# Genotyping of *cagA* and the intermediate region of *vacA* in strains of *Helicobacter pylori* isolated from Colombian adult patients and associations with gastric diseases

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## Abstract

**Objective:** This study characterizes the diversity of *cagA* and *vacA* virulence genes in Colombian patients to determine possible associations between them and the severity of endoscopic findings. It considers all four genotypes reported for the *vacA* gene (s, m and i). **Materials and methods:** *Helicobacter pylori* was detected in biopsies of 62 patients through culturing and by molecular methods. Genotypes of *cagA* and *vacA* (*m/i/s*) were determined by PCR and sequencing. **Results:** One hundred twenty four strains from 62 patients were isolated. Of these, 48.5% (n = 48) were *vacA s2/m2/i2 - cagA* (-) which were mostly found in patients with follicular gastritis; 32.3% (n = 32) were *vacA s1/m1/i1-cagA* (+) which were mostly found in patients with follicular gastritis, chronic gastritis and possible metaplasia. Significant associations were found between the presence of *cagA* and the *vacA s1/m1/i1* genotype and the absence of *cagA* and the *vacA s2/m2/i2* genotype ($p < 0.001$). No significant association was found between the severity of endoscopic findings and the *cagA-vacA* status of the strains. **Conclusion:** We found a low prevalence of *cagA* (+) strains, the *cagA-vacA* status is not a predictor of risk in this population. Moreover, the presence of heterogenous infections without tropism suggests a need for biopsies from both the corpus and the antrum of the stomach in routine clinical practice.

## Keywords

*vacA* subtypes, *cagA*, *Helicobacter pylori,* Colombia.

## INTRODUCTION

Helicobacter pylori are spiral-shaped, microaerophilic gram-negative bacteria that infect more than 50% of the world's population. (1) The infection has been associated with diseases such as gastritis, peptic ulcers, MALT (mucosa-associated lymphoid tissue) lymphoma and gastric adenocarcinoma. (2) H. pylori's most important virulence factors are the cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA). (3) Depending on whether the cagA gene is present, strains are classified as positive (type I) which is more virulent due to the visible gastric damage induced, and negative (type II) which is less virulent and acts more like commensal bacteria than pathogens. (4)

The VacA protein causes a series of alterations in human gastric cells that include formation of cytoplasmic vacuoles, (5, 6) permeabilization of the plasma membrane, (6) mitochondrial secretion of cytochrome c, (7) mitochondrial fragmentation, (8) activation of mitogen-activated kinases. (9) and induction of autophagy. (6) It has been classified as an immunomodulatory protein. (6, 10) The vacA gene has 3 polymorphic regions that are involved in the development of the disease: the signal region (s), the intermediate region (i), and the middle region (m). (2, 3, 6, 11-13)

The *s* region is involved in the efficiency of channel formation, and the *m* region affects tropism towards host cells. (3). Although the *i* region has only recently been described, it has been observed that the vacuolating activity of variant VacA *i*1 is stronger than that of subtype VacA *i*2. (3, 6) Some analyses

 

of the nucleotide and amino acid sequence of this gene have revealed polymorphisms grouped in clusters A, B and C. Of these, the amino acids in clusters B and C are responsible for the vacuolating activity of the protein. (11)

To classify a strain as *i1*, *i2* or *i3*, the amino acid sequence is compared with sequences of reference strains (60190 for *i1* and Tx30a for *i2*). The *i3* variant of vacA includes strains whose B cluster is similar to that of *i1* and whose C cluster is similar to that of *i2*. (11) Different combinations of the 3 regions have been described: vacA *s1/m1* strains have vacuolating activity and pose greater risk of gastric atrophy and adenocarcinoma than do the less virulent vacA *s2/m2* strains. (11, 12) vacA *i1* strains have also been associated with gastric adenocarcinoma. (2, 11, 12)

Recently, epidemiological studies have suggested that the interaction between cagA and vacA virulence factors, (6) factors of host susceptibility such as differences between men and women, (14-16) and environmental factors modulate development of the disease. (4, 12)

Several genotyping studies of H. pylori isolates from different Colombian populations have been published. Taking into account that the intermediate region can change the behavior of the toxin, this study complements data on genotyping of these two toxins including characterization of this region of vacA in isolates from a Colombian Andean population with gastric diseases of varying severity and evaluates correlations between genotype and the development of gastric disease.

## MATERIALS AND METHODS

### Patients and Study Population

We included 62 patients from Bogotá, Colombia who were over the age of 18 years, who were infected with H. pylori, and who had gastric symptoms and indications for endoscopy. Patients who had ingested proton pump inhibitors (PPI) within the 15 days prior to the procedure, antacids within the 12 hours prior to the procedure, or antibiotics in month prior to the procedure were excluded. Also excluded were patients suffering from cardiovascular and respiratory diseases, cancer patients who had undergone radiation therapy or chemotherapy in the six months prior to the procedure, and patients with coagulopathies or amyloidosis.

