Multiple cag genotypes of *Helicobacter pylori* isolates colonize the oesophagus in individual hosts in a Venezuelan population

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Abstract

**Purpose.** Multiple *Helicobacter pylori* strains colonize and coexist in the stomach of one single patient, carrying heterogeneous distributions of cag genotypes. The oesophagus provides a niche for *H. pylori* colonization; however, little is known about its adaptive role.

**Methodology.** Using PCR for cagA, cagE and virB11 genes from cag-pathogenicity island (PAI) and Etest for antimicrobial susceptibility test, we determined cag-PAI genotypes associated with *H. pylori* virulence, when positive cultures were matching in both the stomach and the oesophagus (96 isolates; 8 out of 80 dyspeptic patients).

**Results.** The stomach showed complete cag-PAI islands in 77% of the isolates, whereas the oesophagus showed complete cag-PAI islands only in 44% of the isolates. Expression of CagA and interleukin 8 correlated with inflammatory processes and histopathological changes in the stomach, but not in the oesophagus. Different cag-PAI profiles were found in both mucosae of an individual host, and at least one oesophageal profile corresponded to one profile identified in stomach. The antibiotic resistance profiles showed variability in the colonization by single or mixed *H. pylori* isolates in the gastric and oesophageal mucosa both intra- and inter-individuals.

**Conclusion.** These results demonstrate colonization with multiple *H. pylori* isolates in the oesophageal mucosa, like those found in the stomach of individual hosts. *H. pylori* was characterized by a dominant partial island, low interleukin 8 induction with lower histopathological damage and lower antibiotic resistance, suggesting that the microenvironmental changes in individual hosts select less virulent isolates in the oesophagus than in the stomach. New approaches to ensure effective eradication therapy in multi-resistant *H. pylori* strains must be developed.

INTRODUCTION

*Helicobacter pylori* is a Gram-negative, flagellated, microaerophilic bacterium that selectively colonizes the gastric mucosa, commonly giving rise to peptic ulceration, gastric lymphoma and gastric adenocarcinoma [1]. Around 70% of strains possess the cytoxin-associated gene A (cagA), this being the principal marker of the so-called cag pathogenicity island (PAI), the presence of which has been associated with the development of gastric cancer [2]. Among cag-PAI genes, the most studied is cagA that encodes for the CagA protein. CagA is an oncprotein, which is delivered into gastric epithelial cells via a bacterial type IV secretion system. It can induce adhesion, proliferation, morphological alterations, pro-inflammatory interleukin secretion and apoptosis, which result from multiple transformations in the host signalling pathways [2, 3]. The gene cagE encodes a protein responsible for the induction of interleukin 8 (IL-8), eliciting a mucosal inflammatory response and epithelial cell damage. The gene virB11 encodes a functional adenosine triphosphatase activity and, together with other cag-PAI genes, constitutes the core structure for the type IV secretion system that allows the delivery of CagA protein into gastric cells [4]. Additionally, cagE and virB11 have been studied as markers of cag-PAI, and their presence is related to severe gastroduodenal diseases, such as ulcer and gastric cancer, rather than to gastritis [5].

*H. pylori* exhibits a rather high genetic diversity, greater than most bacteria, with exceptionally high rates of DNA point mutations as well as intra- and inter-genetic recombination,
which allow its adaptation to changing environments [6]. The most significant consequences of these genomic changes are antibiotic resistance and differences in the prevalence or expression of bacterial virulence factors [7]. Antibacterial resistance is the major cause for treatment failure in first eradication regimes. At present, the first line therapy, recommended by the Maastricht consensus report, contemplates the use of a proton pump inhibitor and the antibiotics amoxicillin and clarithromycin or metronidazole. This scheme has been generally adopted worldwide [8].

Studies in developed countries show that most strains of *H. pylori* isolated from different sites in the stomach of individual hosts carry homogeneous DNA profiles [9], different from multiple-strain infections reported in developing countries such as China, India, Mexico and Brazil [9–12]. In Venezuela, a high frequency of gastric *H. pylori* colonization with multiple strains has been demonstrated [13]; however, few studies deal with the oesophageal colonization of *H. pylori* [14–16]. The oesophagus is a potential environment for bacterial colonization: alterations of the microenvironment, such as reflux-related diseases, oesophagitis, Barrett’s oesophagus or oesophageal carcinoma, could affect the bacterial biota and the oesophageal epithelium [17]. The role of *H. pylori* infection in these diseases is controversial; however, *H. pylori* can survive for a certain amount of time in gastric juice in the oesophagus [18]. Recent studies carried out by our group in Venezuelan dyspeptic patients have shown a high frequency of *H. pylori* infection (53–86 %) and heterogeneity of *cag* genotypes in biopsies of the gastroesophageal mucosa [15, 19]. However, the oesophageal colonization by multiple strains of *H. pylori* has not yet been reported.

