Dig Dis Sci (2015) 60:74–85
DOI 10.1007/s10620-014-3319-8

ORIGINAL ARTICLE

Correlation of *Helicobacter pylori* Genotypes with Gastric Histopathology in the Central Region of a South-European Country

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Received: 17 March 2014 / Accepted: 1 August 2014 / Published online: 21 August 2014

Abstract

Background Outcome of *Helicobacter pylori* (*H. pylori*) infection results from interaction of multiple variables including host, environmental and bacterial-associated virulence factors.

Aim This study aimed to investigate the correlation of *cagA*, *cagE*, *vacA*, *iceA* and *babA2* genotypes with gastric histopathology and disease phenotype in the central region of a South-European country.

Methods This prospective study involved 148 infected patients (110 female; mean age 43.5 ± 13.4 years) submitted to endoscopy with corpus and antrum biopsies. *H. pylori* was cultured and DNA extracted from the isolates. Genotypes were determined by PCR. Histopathological features were graded according to the updated Sydney system and OLGA/OLGIM classification. Only patients with single *H. pylori* genotypes and complete histopathological results were included.

Results Antrum samples presented higher degrees of atrophy, intestinal metaplasia, chronic inflammation and neutrophil activity. Genotype distribution was as follows: *cagA*-31.8 %; *cagE*-45.9 %; *vacA* s1a-24.3 %; *vacA* s1b-19.6 %; *vacA* s1c-0.7 %; *vacA* s2-55.4 %; *vacA* m1-20.9 %; *vacA* m2-79.1 %; *vacA* s1m1-18.9 %; *vacA* s1m2-25.7 %; *vacA* s2m1-2 %; *vacA* s2m2-53.4 %; *iceA1*-33.8 %; *iceA2*-66.2 %; *babA2*-12.2 %. *CagA* genotype was significantly associated with higher degrees of intestinal metaplasia, neutrophil activity, chronic inflammation and OLGIM stages. *BabA2* was linked with higher *H. pylori* density. Strains with *vacA* s1m1 or *vacA* s1m1 + *cagA* positive genotypes had a significant association with peptic ulcer and *vacA* s2m2 with iron-deficient anemia.

Conclusions *cagA*, *vacA* s1m1 and *babA2* genotypes are relatively rare in the central region of Portugal. *cagA*-positive strains are correlated with more severe histopathological modifications. This gene is commonly associated

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with *vacA* s1m1, and such isolates are frequently found in patients with peptic ulcer.

Keywords *Helicobacter pylori* · Genotype · Gastritis · Histopathology · *cagA* · *vacA*

Introduction

Helicobacter pylori (*H. pylori*) infection affects an estimated 50 % of the global population [1]. It is associated with multiple gastric pathologies, including gastritis, gastroduodenal ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [2]. Approximately, 84.2 % of Portuguese adults are infected by *H. pylori* [3]. However, there is not a direct correlation between the prevalence of infection and the prevalence of *H. pylori* related manifestations since only approximately 10–20 % of those infected develop clinical disease. This occurs because the severity of disease manifestations is influenced by multiple variables, including host genetic diversity, environmental factors and *H. pylori* genetic heterogeneity [4].

Several genes, such as *cagA*, *cagE*, *vacA*, *iceA*, and *babA2*, have been identified as markers for enhanced pathogenicity of *H. pylori*. The *cagA* gene (cytotoxin-associated gene) is a marker of the pathogenicity island gene (*cag*PAI) presence. Infection with *cagA*-positive strains is associated with a greater inflammatory response and an increased risk of adverse clinical outcomes including atrophic gastritis and gastric cancer [5, 6]. *cagE* is another marker gene for *cag*PAI and has also been associated with a more severe clinical outcome by stimulating the production of several cytokines from infected epithelial cells [7].

The vacuolating cytotoxin gene (*vacA*) is present in all *H. pylori* strains and comprises variable regions (signal-s, middle-m and intermediate-i). VacA protein is a potent cytotoxin that induces host cell vacuolation and, ultimately, cell death [8]. There are different allele variations for this gene, including for the signal (s1a, s1b, s1c, s2) and middle region (m1 and m2). The mosaic combination of these allelic types determines the level of cytotoxin produced and, eventually, the histopathological modifications and gastrointestinal manifestations. Strains with *vacA* s1 and m1 alleles produce a large amount of toxin and epithelial damage, whereas s2 and m2 genotypes produce little or no toxin [8, 9]. Most *vacA* s1 strains are *cagA*-positive, thus the two markers are closely related [9, 10].

The gene *iceA* (induced by contact with epithelium) has two main allelic variants, *iceA1* and *iceA2*. Expression of *iceA1* is upregulated on contact of *H. pylori* with human epithelial cells and may be linked with peptic ulcer disease [11].

The blood group antigen binding adhesin (BabA), a protein encoded by the *babA2* gene, binds to Lewis b antigens and ABO antigen [12]. There are three alleles for *babA* gene but only the *babA2* gene product is functionally active. The presence of this gene has been associated with gastric cancer [13].

The distribution of *H. pylori* genotypes and its correlation with histological modifications and disease outcomes presents geographic differences. The present study aimed to investigate whether there is any association of *cagA*, *cagE*, *vacA*, *iceA* and *babA2* genotypes with clinical presentation and gastric histological modifications in *H. pylori* infected patients from the central region of Portugal.

Patients and Methods

Patients

In this single-center study, consecutive patients with non-ulcer dyspepsia, peptic ulcer, iron-deficient anemia, indication for chronic therapy with proton pump inhibitors (PPI) and/or first-degree relatives with gastric carcinoma were prospectively considered for inclusion. All of them had a positive ^{13}C-urea breath test and indication for upper endoscopy. Exclusion criteria were: age <18 years; pregnancy; lactating and/or fertile women who were not using safe contraceptive methods; history of allergy/hypersensitivity to any antibiotic or PPI; previous gastric malignancy and/or gastric surgery; current use of anticoagulants; marked thrombocytopenia; systemic severe disease (hepatic, cardio-respiratory or renal disease; uncontrolled diabetes; active malignant diseases, coagulopathies); use of antibiotics in the last 4 weeks; use of PPI in the last 2 weeks.

