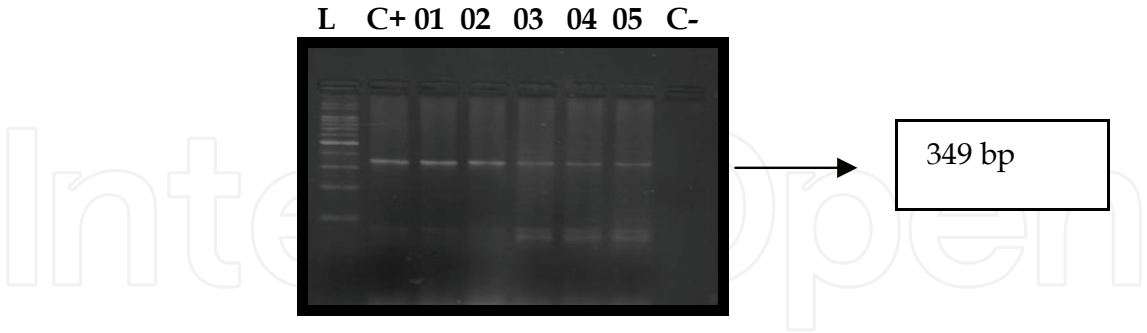
Fig. 8. Amplification of cagA gene of *H. pylori* (349 bp). L, ladder 100 bp; C+, positive control; 01 to 05, positive samples; C-, negative control.
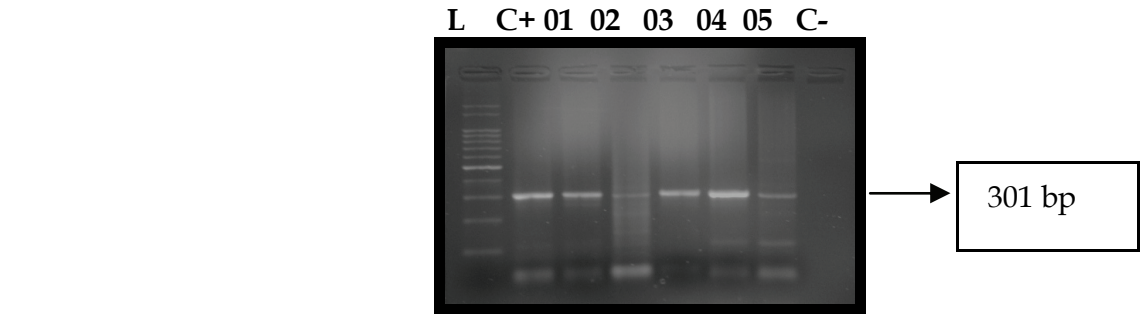

Fig. 9. Amplification of cagT gene of *H. pylori* (301 bp). L, ladder 100 bp; C+, positive control; 01 to 05, positive samples; C-, negative control.
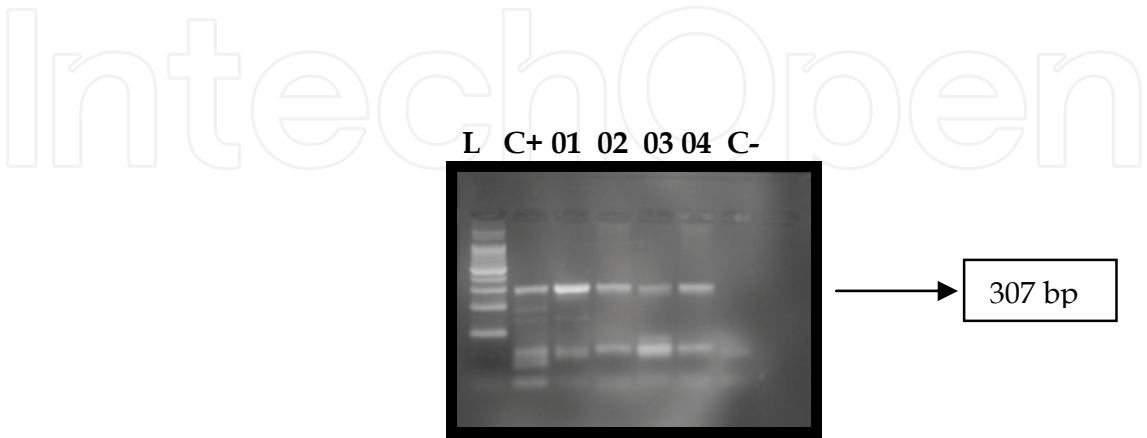