In order to establish if the oesophageal *H. pylori* colonization results from circulating strains in the stomach, we determined *cag*-PAI genotypes associated with *H. pylori* virulence as well as antimicrobial susceptibility profiles of isolates obtained from the gastroesophageal mucosa. Furthermore, we also investigated if the *H. pylori* colonization in oesophagus is related to an inflammatory response through IL-8 expression induced by CagA.

**METHODS**

**Sample collection and bacterial culture**

Patients consulting the Gastroenterology Unit of the Oncology Service of the Hospital del Instituto Venezolano de los Seguros Sociales with gastrointestinal symptoms of dyspepsia (upper abdominal pain, heartburn and reflux), requiring upper-gastrointestinal endoscopy, were recruited into this study. All volunteers signed an informed consent form and the bioethics committees of Instituto Venezolano de Investigaciones Científicas and Hospital del Instituto Venezolano de los Seguros Sociales approved this study. Previous data of *H. pylori* DNA in biopsies from both mucosae (80 patients) were used to obtain *H. pylori* cultures [19]. Only eight subjects (two male and six female), aged 30–60 years (mean age, 44 years), matching *H. pylori*-positive cultures in both mucosae were selected. Endoscopic observation on these eight patients indicated that one (12.5 %) had gastric ulcer, two (25 %) had antral gastritis, one (12.5 %) had only gastritis, four (50 %) had hiatal hernia and four (50 %) had apparently normal gastric mucosa. All patients presented apparently normal oesophageal mucosa. During the endoscopy procedure, three antral and three oesophageal biopsy specimens were collected from each patient as previously described [19]. One biopsy specimen from each mucosa was immediately placed in formalin for histopathology and immunofluorescence studies; the other two biopsies were submitted for *H. pylori* culture.

Gastroesophageal biopsies of eight dyspeptic patients (positive for *H. pylori* infection) were homogenized with 300 µl PBS solution (0.9 %) in 1.5 ml tubes and shaken for 50 s at 5000 r.p.m. in a mini-headbeater-1 (BioSpec Products). A 100 µl volume of each suspension was seeded (duplicate) on chocolate agar selective medium (blood agar base plates, Difco), supplemented with 10 % (v/v) sheep blood and a mixture of antibiotics and fungicide: vancomycin (10 µg ml⁻¹), polymyxin B (2.5 IU l⁻¹), trimethoprim (5 µg ml⁻¹) and amphotericin B (2.5 µg ml⁻¹) (Sigma-Aldrich). The plates were incubated at 37 °C in microaerophilic atmosphere (Becton Dickinson) for 4–7 days. Small translucent colonies of *H. pylori* were identified by morphological characteristics, Gram staining and rapid urease test (Ure-IVIC; [20]). For each positive culture of *H. pylori*, six single colonies were picked for further culturing, and the remaining colonies were pooled and preserved in Brucella broth plus 15 % (w/v) glycerol at −80 °C. The colonies of *H. pylori* selected were sub-cultured and preserved under the same conditions as described before. A total of 96 isolates (six single colonies obtained from each mucosa per patient) were processed separately for antimicrobial susceptibility testing and DNA extraction. The DNA from 96 *H. pylori* isolates was extracted using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer’s instructions and stored at −20 °C.

**Antibiotic susceptibility test**

Five antibiotics against *H. pylori* were tested: clarithromycin, amoxicillin, levofloxacin, tetracycline and metronidazole, through the episolometer method (Etest, bioMérieux). The antibiotic concentrations ranged from 0.0016 to 256 mg l⁻¹ for clarithromycin, amoxicillin, tetracycline and metronidazole and from 0.002 to 32 mg l⁻¹ for levofloxacin. Antibiotic Etest strips were applied individually to five chocolate agar plates, which were inoculated for each isolate. Additionally, one plate was used as growth control without antibiotic. A total of 576 plates were incubated at 37 °C in microaerophilic atmosphere (Becton Dickinson) for 4–7 days. An elliptical zone of inhibition is produced, corresponding to the MIC. The results were read according to the supplier’s recommendations. The resistance breakpoints at which we considered there was growth inhibition of the micro-organism in accordance with the Clinical and Laboratory Standards Institute [21] were as follows: MIC 1 mg l⁻¹ for clarithromycin, MIC 0.5 mg l⁻¹ for amoxicillin, MIC 2 mg l⁻¹ for tetracycline, MIC 1 mg...
l⁻¹ for levofloxacin and MIC 8 mg l⁻¹ for metronidazole as previously described [22]. A strain of H. pylori ATCC 43504 was used as a control strain for antimicrobial susceptibility testing.