Study Design

All patients were submitted to upper endoscopy with four biopsies in the antrum and four in the corpus, always including a biopsy from *incisura angularis*. The first two biopsies from the antrum and from the corpus were immediately placed in independent containers of adequate transport media—Portagerm pylori (bioMérieux Portugal, Linda-A-Velha, Portugal)—at 4 °C, and sent to microbiology laboratory. Urease test and Gram staining of a smear prepared from the biopsy specimen were performed to confirm the presence of *H. pylori*. After manual grinding with disposable material, the samples were distributed directly in agar pylori (bioMérieux Portugal, Linda-A-Velha, Portugal). Cultures were incubated for a minimum of 72 h and a maximum of 10 days at 37 °C under microaerophilic conditions, produced with H_2-CO_2-

generating packs (GENbox Microaer, bioMérieux Portugal, Linda-A-Velha, Portugal). *H. pylori* isolates were identified by colony morphology, characteristic spiral morphology on Gram staining, and positive catalase, urease and oxidase tests.

The other biopsy specimens, for histological studies, were fixed in 10 % formaldehyde. Formalin-fixed, paraffin-embedded tissue, after cutting and adequate deparaffination, was then submitted to traditional staining with hematoxylin + eosin, modified Giemsa and Warthin-Starry. Histological findings were described according to the modified Sydney criteria [14]. Two different pathologists, blinded to the genotype results, independently reviewed all slides and evaluated the following histopathological parameters: *H. pylori* density, chronic inflammation (mononuclear infiltration), polymorphonuclear activity (neutrophil activity), intestinal metaplasia and glandular atrophy. These items were scored on an ordinal scale: 0—absent; 1—mild; 2—moderate; 3—severe. Both pathologists discussed discrepant results and a final grading was established by consensus. For practical reasons, we divided glandular atrophy and intestinal metaplasia in two groups (0—absent; 1 to 3—present) and neutrophil activity, chronic inflammation and *H. pylori* colonization density also in two groups (0 + 1—absent or mild; 2 + 3—moderate or severe). Gastritis stage was also assessed according to the international Operative Link on Gastritis Assessment (OLGA) and Operative Link on Intestinal Metaplasia Assessment (OLGIM) [15–17].

Antrum and corpus samples were processed separately, and we excluded patients with incomplete histological results.

H. pylori Genotyping

DNA extraction from pure culture of *H. pylori* was performed with a special extraction kit (QIAamp® DNA Mini Kits, QIAGEN, Izasa Portugal, Carnaxide, Portugal) according to manufacturer's instructions.

The *cagA*, *cagE*, *vacA* (s1a, s1b, s1c, m1, m2), *iceA* (A1, A2), and *babA2* genotypes were determined with real-time PCR molecular technique by using specific primers selected from previously published works (Table 1) [9, 18–22]. For *cagA*, we used two sets of primers. A *cagA*-positive status was defined when *cagA* gene was detected by at least one of the two primer pairs.

PCR was performed in a volume of 20 µl containing 4.0 µl of LightCycler FastStart DNA MasterPLUS SYBR® Green I (Roche Diagnostics, Mannheim, Germany), 0.5 µM of each specific primers, 13.0 µl of H_20 PCR grade and 2.0 µl of DNA extract. An initial denaturation cycle at 95 °C for 10 min was performed in all cases followed by 45 amplification cycles. Specific denaturation, annealing and extension conditions for each set of primers are expressed in Table 1. PCR products were checked on 2 % agarose gels stained with ethidium bromide and transilluminated with UV light. Base-pair ladder size markers were added (E-Gel® Quantitative DNA Ladder, Invitrogen, Carlsbad, USA), and positive and negative controls were always included. Melting curves were also plotted automatically and analyzed with the LightCycler software. By comparing both results, it was possible to establish specific melting temperatures to identify each gene. To avoid any methodological error a different technician, blinded to previous results, repeated all procedures. Positive and negative controls were included.

Antrum and corpus specimens were processed separately. If multiple strains were identified or if it was not possible to determine *cagA*, *cagE*, *vacA*, iceA and/or *babA2* status the patient was excluded.

Statistical Analysis

The chi-square test and Fisher's exact test were used to assess the relationship between individual genotypes and the presence of neutrophil activity, chronic inflammation, colonization density, atrophy and intestinal metaplasia. The same tests were performed to evaluate the relationship between individual genotypes and clinical manifestations and/or reason for *H. pylori* eradication. For multiple comparisons involving the five genes, a Bonferroni correction was applied and only p values less than 0.01 were considered statistically significant.

For each gene, a comparison of histopathological scores was also performed with Mann–Whitney test. Paired sample Wilcoxon test was used to compare antrum and corpus scores.

The correlation of the ten histopathological phenotypes with clinical variables was also performed with the abovementioned tests, and the Bonferroni-adjusted p value was 0.005.

Logistic regression model was obtained for each histopathological phenotype using genotypes *cagA*, *cagE*, *vacA*, *iceA* and *babA2* as factors. Age, alcohol consumption, tobacco consumption, local of residence and gender were included as possible confounders. Results of logistic regression were expressed as odds ratios (OR) with 95 % confidence intervals (CI), and a p value less than 0.05 was accepted as statistically significant.

Data were analyzed in SPSS version 20.0 (IBM, Illinois, US).