Fig. 10. Amplification of dupA jhp0917 gene of *H. pylori* (307 bp). L, ladder 100 bp; C+, positive control; 01 to 04, positive samples; C-, negative control.
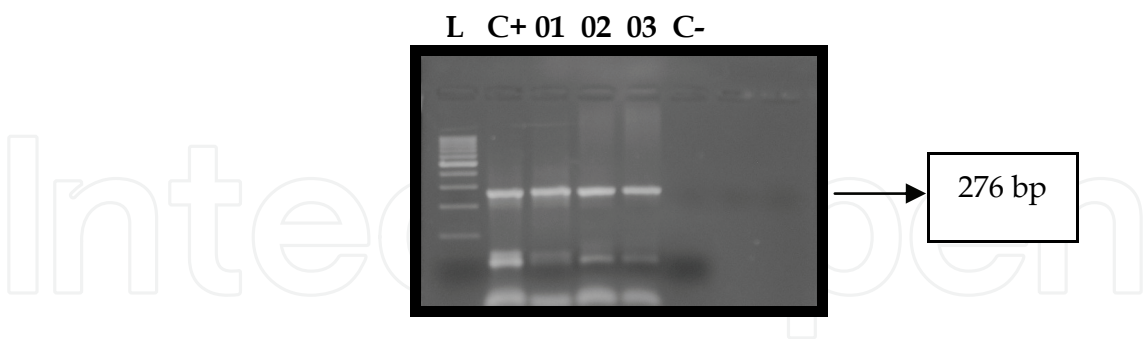
Fig. 11. Amplification of dupA jhp0918 gene of *H. pylori* (276 bp). L, ladder 100 bp; C+, positive control; 01 to 03, positive samples; C-, negative control.

After this analysis, tables with absolute frequencies (n) and percentages (%) were made in order to compare the genotypes combinations. vacA s1m1, cagA positive, cagT positive and dupA negative were, individually, the most frequent genotypes (**Table 3**). In order to specify better the results obtained, the dupA gene was considered positive when its two continuous regions (jhp0917 and jhp0918) were positive simultaneously. So, the combinations of genotypes were made and then compared between the two groups of patients, with early and advanced gastric cancer (**Tables 4 and 5, respectively**). The most prevalent genotype for all the samples, in both groups of patients, was vacA s1m1, cagA positive, cagT positive and dupA negative. The percentage was 29.21%. About the comparison of genotypes combinations between the two groups of samples, there was not a significant difference (p = 0.116).

Genomic region		Frequence (n)	Percentage (%)
vacA s/m gene	s1m1	63	70.79
	s2m1	26	29.21
cagA gene	positive	67	75.28
	negative	22	24.72
cagT gene	positive	55	61.80
	negative	34	38.20
dupA jhp0917 gene	positive	34	38.20
	negative	55	61.80
dupA jhp0918 gene	positive	48	53.93
	negative	41	46.07
dupA jhp0917/0918 gene	positive	28	31.46
	negative	61	68.54

Table 3. Descriptive analysis of genomic regions of *H. pylori*

Genotypes combinations: vacA s/m, cagA, cagT, dupA (jhp0917/0918)	Frequency (n)	Percentage (%)
s1m1 neg neg neg	8	8.99
s1m1 neg neg pos	1	1.12
s2m1 neg neg neg	3	3.37
s2m1 neg neg pos	2	2.25
s1m1 neg pos neg	3	3.37
s1m1 neg pos pos	1	1.12
s2m1 neg pos neg	2	2.25
s2m1 neg pos pos	2	2.25
s1m1 pos neg neg	9	10.11
s1m1 pos neg pos	6	6.74
s2m1 pos neg neg	4	4.49
s2m1 pos neg pos	1	1.12
s1m1 pos pos neg	26	29.21
s1m1 pos pos pos	9	10.11
s2m1 pos pos neg	6	6.74
s2m1 pos pos pos	6	6.74