**Helicobacter genus and H. pylori species PCR assays**

Helicobacter spp. and H. pylori infection were detected by PCR using genus- and species-specific primers for 16S rRNA, glmM and ureA genes [23–25]. PCRs were performed in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems) using Colorless Go Taq Hot Start Master Mix (Promega), using the same cycling conditions as originally described for each pair of primers (Table 1). Each reaction contained all components of the PCR, 2–4 µl extracted DNA, 3 µl primer mix (5 µM) and sterile distilled water to a final volume of 25 µl. Two positive controls were used as templates for each PCR assay: (a) DNA from H. pylori isolated from a Venezuelan patient with gastritis and (b) DNA from H. pylori reference strain 26695 [26]. Subsequently, the amplicons were visualized by running the reaction mixture in a TBE agarose gel [2 % (w/v)], stained with ethidium bromide and observed under a UV transilluminator.

**cag-PAI genotypes**

The cagA, cagE and virB11 presence was investigated in 96 isolates for the detection of genotypes associated with H. pylori cag-PAI in both mucosae, using specific primers described for each gene, as shown in Table 1 [4, 27]. PCRs were performed using Colorless Go Taq Hot Start Master Mix (Promega), as described above. According to the genetic composition of the cag-PAI, we considered a complete cag-PAI when all three genes were present, a partial cag-PAI when at least one gene was lacking and a deleted island when all three genes were absent.

**Histopathology**

Formalin-fixed biopsies were embedded in paraffin. Sections of 4 µm were cut and placed on slides, then deparaffinized in xylene for 5 of 4 µm were cut and placed on slides, then deparaffinized in Formalin-fixed biopsies were embedded in paraffin. Sections and subsequently rehydrated in PBS at pH 7.4 following the protocol previously described [19]. The sections were used for conventional histopathology using haematoxylin–eosin technique according to the Sydney System for the stomach (antrum) [28] and the presence of maliggun cells (clear) as indicative of alterations induced by H. pylori [29]. Criteria similar to the Sydney System were applied to characterize patterns of inflammation in the oesophagus. The presence of the following features in the biopsies was determined: basal hyperplasia, metaplasia, plasma cells, lymphocyte, mononuclear cells and neutrophil infiltration and microabscesses [30], as described previously [19].

**Immunofluorescence**

Samples of gastric and oesophageal biopsies were fixed in PBS containing 4 % (w/v) paraformaldehyde at 4 °C for 6 h. Sections 4–5 µm in thickness were cut and placed onto glass slides. Sections were dehydrated with ethanol, clarified with xylene and embedded in Paraplast (Sigma-Aldrich) [15]. The non-specific binding of antibodies was reduced by previous incubation with 0.1 % (w/v) PBS-T [PBS plus 0.3 % (w/v) Triton X-100] with 3 % (w/v) BSA (Sigma-Aldrich) blocking solution for 1 h at room temperature. Subsequently, the sections were incubated overnight at 4 °C in a humid chamber with specific primary antibodies previously diluted 1 : 1000 in 0.1 % (w/v) PBS-T with 3 % (w/v) BSA : anti-CagA (ab37351, mouse; Abcam) for the visualization of H. pylori cells and a recombinant anti-IL-8 (ab7747, rabbit; Abcam) for the visualization of IL-8-positive cells.

After incubation steps, the slides were washed three times with PBS and incubated for 1 h at room temperature with goat polyclonal secondary antibodies: FITC-labelled anti-rabbit IgG (ab6717; Abcam) and anti-mouse IgG labelled with 5-TRICT (ab6897; Abcam), both diluted 1 : 500 in 0.1 % (w/v) PBS-T with 3 % (w/v) BSA. After the antibody reactions, the slides were washed with PBS and the nuclei, further stained with 4',6-diamidino-2-phenylindole (Molecular Probes, Life Technologies; 1 µg ml⁻¹ for 10 min) and later washed with PBS. A negative control was incubated with blocking buffer

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**Table 1. Primers used in this study**