Ethical Considerations

The study was approved by the ethical committee of our Hospital and the Faculty of Medicine and performed in

Table 1 Specific primers for detection of cagA, cagE, vacA, iceA and babA2 genes

Gene	Primer	Primer sequence (5′–3′)	PCR conditions	PCR product (bp)	Reference
cagA	A1	CCATGAATTTTTGATCCGTTCGG	95 °C, 10 s; 62 °C, 5 s; 72 °C, 16 s	394	[18]
	A2	GATAACAGGCAAGCTTTTGAGGGA			
	A3	ATGGGGAGTCATGATGGCATAGAACC	95 °C, 10 s; 62 °C, 5 s; 72 °C, 16 s	717	[18]
	A4	ATTAGGCAAATTAAAGACAGCCACC			
cagE	Forward	AGACATGCAAAAAGGTAT	95 °C, 10 s; 46 °C, 5 s; 72 °C, 37 s	900	[19]
	Reverse	CAATCTAGTGGGGTGGTA			
vacA s1/s2	Forward	ATGGAAATACAACAAACACAC	95 °C, 10 s; 53 °C, 5 s; 72 °C, 12 s	259 (s1) 286 (s2)	[9, 20]
	Reverse	CTGCTTGAATGCGCCAAAC			
vacA s1a	Forward	GTCAGCATCACACCGCAAC	95 °C, 10 s; 56 °C, 5 s; 72 °C, 8 s	190	[21]
	Reverse	CTGCTTGAATGCGCCAAAC			
vacA s1b	Forward	AGCGCCATACCGCAAGAG	95 °C, 10 s; 56 °C, 5 s; 72 °C, 8 s	187	[21]
	Reverse	CTGCTTGAATGCGCCAAAC			
vacA s1c	Forward	TTAGTTTCTCTCGCTTTAGTRGGGYT	95 °C, 10 s; 60 °C, 5 s; 72 °C, 9 s	220	[19]
	Reverse	CTGCTTGAATGCGCCAAAC			
vacA m1	Forward	GGTCAAAATGCGGTCATGG	95 °C, 10 s; 56 °C, 5 s; 72 °C, 12 s	290	[9, 22]
	Reverse	CCATTGGTACCTGTAGAAAC			
vacA m2	Forward	GGAGCCCCAGGAAACATTG	95 °C, 10 s; 54 °C, 5 s; 72 °C, 15 s	352	[9, 22]
	Reverse	CATAACTAGCGCCTTGCAC			
iceA1	Forward	GTGTTTTTAACCAAAGTATC	95 °C, 10 s; 51 °C, 5 s; 72 °C, 10 s	247	[20, 21]
	Reverse	CTATAGCCASTYTCTTTGCA			
iceA2	Forward	GTTGGGTATATCACAATTTAT	95 °C, 10 s; 51 °C, 5 s; 72 °C, 14 s	229 or 334	[20, 21]
	Reverse	TTRCCCTATTTTCTAGTAGGT			
babA2	Forward	AATCCAAAAAGGAGAAAAAGTATGAAA	95 °C, 10 s; 60 °C, 5 s; 72 °C, 34 s	832	[22]
	Reverse	TGTTAGTGATTTCGGTGTAGGACA			

accordance with the Declaration of Helsinki, the International Conference on Harmonisation Good Clinical Practice Guidelines, and applicable local laws and regulations. Signed informed consent was obtained from each patient.

Results

Epidemiology and Histopathology

A total of 148 patients with infection by a single strain of *H. pylori* and complete histological evaluation were included. Their mean age was 43.5 ± 13.4 years (range 18–78) and 101 (68.2 %) had less than 50 years. Thirty-eight (25.7 %) were male, 84 (56.8 %) lived in a rural area, 33 (22.3 %) and 24 (16.2 %) had history of alcohol and tobacco consumption, respectively. These patients were referred for *H. pylori* eradication for one or more of the following reasons: non-ulcer dyspepsia—65.5 %; chronic therapy with proton pump inhibitors—24.3 %; iron-deficient anemia—20.3 %; first-degree relatives with gastric cancer—18.9 %; peptic ulcer—8.1 %. Gastritis classification according to updated Sydney system is presented in Table 2. The average chronic inflammation, neutrophil activity, glandular atrophy and intestinal metaplasia scores in the antrum were significantly higher than in the corpus. That did not happen with *H. pylori* density scores. We found no cases of epithelial dysplasia.

Older individuals (≥50 years) presented higher degrees of neutrophil activity for body (OR 2.68; 95 % CI 1.24–5.79) and antrum (OR 2.98; 95 %; CI 1.39–6.38), atrophy for antrum (OR 5.13; 95 % CI 1.89–13.94) and OLGA stages (levels I–III: ≥50 years—31.9 % versus <50 years—6.9 %; $p = 0.001$). Residence in urban areas was significantly associated with greater chronic inflammation levels in the antrum (OR 3.04; 95 % CI 1.48–6.22). There was no correlation of histopathological findings with gender, history of former *H. pylori* eradication and alcohol/tobacco consumption.

Table 2 Classification of gastritis according to updated Sydney system

Classification/grade	Antrum (*n*)	Corpus (*n*)	*p*
Atrophy			**0.008**
0	128 (86.5 %)	144 (97.2 %)	
1	19 (12.8 %)	2 (1.4 %)	
2	1 (0.7 %)	1 (0.7 %)	
3	0 (0 %)	1 (0.7 %)	
Chronic inflammation			**<0.0001**
0	0 (0 %)	15 (10.1 %)	
1	101 (68.2 %)	120 (81.1 %)	
2	47 (31.8 %)	11 (7.4 %)	
3	0 (0 %)	2 (1.4 %)	
Neutrophil activity			**<0.0001**
0	3 (2.1 %)	28 (18.9 %)	
1	60 (40.5 %)	83 (56.1 %)	
2	65 (43.9 %)	31 (20.9 %)	
3	20 (13.5 %)	6 (4.1 %)	
Intestinal metaplasia			**0.005**
0	126 (85.1 %)	141 (95.2 %)	
1	18 (12.1 %)	5 (3.4 %)	
2	3 (2.1 %)	1 (0.7 %)	
3	1 (0.7 %)	1 (0.7 %)	
Density of *H. pylori* colonization			0.571
0	2 (1.4 %)	1 (0.7 %)	
1	106 (71.6 %)	111 (75 %)	
2	30 (20.3 %)	28 (18.9 %)	
3	10 (6.7 %)	8 (5.4 %)	

Bold values are statistically significant

Genotype Distribution

Genotype distribution according with real-time PCR is presented in Table 3. Twenty-four of the 28 isolates (85.7 %) with genotype *vacA* s1m1 were *cagA* positive, and this relation was statistically significant (OR 25.30; 95 % CI 7.99–80.07). An inverse relationship was found between *cagA* and *vacA* s2m2 (OR 0.06; 95 % CI 0.02–0.15). Thirteen of the 18 strains (72.2 %) positive for *babA2* also had *cagA* (OR 7.34; 95 % CI 2.44–22.12).