### Strains and DNA Purification

Biopsies were macerated and a $10^{-1}$ dilution was seeded on GC agar (Oxoid, Germany) supplemented with cholesterol (1X, Gibco) and DENT (Oxoid, Germany). (17) They were incubated at 37° C in a controlled atmosphere with 10% carbon dioxide ($CO_2$) for four to ten days. The Quick-gDNA Miniprep kit (Zymo Research, California, United States) was used to extract DNA in accordance with the manufacturer's instructions. The strains were selected by culture and confirmed as H. pylori by polymerase chain reaction (PCR) of the 23S gene with the HPY-S and HPY-A primers previously reported. (18)

### Detection of cagA

PCR was performed with the primers designed in this study and the previously reported protocol as modified and described in Table 1. (19) The PCR had a final volume of 25 µL with GoTaq Green Master Mix 1X (Promega, Wisconsin, United States ) and 0.25 mM of each primer. Visualization was performed on 1.5% agarose gels.

### vacA Genotyping

The 3 polymorphic regions of the vacA gene were characterized by means of PCR with a final volume of 25 µL containing GoTaq Green Master Mix 1X (Promega, Wisconsin, United States) and 0.25 mM of the corresponding primers for each region according to the previously reported protocols as modified in this study (Table 1).

For the visualization of the products, 2% agarose gels were used for the *m* and *i* regions and 10% polyacrylamide was used for the *s* region. (29: 1) Polyacrylamide gels were stained with a 3X GelRed solution (15 µL of Gelred 10,000X and 5 mL of sodium chloride [NaCl] 1M) for 1 hour under constant stirring at room temperature.

Samples that tested positive for both subtypes (vacA *i1* and *i2*) were sequenced (Macrogen, Seoul, Korea). Sequence analysis was performed with CLC Genomics Workbench 10, and amino acid sequences were compared with reference sequences 60190 (GenBank No. U05676) and Tx30a (GenBank No. U29401). Strains were defined as *i3* when the B cluster was similar to that of reference strain *i1* and the C cluster was similar to reference strain *i2*. (11)

### Statistical Analysis

Fisher's exact test was used to determine possible associations between the cagA status and the vacA genotype of the strains, and a generalized linear model was used to determine which variables had significant associations. The same statistical analysis was performed for polymorphic regions of the vacA gene and cagA status and between and among cagA status, vacA gene subtypes and severity of endoscopic findings. A chi squared ($\chi 2$) test was used to determine associations between the patient's sex and cagA status, and the patient's sex and severity of endoscopic findings. Endoscopic findings were classified as either

**Table 1.** Primers and protocol used for amplification of cagA and vacA

| Region | Primer | Sequence | Product size (bp) | PCR protocol |
|---|---|---|---|---|
| *cagA* | DR1 | GATAACAGGCAAGCTTTTGAGG | 179 | One 4 min cycle at 94° C, Thirty-two cycles at 94° C for 30 s, 52° C for 30 s and 72° C for 1 min, and one cycle of 72° C for 10 min (19) |
| | DR2 | CTGCAAAAGATTGTTTGGCAGA | | |
| *vacA s* (19) | Va1-F | ATGGAAATACAACAAACACAC | s1: 259 | One 4 min cycle at 94° C, Thirty cycles at 94° C for 30 s, 58° C for 30 s and 72° C for 1 min, and one cycle of 72° C for 10 min (19) |
| | Va1-R | CTGCTTGAATGC GCCAAAC | s2: 289 | |
| *vacA m1* (19) | Va3-F | GGTCAAAATGCGGTCATGG | 290 | One 4 min cycle at 94° C, Thirty cycles at 94° C for 30 s, 55° C for 30 s and 72° C for 1 min, and one cycle of 72° C for 10 min (19) |
| | Va3-R | CCATTGGTACCT GTAGAAAC | | |
| *vacA m2* (19) | Va4-F | GGAGCCCCAGGAAACATTG | 352 | |
| | Va4-R | CATAACTAGCGTCTTGCAC | | |
| *vacA i1* (2) | VacF1 | GTTGGGATTGGGGGAATGCCG | 426 | One 4 min cycle at 95° C, Thirty-five cycles at 95° C for 30 s, 55° C for 60 s and 72° C for 30 s, and one cycle of 72° C for 5 min (2) |
| | C1R | TTAATTTAACGCTGT TTGAAG | | |
| *vacA i2* (2) | VacF1 | GTTGGGATTGGGGGAATGCCG | 432 | |
| | C2R | GATCAACGCTCTGAT TTGA | | |

bp: base pairs

mild gastric disease and severe gastric disease (Table 2). R software (20) was used for analysis and a p value <0.05 was considered significant.