<table>
<thead>
<tr>
<th>Gen</th>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
<th>Size of PCR product (bp)</th>
<th>Reference</th>
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<td>glmM</td>
<td>glmMF</td>
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<td>[25]</td>
</tr>
<tr>
<td></td>
<td>glmMR</td>
<td>GCTTACCTTCAACGCTAAGCGGC</td>
<td>128</td>
<td>[27]</td>
</tr>
<tr>
<td>ureA</td>
<td>ureAF</td>
<td>GGAATGTTAAATAGGTT</td>
<td>128</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>ureAR</td>
<td>CTCTTAGTTGTTTCTAC</td>
<td>508</td>
<td>[4]</td>
</tr>
<tr>
<td>CagA</td>
<td>cagAF</td>
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<td>294</td>
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<td>cagAR</td>
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<td>128</td>
<td>[27]</td>
</tr>
<tr>
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<td>cagER</td>
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<td>508</td>
<td>[4]</td>
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<td>virB11</td>
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<td></td>
<td>virB11R</td>
<td>GATAATAAGTGTATTTACCCGCTTC</td>
<td>491</td>
<td>[4]</td>
</tr>
</tbody>
</table>

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without primary antibodies and then with secondary antibodies. Finally, the slides were embedded with Immunomount (Thermo) to be observed with a laser scanning confocal microscope (Fluo-View 1000; Olympus America). All images were acquired using the FV10-ASW version 02.01.01.04 software (Olympus). Image J software (National Institutes of Health) was used for image processing and adjustment of contrast and brightness. Epifluorescence images were obtained with a Nikon microscope (Nikon). *H. pylori* density was measured in 10 fields for each biopsy from eight patients in order to estimate the level of expression of CagA (red dye) and IL-8 (green dye) in 160 fields total. CagA expression was measured by counting the number of gastric (*n*=2496) and oesophageal (*n*=576) bacteria, whereas IL-8 expression was measured by counting the number of gastric (*n*=2208) and oesophageal (*n*=1344) cells in the cytoplasm, in the same field where *H. pylori* CagA-positive cells were estimated.

**Statistical analysis**

All statistical analysis and graphs were conducted using Origin 8.1 Pro software (Origin Lab). Data are expressed as mean±SEM. The statistical significance of data was estimated by Student’s *t*-test for two group data (values of *P*≤0.05 were considered significant). The associations between data were analysed using Pearson’s correlation analysis.

**RESULTS**

**Detection of *H. pylori* by PCR and presence of cag-PAI (cagA, cagE and virB11) in single-colony cultures**

*H. pylori* infection was detected in single-colony cultures from eight dyspeptic patients by amplification of the 16S RNA, glmM and ureA genes, confirming the presence of *H. pylori* DNA in 96 isolates (48 obtained from antrum, and 48 from oesophagus). Thus, when the cag-PAI gene composition was studied by individual mucosa in these 96 *H. pylori*-positive isolates, we found that 94 % (45/48) were cagA positive, 92 % (44/48) were cagE positive and 90 % (43/48) were virB11 positive in the gastric mucosa. In the oesophagus, cagA, cagE and virB11 were found in 42 (20/48), 100 (48/48) and 94 % (45/48), respectively. The frequency analysis of these cag genes in both mucosae showed that cagA had a higher frequency in the stomach antrum (93.7±3.5 %) than in the oesophagus (41.7 ±7.2 %; *P*≤3.80e−9), while cagE and virB11 were less variable (Fig. 1a). The correlation analysis (*r*) between antrum and oesophagus for cagA, cagE and virB11 was 0.28 (*P*<0.136), 0.466 (*P*≤8.37e−4) and 0.81 (*P*≤4.05e−12), respectively, indicating a statistically significant correlation for cagE and virB11, but not for cagA. These results indicate a clear association of cagE and virB11 genes between antrum and oesophagus, while there is no correlation for the cagA gene. This suggests that cagA isolates found in the stomach would not necessarily be present in the oesophagus.

A complete cag-PAI profile was detected in 77.08 % (37/48) of antrum isolates and 43.75 % (21/48) of oesophagus isolates, whereas different partial cag-PAI profiles were present in 22.92 % (11/48) of antrum isolates and 62.50 % (30/48) of the oesophagus isolates (Fig. 1b). These results indicate that the distribution of cag-PAI genotype profiles changes dramatically depending on the epithelium, suggesting that *H. pylori* strains can adapt to different microenvironmental conditions leading to greater genetic diversity.

When we examined the distribution of the cag genotype profiles in the gastroesophageal mucosa of individual hosts (Fig. 2), we found one patient carrying a single genotype with the same genetic composition of cag-PAI in both mucosae (patient 7). The other seven patients carried mixed genotypes with different cag-PAI composition in the antrum and oesophagus. Mixed and unique profiles were found in both mucosae from the same individual host. From the seven
patients with different cag-PAI compositions, four had mixed genotypes in the stomach and single genotype in the oesophagus (patients 1, 2, 4 and 8), two patients had single genotype in the stomach and mixed genotypes in the oesophagus (patients 3 and 5) and only one patient had mixed genotypes in both mucosa (patient 6), suggesting that the isolates found in the oesophagus could come from circulating strains in the stomach, through recombination with colony variants of the same strain.