Correlation of Genotype with Clinical Manifestations, Histopathology and Epidemiological Factors

Correlation between specific genotypes and clinical manifestations/indication for *H. pylori* eradication is presented in Table 4.

When we compared epidemiological characteristics with *H. pylori* genotypes, we found a significant association of male sex with *vacA* m1 (OR 3.23; 95 % CI 1.39–7.14) and *vacA* s1m1 (OR 3.33; 95 % CI 1.39–7.69).

Table 3 Genotype distribution for all 148 *H. pylori* isolates

Genotype	*n* (%)
cagA positive	47 (31.8 %)
cagE positive	68 (45.9 %)
vacA s	
vacA s1	66 (44.6 %)
vacA s1a	36 (24.3 %)
vacA s1b	29 (19.6 %)
vacA s1c	1 (0.7 %)
vacA s2	82 (55.4 %)
vacA m	
vacA m1	31 (20.9 %)
vacA m2	117 (79.1 %)
vacA s + m	
vacA s1m1	28 (18.9 %)
vacA s1m2	38 (25.7 %)
vacA s2m1	3 (2 %)
vacA s2m2	79 (53.4 %)
iceA	
iceA1	50 (33.8 %)
iceA2	98 (66.2 %)
babA2	18 (12.2 %)

An overview of genotype distribution and its relationship with histopathological characteristics is presented in Tables 5 and 6. Distribution according to OLGA and OLGIM staging systems is presented in Table 7. For OLGA, there were no cases of patients in stages IV and the same happened for OLGIM in stage III so we suppressed such categories from the tables. Values for *vacA* m2 and *iceA2* are the opposite ones from *vacA* m1 and *iceA1*, and we also removed it from the tables. The same happened with *vacA* s1c since we had only one positive isolate with this s1 subtype.

Combined genotype *cagA* positive + *vacA* s1m1 was more common in patients with peptic ulcer (OR 6.56; 95 % CI 1.91–22.55), higher degrees of neutrophil activity in the body (OR 3.96; 95 % CI 1.59–9.86) and antrum (OR 4.54; 95 % CI 1.47–14.05).

Genotype *cagA* positive + *vacA* s1m1 + *babA2* positive occurred in 7 cases (4.7 %). It was significantly related with higher degrees of *H. pylori* colonization in the body (OR 22.20; 95 % CI 2.57–191.55) and antrum (OR 7.57; 95 % CI 1.41–40.78) and was more frequent in patients with peptic ulcer (OR 11.00; 95 % CI 2.13–56.83).

In a logistic regression analysis including epidemiological factors as potential confounders, we recognized the subsequent significant associations: presence of intestinal metaplasia in the antrum with *cagA* (OR 3.76; 95 % CI 1.26–11.24); higher levels of neutrophil activity in the antrum with *cagA* (OR 2.58; 95 % CI 1.12–5.95);

Table 4 Relationship of bacterial genotypes with clinical manifestations and/or other indications for *H. pylori* eradication

	Non-ulcer dyspepsia		Peptic ulcer		Iron-deficient anemia		GERD/Chronic therapy with PPI		First-degree relatives with gastric cancer	
	Present $n = 97$	Absent $n = 51$	Present $n = 12$	Absent $n = 136$	Present $n = 30$	Absent $n = 118$	Present $n = 36$	Absent $n = 112$	Present $n = 28$	Absent $n = 120$
cagA	35 (36.1 %)	12 (23.5 %)	7 (58.3 %)	40 (29.4 %)	**3 (10 %)**	**44 (37.3 %)***	9 (25 %)	38 (33.9 %)	7 (25 %)	40 (33.3 %)
cagE	46 (47.4 %)	22 (43.1 %)	7 (58.3 %)	61 (44.9 %)	11 (36.7 %)	57 (48.3 %)	18 (50 %)	50 (44.6 %)	15 (53.6 %)	53 (44.2 %)
vacA s1a	26 (26.8 %)	10 (19.6 %)	3 (25 %)	33 (24.3 %)	**2 (6.7 %)**	**34 (28.8 %)***	8 (22.2 %)	28 (25 %)	7 (25 %)	29 (24.2 %)
vacA s1b	21 (21.6 %)	8 (15.7 %)	6 (50 %)	23 (16.9 %)	4 (13.3 %)	25 (21.2 %)	6 (16.7 %)	23 (20.5 %)	9 (32.1 %)	20 (16.7 %)
vacA s2	49 (50.5 %)	33 (34.7 %)	3 (25 %)	89 (58.1 %)	**24 (80 %)**	**58 (49.2 %)***	22 (61.1 %)	60 (53.6 %)	12 (42.9 %)	70 (58.3 %)
vacA m1	24 (24.7 %)	7 (13.7 %)	6 (50 %)	25 (18.4 %)	2 (6.7 %)	29 (24.6 %)	3 (8.3 %)	28 (25 %)	6 (21.4 %)	25 (20.8 %)
vacA s1m1	21 (21.6 %)	7 (13.7 %)	**6 (50 %)**	**22 (16.2 %)***	2 (6.7 %)	26 (22 %)	3 (8.3 %)	25 (22.3 %)	6 (21.4 %)	22 (18.3 %)
vacA s1m2	27 (27.8 %)	11 (21.6 %)	3 (25 %)	35 (25.7 %)	4 (13.3 %)	34 (28.8 %)	11 (30.6 %)	27 (24.1 %)	10 (35.7 %)	28 (23.3 %)
vacA s2m1	3 (3.1 %)	0 (0 %)	0 (0 %)	3 (2.2 %)	0 (0 %)	3 (2.5 %)	0 (0 %)	3 (2.7 %)	0 (0 %)	3 (2.5 %)
vacA s2m2	46 (47.4 %)	33 (64.7 %)	3 (25 %)	76 (55.9 %)	**24 (80 %)**	**55 (46.6 %)***	22 (61.1 %)	57 (50.9 %)	12 (42.9 %)	67 (55.8 %)
iceA1	34 (35.1 %)	16 (31.4 %)	4 (33.3 %)	44 (33.8 %)	10 (33.3 %)	40 (33.9 %)	9 (25 %)	41 (36.6 %)	7 (25 %)	43 (35.8 %)
babA2	12 (12.4 %)	6 (11.8 %)	4 (33.3 %)	14 (10.3 %)	0 (0 %)	18 (15.3 %)	5 (13.9 %)	13 (11.6 %)	5 (17.9 %)	13 (10.8 %)