Table 4. Genotype combinations, according to the results obtained for vacA s/m, cagA, cagT and dupA (jhp0917/0918) genes of *H. pylori*

Genotype combinations: vacA s/m, cagA, cagT, dupA (jhp0917/0918)	Early gastric câncer	Advanced gastric cancer	Total
s1m1 neg neg neg	4 12.90	4 6.90	8
s1m1 neg neg pos	1 3.23	0 0.00	1
s2m1 neg neg neg	1 3.23	2 3.45	3
s2m1 neg neg pos	0 0.00	2 3.45	2
s1m1 neg pos neg	2 6.45	1 1.72	3
s1m1 neg pos pos	1 3.23	0 0.00	1
s2m1 neg pos neg	2 6.45	0 0.00	2
s2m1 neg pos pos	1 3.23	1 1.72	2
s1m1 pos neg neg	3 9.68	6 10.34	9
s1m1 pos neg pos	2 6.45	4 6.90	6
s2m1 pos neg neg	2 6.45	2 3.45	4

Genotype combinations: vacA s/m, cagA, cagT, dupA (jhp0917/0918)	Early gastric câncer	Advanced gastric cancer	Total
s2m1 pos neg pos	1 3.23	0 0.00	1
s1m1 pos pos neg	5 16.13	21 36.21	26
s1m1 pos pos pos	4 12.90	5 8.62	9
s2m1 pos pos neg	2 6.45	4 6.90	6
s2m1 pos pos pos	0 0.00	6 10.34	6
Total	31	58	89

p = 0.116

Table 5. Comparison of genotypes combinations between early and advanced gastric cancer.

3. Conclusions

The understanding of gastric carcinogenesis has advanced considerably in recent decades, especially as regards to the role of *Helicobacter pylori* in the progression of chronic gastritis in precancerous lesions and cancer (de Vries et al., 2007). The risk of development of gastric cancer is also related to genetic characteristics of the host and environmental factors, which, associated with specific bacterial strain characteristics, influence the severity of the chronic inflammatory response (Peek et al., 1999). 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 (Blaser & Atherton, 2004). This leads to the conclusion that some strains are more virulent than others (Kusters et al., 2006), expressing, in different manners, specific bacterial products.

In the present study, we compared the *H. pylori* strains obtained from patients with early and advanced distal type intestinal gastric adenocarcinoma with the aim to identify possible differences among strains present in one or other case, using the polymerase chain reaction (PCR) for several bacterial genome regions. The primary detection of *H. pylori* in the samples was performed using the urease C region, present in all strains. After this, PCR was used for amplifying two genes from the cagPAI (cagA and cagT), the vacA gene (regions s and m) and the dupA gene (jhp0917/0918).

Strains with an s1-type signalling-sequence allele produce functional VacA toxin, whereas those with an s2-type signalling sequence have little cytotoxic activity (Peek & Blaser, 2002). Moreover, mosaic s1m1 strains are more toxic than s1m2 strains and are associated with more severe forms of gastritis, atrophy and intestinal metaplasia (Björkholm et al., 2003). The presence of s1 m1 vacA alleles is also strongly correlated with the expression of the cag-PAI (Covacci et al., 1993; Tummuru et al., 1993). Although located on a different loci of the *H. pylori* chromosome, cagA is strongly linked with VacA cytotoxic activity (Hocker & Hohenberger, 2003) and strains expressing the combination of these alleles and cag-PAI show enhanced epithelial cell injury (Ghiara et al., 1995). In our study, for patients with advanced gastric cancer, we found prevalent cagA positive (82.8%) and vacA s1m1 (70.7%) strains, corroborating the results of other studies (Miehlke et al., 2000; Leanza et al., 2004),

and demonstrating that this combination can result in more virulent strains. These results are also similar to those obtained in China (Qiao et al., 2003), where *H. pylori* strains were cagA positive in 95% of samples in the gastric cancer group and the expression of type s1 vacA was more than type s2. Nevertheless, with respect to type m, the expression of type m1 was equal to type m2, even in the gastric cancer group. In our study we did not find a positive result for m2.