**Antibiotic susceptibility and resistance test**

Etest analysis from single colonies can evidence the phenotypic variability of *H. pylori* isolates in individual hosts (Table 2). The rates of susceptibility for the 48 isolates from the gastric mucosa of eight patients were 100 % for clarithromycin, 100 % for amoxicillin, 60 % for levofloxacin, 77 % for tetracycline and 23 % for metronidazole, while susceptibility for the 48 isolates from the oesophagus was 98, 100, 81, 83 and 21 %, respectively.

We found six resistance profiles classified according to the combination of antibiotic resistance by isolate (Table 2). The most resistant profile in isolates from the gastric mucosa was Profile 1, found in 6 % (3/48 isolates), which showed resistance to levofloxacin, tetracycline and metronidazole. On the other hand, the most common profile was Profile 4 found in 42 % (20/48 isolates), showing resistance only to metronidazole. Profile 2 was observed in 13 % (6/48 isolates) and Profile 3 was observed in 17 % (8/48 isolates) showing resistance to levofloxacin and metronidazole or tetracycline and metronidazole. Profile 6 (2 %) showed susceptibility to all antibiotics (no resistance) in only one of 48 isolates. A similar pattern was observed in isolates from the oesophagus with the addition of a seventh profile where susceptibility to all antibiotics tested was found in seven of the 48 isolates (Table 2). The phenotypic variability of *H. pylori* isolates on this study indicates that one patient can be co-infected with multiple *H. pylori* strains (single and/or mixed genotypes) in both mucosae at the same time (Table 2). The *H. pylori* variability in individual hosts may be associated with the recurrence of infection and antibiotic resistance.

**Histopathology of the gastroesophageal mucosa**

Histopathological analysis of the antrum gastric mucosa was performed in six of the eight dyspeptic patients (in two patients, the amount of the tissue was insufficient) and classified according to the Sydney System. Fig. 3 shows the histopathological alterations identified and their frequencies both in the gastric and oesophageal mucosa. In the antrum biopsy samples, all patients had chronic gastritis with dysplasia accompanied with monocyte and neutrophil

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**Table 2. Profiles of susceptibility and antibiotic resistance of *H. pylori* isolates from six single colonies in the gastric (n=48) and oesophageal mucosa (n=48) in individual hosts**

<table>
<thead>
<tr>
<th>No. of profiles in the gastric mucosa</th>
<th>Etest analysis with five antibiotics against <em>H. pylori</em> isolates</th>
<th>Resistance profiles (n)</th>
<th>No. of profiles in the oesophageal mucosa</th>
<th>Etest analysis with five antibiotics against <em>H. pylori</em> isolates</th>
<th>Resistance profiles (n)</th>
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<tr>
<td></td>
<td>CLR</td>
<td>AMX</td>
<td>LVX</td>
<td>TET</td>
<td>MTZ</td>
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<tr>
<td><em>H. pylori</em> ATCC 43504</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S</td>
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<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>4</td>
<td>S</td>
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<td>5</td>
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<tr>
<td>6</td>
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<tr>
<td></td>
<td>7</td>
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<tr>
<td>Resistance (%)</td>
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<td>0</td>
<td>40</td>
<td>23</td>
<td>77</td>
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<tr>
<td>Sensitivity (%)</td>
<td>100</td>
<td>100</td>
<td>60</td>
<td>77</td>
<td>23</td>
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</tbody>
</table>

CLR, clarithromycin; AMX, amoxicillin; LVX, levofloxacin; TET, tetracycline; MTZ, metronidazole; S, sensitive; R, resistant.
infiltration and bacteria as the most frequent alterations (range from 66 to 100%), whereas in oesophageal biopsies (7/8 patients), bacteria, microabscesses and neutrophil infiltration were the most common alterations (range from 40 to 57%), suggesting a cellular immune response. Other changes such as hyperplasia, plasmocytes, dysplasia and metaplasia were also observed (Fig. 3). The mean frequency of all alterations in the antral biopsies was significantly higher than that of the oesophageal biopsies (66.63±9.39 versus 34.71±6.88, \(P<0.05\)). These results may suggest that the high frequency of histopathological damages in the gastric mucosa is related to a higher proportion of complete \(cag\)-PAI in the antrum isolates.