Bold values are statistically significant

GERD gastroesophageal reflux disease

* $p < 0.01$

Table 5 Association between *H. pylori* genotype and gastric body histopathology

Genotype	Glandular atrophy		Intestinal metaplasia		Neutrophil activity		Chronic inflammation		Colonization density	
	0 $n = 144$	1–3 $n = 4$	0 $n = 141$	1–3 $n = 7$	0–1 $n = 111$	2–3 $n = 37$	0–1 $n = 135$	2–3 $n = 13$	0–1 $n = 112$	2–3 $n = 36$
cagA	45 (31.2 %)	2 (50 %)	43 (30.5 %)	4 (57.1 %)	**28 (25.2 %)**	**19 (51.4 %)***	39 (28.9 %)	8 (61.5 %)	34 (30.4 %)	13 (36.1 %)
cagE	67 (46.5 %)	1 (25 %)	66 (46.8 %)	2 (28.6 %)	49 (44.1 %)	19 (51.4 %)	61 (45.2 %)	7 (53.8 %)	51 (45.5 %)	17 (47.2 %)
vacA s1a	35 (24.3 %)	1 (25 %)	34 (24.1 %)	2 (28.6 %)	25 (22.5 %)	11 (29.7 %)	32 (23.7 %)	4 (30.8 %)	27 (24.1 %)	9 (25 %)
vacA s1b	27 (18.8 %)	2 (50 %)	26 (18.4 %)	3 (42.9 %)	17 (15.3 %)	12 (32.4 %)	25 (18.5 %)	4 (30.8 %)	22 (19.6 %)	7 (19.4 %)
vacA s2	81 (56.2 %)	1 (25 %)	80 (56.7 %)	2 (28.6 %)	68 (61.3 %)	14 (37.8 %)	77 (57 %)	5 (38.5 %)	62 (55.4 %)	20 (55.6 %)
vacA m1	30 (20.8 %)	1 (50 %)	28 (19.9 %)	3 (42.9 %)	19 (17.1 %)	12 (32.4 %)	26 (19.3 %)	5 (38.5 %)	22 (19.6 %)	9 (25 %)
vacA s1m1	27 (18.8 %)	1 (25 %)	25 (17.7 %)	3 (42.9 %)	16 (14.4 %)	12 (32.4 %)	23 (17 %)	5 (38.5 %)	20 (17.9 %)	8 (22.2 %)
vacA s1m2	36 (35 %)	2 (50 %)	36 (25.5 %)	2 (28.6 %)	27 (24.3 %)	11 (29.7 %)	35 (25.9 %)	3 (23.1 %)	30 (26.8 %)	8 (22.2 %)
vacA s2m1	3 (2.1 %)	0 (0 %)	3 (2.1 %)	0 (0 %)	3 (2.7 %)	0 (0 %)	3 (2.2 %)	0 (0 %)	2 (1.8 %)	1 (2.8 %)
vacA s2m2	78 (54.2 %)	1 (25 %)	77 (54.6 %)	2 (28.6 %)	65 (58.6 %)	14 (37.8 %)	74 (54.8 %)	5 (38.5 %)	60 (53.6 %)	19 (52.8 %)
iceA1	48 (33.3 %)	2 (50 %)	47 (33.3 %)	3 (42.9 %)	38 (34.2 %)	12 (32.4 %)	47 (34.8 %)	3 (23.1 %)	36 (32.1 %)	14 (38.9 %)
babA2	18 (12.5 %)	0 (0 %)	17 (12.1 %)	1 (14.3 %)	12 (10.8 %)	6 (16.2 %)	16 (11.9 %)	2 (15.4 %)	**9 (8 %)**	**9 (25 %)***

Bold values are statistically significant

* $p < 0.01$

Table 6 Association between *H. pylori* genotype and gastric antrum histopathology