## RESULTS

### Patients and Isolation of H. pylori

We analyzed 124 H. pylori strains from 62 patients who had a variety of symptoms (Table 3). Their average age was 50.2 ± 16.2 years, and the male-female ratio was 1.48/1.

### Genotyping of cagA and vacA

PCR with corresponding primers established that 52 (48.1%) of the 124 strains analyzed were positive for cagA. No significant associations were found between the severity of endoscopic findings and the presence of cagA ($\chi 2$, $p > 0.05$) (Table 4). There were also no significant associations between the sex of the patients and the presence of cagA ($\chi 2$, $p > 0.05$), between the severity of the endoscopic findings and sex (Fisher's exact test, $p > 0.05$), or between the severity of the endoscopic findings and the presence of cagA ($\chi 2$, $p > 0.05$) (Table 4).

In the case of vacA, 16 strains were discarded (n = 8 patients) because it was not possible to determine the genotype for the *s* region in three cases (37.5%), the *m* region in three cases (37.5%), the *s* region in one case (12.5%) and for all three regions in one other case (12.5%). Of the remaining 108 strains, fifty strains were *s1*, fifty-eight were *s2*, forty-one were *m1*, and sixty-seven were *m2*. Thirty-four strains were *i1* and 55 *i2*. Nineteen of the samples sequenced (17.6%) showed amplification for both vacA *i1* and vacA

**Table 2.** Classification of endoscopic findings*

| Severity | Endoscopic findings (%) | Average age (years) |
|---|---|---|
| Mild | Suspected gastroparesis (1.61) | 89 |
| | Chronic and acute gastritis (12.9) | 53.7 ± 9.66 |
| | Chronic gastritis (16.1) | 58.8 ± 17.3 |
| | Follicular gastritis (27.4) | 39.8 ± 12.4 |
| | Petechial gastritis (4.84) | 48.6 ± 24.1 |
| Severe | Erosive gastritis (17.7) | 52.5 ± 14.6 |
| | Atrophic gastritis (6.45) | 58.5 ± 19.3 |
| | Gastric ulcer (1.61) | 58 |
| | Suspicion of metaplasia (11.2) | 45.1 ± 13.1 |

* Prevalence of endoscopic findings is shown with classification as either mild or severe according to medical criteria.

**Table 3.** Distribution of gastric symptoms reported by patients*

| Symptom | Number of Patients (%) |
|---|---|
| Epigastric pain | 37 (59.7) |
| Regurgitation | 32 (51.6) |
| Abdominal pain | 31 (50.0) |
| Sternal pain | 8 (12.9) |
| Dysphagia | 6 (9.68) |
| Postprandial filling | 10 (16.1) |
| Loss of appetite | 1 (1.61) |
| Weight loss | 9 (14.5) |
| Heartburn | 28 (45.2) |
| Reflux | 32 (51.6) |

* Patients reported various gastric symptoms before digestive endoscopy.

*i2* subtypes (GenBank No. MF457450 to MF457477). Of these, four were classified as *i1*, three as *i2*, three as *i3* and nine as *i1i2*. The last group was eliminated in subsequent analyses. No significant associations were found the severity of the endoscopic findings and either vacA subtypes or the full vacA genotype ($\chi 2$, $p > 0.05$) (Table 4).

Twenty-eight strains (22.6%) were discarded from the evaluation of the relationship between the two genes because they had different genotypes of cagA and vacA between antrum and corpus. No significant tropism or correlation was found between the genotype and location within the stomach of these strains. However, a significant association was found between cagA status and the vacA gene subtypes looked at separately (Fisher's exact test, $p < 0.05$) (Figure 1) and combined as follows: *s1/m1/i1*-cagA positive (32.3%), *s1/m2/i1*-cagA positive (2.02%) and *s1/m2/i2* (7.07%). Of these 71.4% were cagA positive, 2.02% were *s1/m1/i3*-cagA positive, 1.01% were *s1/m2/i3*-cagA positive, 2.02% were *s2/m1/i1*-cagA positive, and 48.5% were *s2/m2/i2*-cagA negative (Fisher's exact test, $p < 0.001$, generalized linear model binomial $p < 0.05$). In addition, all vacA *i3* strains were cagA positive.