**Expression of \(cag\)A and IL-8 in the gastroesophageal mucosa**

Immunofluorescence was performed on eight gastric and eight oesophageal biopsies, evaluating 10 fields per biopsy using epifluorescence microscopy for statistical analysis (total of 160 images). Additionally, six images per biopsy were obtained by confocal microscopy (laser scanning confocal microscopy) with immunofluorescence staining for \(cag\)A and IL-8 in the gastroesophageal mucosa (Fig. 4). \(cag\)A expression was observed in the \(H. pylori\) cells (red dye) invading the mucosa and submucosa between the gastric cells [Fig. 4a, b, differential interference contrast (DIC) images, white arrows] and in the submucosa of oesophagus (Fig. 4e, f). Expression of IL-8 (green dye) was observed in the cytoplasm of the gastric and oesophageal cells (Fig. 4c, g), showing higher expression of IL-8 in the stomach (Fig. 4d, white arrows) than in the oesophagus (Fig. 4h, white arrows).

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**Fig. 3.** Histopathological observations in gastroesophageal mucosal biopsies from individual hosts. The columns represent the frequency of the histopathological alterations by mucosa.

**Fig. 4.** Laser scanning confocal microscopy of two biopsies (gastric and oesophageal) positive for \(cag\)A and IL-8 by immunofluorescence and DIC. (a) DIC–tetramethylrhodamine isothiocyanate (TRITC) merged image showing \(cag\)A-positive \(H. pylori\) cells in the antrum stained in red (arrows). (b) Higher magnification of area in (a) depicted by the white square. (c) Merged FITC–TRITC image showing IL-8 stained in green in the cytoplasm of gastric cells and \(cag\)A-positive \(H. pylori\) in yellow (colocalization of rhodamine and FITC) indicated by arrows. (d) Higher magnification of area in (c) depicted by the white square, showing IL-8 stained in green, indicated by arrows. (e) DIC–TRITC merged image showing \(cag\)A-positive \(H. pylori\) cells in the oesophagus marked in red (white arrows). (f) Higher magnification of area in (e) depicted by the white square. (g) Merged FITC–TRITC image showing IL-8 stained green in the cytoplasm of oesophageal cells and \(cag\)A-positive \(H. pylori\) cells in yellow (colocalization of rhodamine and FITC) indicated by arrows. (h) Higher magnification of area in (e) depicted by the white square, showing IL-8 stained in green indicated by arrows. These images show high expression of \(cag\)A and IL-8 in the stomach and presence of \(cag\)A with lower expression of IL-8 in the oesophagus.
The frequency analysis for CagA-stained H. pylori cells and IL-8-stained cells by mucosa showed values in the gastric mucosa of 65±5.3% and 57±5.5%, respectively. Lower expression was observed in the oesophagus, with values of 7.5±3.0% and 17.5±4.3%, respectively (Fig. 5). The difference in expression in the stomach and the oesophagus was statistically significant for both CagA (P<8.00e−014) and IL-8 (P<5.66e−08) (Fig. 5). The association analysis between CagA and IL-8 showed a significant correlation value of 0.641 (P<1.45e−10) in the stomach, while it did not reach significant values in the oesophagus (0.118; P<0.294). These findings suggest that CagA induces higher IL-8 expression in the stomach than in the oesophagus.

**DISCUSSION**

The present study demonstrates the colonization with multiple H. pylori isolates in the oesophagus, like those found in the stomach, but with different cag-PAI genotypes in individual hosts in a Venezuelan population. Comparative analysis of the cag-PAI genotypes showed variations in the profiles obtained from colonies in the gastroesophageal mucosa. The genetic distribution of cag genotypes in the oesophagus displayed a large proportion of partial islands (62.50% of isolates) in relationship to complete cag-PAI island (37.50%), whereas in the stomach, complete cag-PAI island was detected in 77.08%, and partial island in 22.92% of isolates. The complete and partial island values in the stomach are in accordance with previous reports of single-colony isolates and gastric biopsies from dyspeptic patients in a range from 58 to 90% for a complete genotype and 4 to 85% for a partial genotype [4,10,19,31–33]. The variability observed evidences the heterogeneity of H. pylori through diverse genetic profiles with dominance of partial cag-PAI genotype in the oesophagus and complete cag-PAI in the stomach.

The frequency of the cagA gene in antrum isolates was higher (93.7±3.5%), together with virB11 (98.8±2.9%) and cagE (95.8±2.9%), which correlated with severe histopathological alterations found in the gastric biopsies. In the oesophagus isolates, by contrast, we found a lower frequency of cagA (41.7±7.2%) and similar frequencies for cagE (83.3±5.5%) and virB11 (93.7±3.5%). This suggests that cagA is the gene that establishes the difference between a complete and a partial cag-PAI profile in the presence of the other two genes, which, in turn, results in less virulent strains associated with less severe inflammation in the oesophageal mucosa as evidenced in the histopathological outcomes of this study.