Genotype	Glandular atrophy		Intestinal metaplasia		Neutrophil activity		Chronic inflammation		Colonization density	
	0 $n = 128$	1–3 $n = 20$	0 $n = 126$	1–3 $n = 22$	0–1 $n = 63$	2–3 $n = 85$	0–1 $n = 101$	2–3 $n = 47$	0–1 $n = 108$	2–3 $n = 40$
cagA	39 (30.5 %)	8 (40 %)	35 (27.8 %)	12 (54.5 %)	13 (20.6 %)	34 (40 %)	31 (30.7 %)	16 (34 %)	32 (29.6 %)	15 (37.5 %)
cagE	59 (46.1 %)	9 (45 %)	56 (44.4 %)	12 (54.5 %)	24 (38.1 %)	44 (51.8 %)	45 (44.6 %)	23 (48.9 %)	47 (43.5 %)	21 (52.5 %)
vacA s1a	31 (24.2 %)	5 (25 %)	27 (21.4 %)	9 (40.9 %)	17 (27 %)	19 (22.4 %)	23 (22.8 %)	13 (27.7 %)	24 (22.2 %)	12 (30 %)
vacA s1b	23 (18 %)	6 (30 %)	24 (19 %)	5 (22.7 %)	7 (11.1 %)	22 (25.9 %)	19 (18.8 %)	10 (21.3 %)	21 (19.4 %)	8 (20 %)
vacA s2	73 (57 %)	9 (45 %)	74 (58.7 %)	8 (36.4 %)	39 (61.9 %)	43 (50.6 %)	58 (57.4 %)	24 (51.1 %)	62 (57.4 %)	20 (50 %)
vacA m1	27 (21.1 %)	4 (20 %)	27 (21.4 %)	4 (18.2 %)	10 (15.9 %)	21 (24.7 %)	18 (17.8 %)	13 (27.7 %)	22 (20.4 %)	9 (22.5 %)
vacA s1m1	24 (18.8 %)	4 (20 %)	24 (19 %)	4 (18.2 %)	8 (12.7 %)	20 (23.5 %)	16 (15.8 %)	12 (25.5 %)	20 (18.5 %)	8 (20 %)
vacA s1m2	31 (24.2 %)	7 (35 %)	28 (22.2 %)	10 (45.5 %)	16 (25.4 %)	22 (25.9 %)	27 (26.7 %)	11 (23.4 %)	26 (24.1 %)	12 (30 %)
vacA s2m1	3 (2.3 %)	0 (0 %)	3 (2.4 %)	0 (0 %)	2 (3.2 %)	1 (1.2 %)	2 (2 %)	1 (2.1 %)	2 (1.9 %)	1 (2.5 %)
vacA s2m2	70 (54.7 %)	9 (45 %)	71 (56.3 %)	8 (36.4 %)	37 (58.7 %)	42 (49.4 %)	56 (55.4 %)	23 (48.9 %)	60 (55.6 %)	19 (47.5 %)
iceA1	40 (31.2 %)	10 (50 %)	42 (33.3 %)	8 (36.4 %)	23 (36.5 %)	27 (31.8 %)	32 (31.7 %)	18 (38.3 %)	37 (34.3 %)	13 (32.5 %)
babA2	15 (11.7 %)	3 (15 %)	14 (11.1 %)	4 (18.2 %)	10 (15.9 %)	8 (9.4 %)	13 (12.9 %)	5 (10.6 %)	11 (10.2 %)	7 (17.5 %)

* $p < 0.01$

advanced levels of chronic inflammation in the body with *cagA* (OR 4.61; 95 % CI 1.37–15.63); higher *H. pylori* density scores in the body with *babA2* (OR 7.46; 95 % CI 2.15–26.32).

Discussion

Although *H pylori* affects approximately 50 % of all humans only a minority develop manifestations associated with this infection. *H pylori* exceptional genetic variability and intraspecies diversity contributes to this discrepancy [23]. There is a geographic variability of *H. pylori* genotypes [20, 24]. Patients infected with more virulent strains have higher probabilities of developing complications [5]. Conclusions about the relation between *H. pylori* genotypes and different clinical outcomes, if derived from a single geographic region, may not be true for other geographic locations [19]. It is important to determine the most prevalent genotypes in a specific region and to confirm whether there is a correlation between putative virulence genes and histological markers of severity. In areas of high *H. pylori* infection prevalence, this information may potentially help us to predict whether there is an increased risk of gastric adenocarcinoma.

To our knowledge, this paper presents the largest characterization to date of *H. pylori* isolates and associated histopathological modifications collected from persons living in the central region of Portugal. Our study population is rather young but, even so, we demonstrated that glandular atrophy, neutrophil activity, and OLGA stages were significantly higher in older patients. Infection generally occurs during childhood and development of more severe histopathological manifestations, such as intestinal metaplasia and glandular atrophy, are linked to prolonged infection and consequently to patients' age [25, 26]. A study performed in the northern part of the country also revealed that patients with atrophic gastritis were significantly older than the ones with no atrophy [5].

A remarkable result was the higher degrees of intestinal metaplasia, glandular atrophy, neutrophil activity and chronic inflammation in the antrum comparatively to the body confirming that *H. pylori* infection in our patients induces more histological changes in the antrum. According to the classification of chronic gastritis, antral predominant non-atrophic gastritis was the dominant pattern in our cohort [17]. Most patients affected by this condition have only a minimally increased risk of adenocarcinoma of the distal stomach when compared with uninfected individuals [17, 27].

One of the most important virulence factors of *H. pylori* is *cagA* that is present in about 60–70 % of the Western strains and in more than 90 % of the East Asian ones [7,

Table 7 Relationship between *H. pylori* genotype and OLGA/OLGIM staging systems

Genotype (%)		*n*	OLGA				*p*	OLGIM				*p*
			0	I	II	III		0	I	II	IV	
cagA	+	47	83	10.6	4.3	2.1	NS	68.1	21.3	8.5	2.1	**0.001**
	−	101	86.1	13.9	0	0		88.1	11.9	0	0	
cagE	+	68	85.3	14.7	0	0	NS	83.8	12.5	2.5	1.2	NS
	−	80	85.1	11.2	2.5	1.2		79.4	17.6	3	0	
vacA s1a	+	36	86.1	11.1	2.8	0	NS	72.3	19.4	8.3	0	NS
	−	112	84.8	13.4	0.9	0.9		84.8	13.4	0.9	0.9	
vacA s1b	+	29	75.9	17.3	3.4	3.4	NS	75.9	17.3	3.4	3.4	NS
	−	119	87.4	11.8	0.8	0		83.2	14.3	2.5	0	
vacA s2	+	82	87.8	12.2	0	0	NS	87.8	12.2	0	0	NS
	−	66	81.8	13.6	3.1	1.5		74.2	18.2	6.1	1.5	
vacA m1	+	31	87.1	9.7	3.2	0	NS	77.4	19.4	3.2	0	NS
	−	115	84.6	13.6	0.9	0.9		82.8	13.7	2.6	0.9	
vacA s1m1	+	28	85.7	10.7	3.6	0	NS	75	21.4	3.6	0	NS
	−	120	85.1	13.3	0.8	0.8		83.3	13.4	2.5	0.8	
vacA s1m2	+	38	79	15.8	2.6	2.6	NS	73.7	15.8	7.9	2.6	NS
	−	110	87.3	11.8	0.9	0		84.5	14.5	1	0	
vacA s2m1	+	3	100	0	0	0	NS	100	0	0	0	NS
	−	145	84.8	13.1	1.4	0.7		81.4	15.2	2.8	0.7	
vacA s2m2	+	79	87.3	12.7	0	0	NS	87.3	12.7	0	0	NS
	−	69	82.6	13	2.9	1.4		75.4	17.4	5.8	1.4	
iceA1	+	50	80	14	4	2	NS	82	12	4	2	NS
	−	98	87.8	12.2	0	0		81.6	16.3	2	0	
babA2	+	18	83.3	11.1	5.6	0	NS	72.2	16.7	11.1	0	NS
	−	130	85.4	13.1	0.8	0.8		83.1	14.6	1.5	0.8	