With regards to early gastric cancer patients, unless we found strains with the same characteristics to those of advanced gastric cancer, some samples (cagA positive for 61.3% of samples and vacA s1m1 for 71.0% of samples), were positive for cagA and vacA s2m1 (29.0%), which may contribute for the maintenance of the early stage of disease. In addition, one possible explanation would be that the strains found in early lesions, in relation to the cagA gene, could be different which respects to their EPYIA motifs (polymorfisms of the cagA gene) (Nguyen et al., 2009; Truong et al., 2009), leading to the maintenance of early stage disease, without its progression to advanced lesions.

As regards to the cagA gene, in an isolate 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 (El-Omar et al., 2000). Some studies have shown that patients with strains that express CagA are three times more likely to develop gastric cancer [Parsonnet et al., 1997; Huang et al., 2003) than those infected with cagA negative strains (Husson et al., 1995; Yamaoka et al., 1999). 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 (OR = 3.032, 95%CI). Besides, studies conducted in Western countries (Crabtree et al., 1994; Blaser et al., 1995) and in Asian countries (Mizushima et al., 2001) reported that most patients with gastric cancer are infected with *H. pylori* cagA positive strains.

Like the cagA gene, the cagT also belongs to the cag pathogenicity island and it is assumed that it 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 (Rohde et al., 2003). 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 (Mattar et al., 2007). 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 a significant result, showing that this gene, like cagA, or acting together with cagA, may be related to gastric cancer development.

Finally, for the dupA gene, which was originally considered a marker for duodenal ulcer disease and a protective factor against gastric cancer in strains isolated from Japan, Korea and Colombia (Lu et al., 2005), we did not find any statistically significant difference between the two studied groups. Thus, although the dupA gene can not be considered as a marker for duodenal ulcer disease, the presence of this gene was found in few samples of patients with gastric cancer, suggesting that it is not an important gene for the development of this disease, by itself. Conversely, dupA genotyping of samples obtained from four populations (Argent et al., 2008), from Belgium, South Africa, China and the United States of America, showed no association of this gene with duodenal ulcer, but suggested an association with gastric cancer. Another study carried out in Brazilian patients (Gomes et al., 2008) also did not find any association between dupA and either of the two diseases. Finally, in India, a study demonstrated a significant association of dupA gene with duodenal ulcer

disease in the population (Arachchi et al., 2007). These different results demonstrate that further studies are required to elucidate the roles of dupA gene in the development of gastric diseases.

There are a number of studies that have investigated the virulence factors of *H. pylori* strains, but a gene marker for gastric cancer disease has not yet been found. Our study has demonstrated an interaction between some virulence factors found in gastric tissue samples obtained by early and advanced distal type intestinal gastric adenocarcinoma and a significant statistically value for cagA gene, which is more present in patients with advanced cancer. In the group gastric cancer as a whole, we found a prevalence of cagA positive, cagT positive, vacA s1m1 strains genotypes and a low number of dupA positive samples. Between the two groups, there were no statistically differences about the most prevalent combination of genotypes. In conclusion, the identification of a disease specific *H. pylori* virulence factor could be useful in clinical practice, helping to identify patients infected with strains that are able to develop a serious disease, such as gastric cancer.

4. Acknowledgment

5. References

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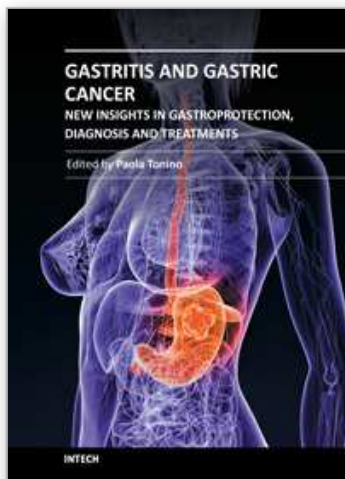
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