The variability between H. pylori isolates was also observed when the cag genotype profiles in both mucosae were analysed in individual hosts. Samples from only one patient showed a single common genotype in both mucosae (patient 7, Fig. 2), whereas the other seven patients displayed mixed and/or single genotypes in both mucosae. This may suggest that strains in the oesophagus could originate from circulating strains in the stomach, through frequent genetic exchange as well as co-evolution of this bacterium to colonize different niches in the host. Our results demonstrate the genetic diversity of H. pylori in a single individual host with multiple strains of H. pylori in each mucosa. Each mucosa, antral or oesophageal, is dominated by a specific cag genotype, suggesting that adaptive host-induced changes occur during H. pylori infection. The existence of multiple H. pylori strains in the stomach with different genetic polymorphisms in an individual host has been demonstrated in numerous studies. Some authors correlate complete cag-PAI with severe clinical diseases associated with gastric and peptic ulcers, atrophic gastritis and gastric cancer [4,5,10,12,19,32–34] and partial cag-PAI or deleted island with lower pathological damages in the gastric mucosa [31,35].

The phenotypic variability of H. pylori was also tested in antibiotic susceptibility and resistance assays using five antibiotics. These five antibiotics are the most recommended by international consensus (Maastricht IV) to be used in triple or quadruple therapy for eradication of H. pylori infection. Our tests showed 100% sensitivity to clarithromycin and amoxicillin in isolates from antrum and oesophagus (98% sensitivity for clarithromycin and 100% for amoxicillin). These results agree with previous studies in stomach isolates, which pointed out that prevalence of resistance varied from 0 to 38% for amoxicillin and from 2 to 63% (mean 14% in 11 countries) for clarithromycin in Latin America [36]. In Venezuela, one study reported 7% resistance [37]. Low percentage of resistance in our study may be attributed to low previous utilization of this antibiotic for the treatment of other infections or variability in resistance rates in different geographical areas of the same
country, as previously reported [38]. However, the primary prevalence of clarithromycin resistance is increasing worldwide, particularly in developed countries such as Italy (35.2 %), France (26 %) and Spain (27.2 %) [39]. A higher resistance to levofloxacin was obtained in isolates from the gastric mucosa (40 %), as compared to the isolates from oesophagus (19 %). The primary H. pylori resistance to fluoroquinolone has been reported according to different countries or regions. A high resistance (over 10 %) has been reported in Taiwan (11.8 %), Japan (15 %), Senegal (15 %), Nigeria (15.6 %), Korea (15.7 %), Egypt (25 %), China (26.6 %) and in European countries such as France (12 %), Spain (14.3 %) and Italy (22.1 %), whereas lower resistance rates have been found in Iran (5.3 %), Western Australia (6 %) and Malaysia (6.8 %) [22, 40, 44, 46]. Global difference in resistance may be related to over-the-counter purchase of drugs, the use of substandard drugs and the consumption rate of these antibiotics. Fluoroquinolone resistance is easily acquired by inhibition of DNA gyrase, through point mutations in the quinolone resistance-determining region of the gyrA and gyrB genes, and topoisomerase [46, 47]. Additionally, fluoroquinolones may induce overexpression of multidrug efflux pumps as resistance mechanisms present in H. pylori [48]. These mechanisms could be acting in parallel to increase the resistance of this bacterium to the antibiotic. In this study, isolates from both mucosae also showed a high rate of resistance to levofloxacin, which may be associated with the increased use of this antibiotic in clinical practice.

Tetracycline resistance in oesophagus and antrum was 17 and 23 %, respectively. This phenotypic variability differs among isolates in both mucosae. Other studies report that tetracycline resistance is very low or even absent worldwide [22, 44, 49]. Some cases of low resistance have been reported in Spain (0.7 %), United Kingdom (0.5 %) and Hong Kong (0.5 %), while higher rates were reported in Korea (5.3 %) and in some developing and emerging countries such as Brazil (9.0 %), Bangladesh (15 %) and Chile (27 %) [22, 49]. Tetracycline resistance, like other antibiotics, increases with the use of the drug due to selective pressure. We found high resistance similar to that reported in some developing and emerging countries and even higher than in another report in Venezuela where the rate of tetracycline resistance was 7 % in isolated strains from gastric biopsies between 1998 and 1999 [37]. This difference in resistance could be due to increased use of this antibiotic in past years in the Venezuelan population.