Bold value is statistically significant

NS ($p > 0.01$)

28, 29]. In our study *cagA* genotype, albeit determined by real-time PCR using two different sets of primers, was identified in only 31.8 % of isolates. This is considerably less than it was previously published for our country [5, 30]. There are different possible explanations for this finding. First, *cagA* is more common in *H. pylori* infection associated with peptic ulcer or gastric carcinoma. In our study, 65.5 % of patients had non-ulcer dyspepsia and only 8.1 % had peptic ulcer. There were no cases of gastric carcinoma. Van Doorn et al. [24] also presented a prevalence of *cagA*-positive strains of only 35.7 % in Egypt, where most of the isolates were from non-ulcer patients. Another possible explanation is that there is a national and regional heterogeneity in distribution of *H. pylori* genotypes and in the Central region of Portugal *cagA* negative strains might be more common. Some authors already stated that there are regional bacterial populations with particular attributes making it difficult to establish universal virulence markers [31]. A recent study performed in a region with one of the highest incidence and mortality from gastric carcinoma in Spain and Western Europe revealed a low prevalence of *cagA*-positive strains (47.7 % of infected patients) [32]. Another recent study, from a different Mediterranean country, also revealed a low prevalence of *cagA*-positive strains (42.3 %) [33]. In a Jordanian study, only 26.9 % of *H. pylori* isolates were *cagA* positive [34]. However, albeit relatively rare in our study population, *cagA* maintained a significant relation with *vacA* s1m1, as already stated by multiple articles [10, 32, 33, 35]. Although *cagA* was found in only 31.8 % of isolates, *cag*PAI was more frequent since *cagE* was present in 45.9 %. A recent study, from Brazil, also reported higher prevalence of *cagE* comparatively to *cagA* [31]. *H. pylori* microevolution can determine loss of all or part of the *cag*PAI [23]. In Japanese and French studies, *cagE* was found to be a better marker for the presence of *cag*PAI than *cagA* [36, 37]. We did not perform detection of *cag*PAI empty site, and this is a limitation of our work. It is interesting to notice that a recent study, in Portuguese children, demonstrated a low prevalence (16.7 %) of *cag*-PAI in patients with non-ulcer dyspepsia comparatively to patients with peptic ulcer disease (75.4 %) [38].

In European and North American populations, *cagA* has been associated with more severe disease such as gastric mucosal atrophy, intestinal metaplasia and gastric cancer [6, 10, 39, 40]. We think the same is valid for the central

region of Portugal since *cagA*-positive status was independently associated with presence of intestinal metaplasia in the antrum, higher degrees of neutrophil activity in the antrum and chronic inflammation in the body as well as higher OLGIM stages. So, this genotype, although rare in our population, was the most frequently related with worrisome histopathological features.

Gene *cagE* is also within the cag PAI and has been associated with a more severe clinical outcome [7]. In the central region of Portugal, *cagE* is more prevalent than *cagA* and has no relation with clinical presentation or gastric histopathology. In our population, this marker of *cag*PAI, although more frequent, is clearly less pathogenic than the *cagA*. Proença Modena et al. [31] also found no correlation of *cagE* with disease outcomes.

There are clear geographic differences in the distribution of both *vacA* s and m subtypes [24, 33]. The *H. pylori vacA* s1m1 genotype is the most common in American, European and East Asian countries [24, 29, 41–43]. The *vacA* s2m2 genotype is apparently less common and another study in Portugal, performed in patients submitted to a screening program, revealed a prevalence of 29.7 % for such allele combination (42.9 % if multiple *vacA* genotypes were excluded). The *vacA* s2m2 prevalence found in our study is among the highest reported [24]. This was expected given the high prevalence of *cagA* negative strains and the association of such genetic profile with *vacA* s2 and *vacA* m2 [24]. This reflects the presence of less virulent strains in the central region of Portugal. However, we cannot forget that we have few cases with peptic ulcer disease and none with gastric cancer. A selection bias cannot be completely excluded, and this is a limitation of our work. Even so, it is interesting to notice that Boukhris et al. [33] identified a higher prevalence (59.2 %) of *vacA* s2m2 genotype in 145 Moroccan isolates with a single association of *vacA* s and m alleles. The same happened in another study in our neighbour, Spain, also with *vacA* s2m2 being the dominant genotype [32].

Mosaicism *vacA* s2m1 was detected in only 3 patients, confirming that this genotype is very rare [9, 10, 24]. Van Doorn et al. established that in the Iberian Peninsula subtype s1b was the dominant one [24, 44, 45]. By the contrary, our study demonstrates that in the central region s2 is the more common allele followed by s1a. It is known that *vacA* s1m1 strains determine higher levels of inflammation in the gastric mucosa and both *vacA* s1m1 and s1m2 strains may be disease-associated but patients with gastric cancer usually have the s1m1 type [5, 10, 28, 39, 44, 46]. In our population, we found no correlations of *vacA* genotypes with gastric histopathological modifications. The last published data about gastric carcinoma epidemiology in Portugal revealed a low incidence rate in the central region comparatively to the south and north [47]. Predominance of less virulent strains in this part of the country could partially explain such differences. However, the relatively small number of isolates with s1m1 and s1m2 and the absence of patients with gastric carcinoma cannot allow us to confirm or deny this association. A larger, epidemiological population-based study would be necessary to clarify these doubts.

We identified an isolate with subtype s1c. This allelic variant is essentially detected in East Asian patients but some cases were already described in Westerners [7, 40, 41, 48].

The m1 allele is relatively rare compared with m2. This is also the opposite of what was previously established for the Iberian Peninsula but similar to the results presented more recently by González et al. for a specific Spanish region [24, 32].

The *iceA1* allelic variant of *iceA* may be linked with the development of peptic ulcers since *iceA1* positive strains produce more proinflammatory factor IL-8 [7, 49]. In our study, population *iceA2* subtype was the dominant one, as previously stated for Western patients [7, 11, 20, 22]. The relationship of *iceA* with gastric histopathology and clinical presentation is also very different from study to study [13, 20, 45]. In our work, we did not find any specific association of *iceA* allelic variants with pathological or clinical phenotype. Another study, involving patients from the northern region of the country, also failed to found a relation between the *iceA* allelic variants and clinical outcome of *H. pylori* infection [45].

The *babA2* gene has been strongly associated with *cagA* and *vacA* s1 in some populations [12]. Prevalence of *babA2* positive strains is very heterogeneous and can be as high as 92.3 % [7, 13, 22]. This genotype can determine a higher risk of ulcer and gastric adenocarcinoma but this is controversial [7]. In our study, *babA2* was very rare and only associated with higher *H. pylori* density scores in the gastric body. To confirm the real prevalence of *babA*, some authors recommend immunoblot as well as the use of several primer pairs by PCR because of possible sequence variations [50, 51].

Strains with *vacA* s1m1 as well as genotype combinations *cagA* positive + *vacA* s1m1 and *cagA* positive + *vacA* s1m1 + *babA2* were associated with peptic ulcer. The correlation of such clinical manifestation with these genotypes is consistent with earlier studies [35, 41, 52]. This is explained by the higher virulence and mucosal injury determined by such strains. On the contrary, the less virulent *H. pylori* genotype *vacA* s2m2 was more common in asymptomatic patients, with iron-deficient anemia.

Traditionally, less aggressive *vacA* s2m2 strains are more common in patients with functional dyspepsia although some studies reported the opposite correlation

[53, 54]. In our study, no significant association with this clinical manifestation was detected.

A major limitation of our work is that we studied only a few genes and, as we know, the average size of an *H. pylori* genome is estimated at 1.62 Mb consisting of 1,590 open-reading frames encoding 1,532 proteins [55]. Probably, in a near future, with increased availability of next-generation sequencing, multiple *H. pylori* complete genomes will be studied and we will discover and/or relate other genes and respective proteins as major factors for specific clinical manifestations of *H. pylori* infection as well as histological parameters of *H. pylori* related gastritis.

Another limitation was the relatively low number of patients with specific histopathological modifications (e.g., corpus glandular atrophy) and clinical manifestations. We had only 12 patients with peptic ulcer and none with gastric cancer. We even had a large number of patients referred for *H. pylori* eradication only because they had first-degree relatives with gastric cancer. Our hospital is a tertiary-referral center, and we included only patients from ambulatory clinical practice. Obviously, the ones with complicated peptic ulcers or gastric cancers are referred for admission and specific treatment. A population-based study could, eventually, overcome this problem.

Genetic factors for infected host can be a very important determinant of infection result. Not including this variable in our work is a major restraint.

H. pylori induces chronic gastritis in virtually all infected patients [26, 32]. However, gastritis and preneoplastic lesions such as glandular atrophy and intestinal metaplasia also occur in uninfected patients. Our work did not involve *H. pylori* negative controls and so we do not know the real correlation of gastric histopathological modifications with *H. pylori* infection.

Finally, most authors that study this problem consider all patients, including the ones with multiple *H. pylori* genotypes. Previous studies in Portugal revealed a high prevalence of patients harboring multiple *H. pylori* strains [5, 21, 45]. However, for correlation of *H. pylori* genotypes with gastric histopathology, most authors exclude patients with multiple isolates. In our opinion, this strategy can be somehow confuse and difficult to interpret so we decided to consider only patients with single *H. pylori* genotypes.

Conclusions

Higher scores of intestinal metaplasia and gastric mucosa inflammation are detected in patients infected by *cagA*-positive *H. pylori* isolates confirming the increased virulence of such strains and the potentially worst outcomes in these cases. *IceA2* allelic variant is the predominant one and has no correlation with disease phenotype. Gene *babA2* is rare and associated with higher *H. pylori* colonization density in the corpus.

Patients with peptic ulcer are frequently infected by *H. pylori* with the *vacA* s1m1 allelic variant or the combination *cagA* + *vacA* s1m1 and *cagA* positive + *vacA* s1m1 + *babA2*.

In the central region of Portugal, there is a predominance of less virulent strains while maintaining the usual correlation with gastric histopathology. This implies low prevalence of gastric atrophy and/or intestinal metaplasia and hypothetically, of gastric adenocarcinoma. Our results, compared with earlier published ones, from other regions, demonstrate that, in the same country, *H. pylori* genotype may have a marked regional variability.

Acknowledgments We are deeply indebted to Professor Javier Gisbert for all comments and suggestions about this manuscript. We also want to thank Dr João Casalta for all support provided in statistical analysis. This work was part of a project designed to study primary and secondary resistance of *Helicobacter pylori* to antimicrobial agents, supported by the Portuguese Institute Fundação para a Ciência e a Tecnologia (PIC/IC/83122/2007).

Conflict of interest There are no conflicts of interest to disclose.

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