Resistance to metronidazole was rather high in both antrum (77 %) and oesophagus (79 %), agreeing with results of other studies in Venezuela (67 %) [37]. Although this antibiotic is recommended by international consensus, high resistance has been reported worldwide [45, 49, 50]. The prevalence of H. pylori resistance to metronidazole varies from Europe to America, Asia and Africa. It is known that its prevalence is highest in developing countries (50–80 %), such as Mexico (76 %) [51] and Venezuela (this study; [37]), while it is lowest, around 17 %, in European countries [22, 44, 50, 52]. When other factors that influence the resistances were analysed, the use of metronidazole to treat parasitic infections in developing countries and nitroimidazoles for gynecological or dental infections in developed countries were the most prominent [49]. This is probably a risk factor for H. pylori resistance to metronidazole in patients with infections other than this bacterium. Other studies have shown that H. pylori is able to multiply within infected epithelial cells. This may explain the treatment failure in some cases and suggest that the difficulty in eradication of the disease is not caused only by the recurrence of antibiotic-resistant mutants [53]. Moreover, the coccid form of H. pylori on the plasma membrane (latent) is resistant to antibiotics and can spread to infect other cells in the absence of effective antibiotic concentration. Therefore, the antibiotic concentration must be high and prolonged in time, not only for eliminating H. pylori extracellularly but also for penetrating into epithelial cells to eliminate intracellular H. pylori [53]. Additionally, proton pump inhibitors must accompany therapy regimens against H. pylori due to the fact that the efficacy of some antibiotics is diminished at low pH. Therefore, proton pump inhibitors are used with antibiotics in the eradication of H. pylori with higher cure rates than without them [54].

The H. pylori resistance profiles in both mucosae presented resistance from one to three antibiotics in 47 stomach isolates and 41 oesophagus isolates. This is the first study showing colonization of H. pylori from single colonies in the oesophageal mucosa. The diversity of resistance profiles allows us to confirm colonization by multiple strains of H. pylori of both inter- and intra-individual mucosa, which is also driven by the genetic variability of the cag genotypes found in single-colony isolates of H. pylori. The detection of strains with multiple resistance in individual hosts from Venezuela must be carried out to establish an effective treatment regimen. Clarithromycin and amoxicillin were the most effective antibiotics in this study, and they must be used with a proton pump inhibitor as first line therapy for the eradication of H. pylori infection.

When we evaluated the relationship between the integrity of the cag island and histopathological alterations, antral chronic gastritis was the most representative alteration accompanied by neutrophil and monocyte infiltration and bacteria. This indicates that cagA-positive island is able to induce an increased inflammatory response and mucosal damage. Assays carried out in vitro with three human gastric cancer cell lines have shown that CagA-positive H. pylori induces significantly more IL-8 secretion than CagA-negative strains [55]. Furthermore, CagA-positive H. pylori can also induce the secretion of IL-8 by oesophageal squamous carcinoma cells in vitro [56]. In this study, immunohistochemical detection of CagA showed marked expression, accompanied by increased induction of IL-8. There was a significant correlation between both expression in the
antrum, together with histopathological changes of the gastric mucosa associated with inflammatory processes.

CagA and IL-8 expression in the oesophageal biopsies was low with minor histopathological and inflammatory alterations, suggesting that there are fewer strains expressing CagA in the oesophagus and therefore less IL-8 is induced. This result might indicate that IL-8 secretion differs among H. pylori strains (complete or partial cag-PAI) and is dependent on the host epithelial cells. The association analysis between CagA and IL-8 in the gastric mucosa was marked, significant with dominance of a complete cag-PAI, while the association in the oesophagus was mild, nonsignificant, but with dominance of a partial cag island. Perhaps cagA-negative strains are more likely to colonize the oesophagus than cagA-positive strains. Presumably, cagA-positive strains that induce a high inflammatory response are likely to be eradicated. To confirm these results, new studies using quantitative methods are necessary for determination of IL-8 expression induced by CagA-positive H. pylori in gastric and oesophageal cell lines stimulated with gastric and oesophageal H. pylori isolates and to examine whether there are substantial differences among epithelia.

Finally, the results both of cag genotypes and antibiotic resistance profiles demonstrate multiple H. pylori isolate colonization in the oesophageal mucosa. H. pylori in the oesophagus was characterized by a dominant partial island, low IL-8 induction with low histopathological alterations and low antibiotic resistance, whereas a complete cag-PAI, high induction of IL-8 with greater histopathological damage, and higher antibiotic resistance was found in the stomach antral mucosa. These results challenge the contention of the relationship between H. pylori infection and oesophageal disease and, therefore, make more studies necessary to understand the relationship between the H. pylori colonization and gastroesophageal reflux disease, Barrett’s oesophagus and oesophageal adenocarcinoma.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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