## DISCUSSION

This study characterizes the diversity of virulence genes cagA and vacA in Colombian patients to determine possible associations between these two genes and the severity of the endoscopic findings. It considers all three genotypes reported for the vacA gene: *s, m* and *i*. The combination of genetic variability, host factors and environmental factors plays a fundamental role in the development of pathologies during H. pylori infections. (3, 4, 6, 12, 21-23). It has been reported that patients infected with vacA *s1/m1/i1*-cagA (+) strains are at greater risk of developing gastric carcinomas than are patients infected with the less virulent vacA *s2/m2/i2*-cagA (-). (2, 4, 11-13, 19, 24)

In the Colombian population studied, a prevalence of 48.1% was found for infections with cagA positive strains. This is lower than previously reported in studies conducted in Bogotá, Colombia which found a prevalence of 73%, (24) but it is similar to that reported in patients from Tolima, Colombia for whom prevalence of cagA positive strains was 43%. (25) The prevalence reported here also differs from those found in other regions of the world such as Senegal (73.3%), (26) the Middle East (100%), the United States (80%), and Western Europe and Latin America (60% to 70%). (11) This is probably due to the fact that the selected population was of medium and high socioeconomic class since it has been seen that the prevalence of the infection is higher in lower socioeconomic levels with poor sanitary conditions. (27, 28)

This study also shows that 22.6% of the patients were infected with two different strains of either or both cagA

**Table 4.** Association between severity of endoscopic findings, cagA status and polymorphic regions of the vacA gene*

| Severity of endoscopic finding | | Mild n (%) | Severe n (%) |
|---|---|---|---|
| *cagA* | Positive | 11 (27.5) | 6 (15.0) |
| | Negative | 15 (37.5) | 8 (20.0) |
| *vacA* | *s1* | 10 (25.0) | 8 (20.0) |
| | *s2* | 16 (40.0) | 6 (15.0) |
| | *m1* | 9 (22.5) | 6 (15.0) |
| | *m2* | 17 (42.5) | 8 (20.0) |
| | *i1* | 10 (25.0) | 6 (15.0) |
| | *i2* | 16 (40.0) | 8 (20.0) |
| | *s1/m1/i1* | 8 (20.0) | 6 (15.0) |
| | *s1/m2/i1* | 1 (2.50) | 0 (0.0) |
| | *s1/m2/i2* | 1 (2.50) | 2 (5.0) |
| | *s2/m1/i1* | 1 (2.50) | 0 (0.0) |
| | *s2/m2/i2* | 15 (37.5) | 6 (15.0) |

* No significant relationships were found between the severity of endoscopic findings and cagA or vacA status. Patients who had different genotypes in the gastric antrum and corpus were excluded. Fisher's exact test and a generalized linear binomial model ($p > 0.05$) were used.

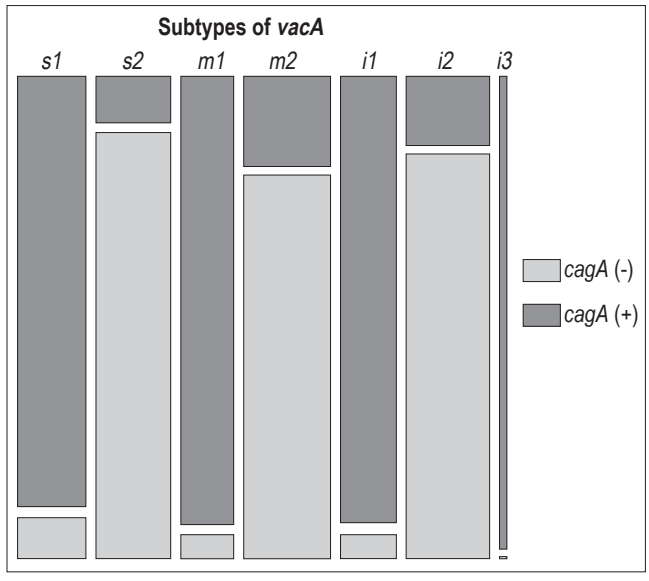


**Figure 1.** Association between cagA status and vacA subtypes. There is a significant association between cagA status and vacA subtypes. Patients with cagA status or vacA genotypes that differed between the antrum and corpus were excluded. Fisher's exact test and a generalized linear binomial model ($p < 0.001$) were used.

and vacA in the gastric antrum and corpus but without any tropism. It is possible that these patients have been infected on more than one occasion by different strains of H. pylori because the prevalence of multiple infections seems to be higher in places like Colombia where the risk of H. pylori infection is high. (29, 30) The presence of these types of infections reaffirms the need to routinely take biopsies from both the corpus and antrum.

The relationship between the presence of cagA and the vacA *s1* genotype has been described by several authors. (31, 32) In this study, 68.1% of positive cagA strains were vacA *s1/m1/i1* and only 6.4% were vacA *s2/m2/i2*. Thus, the vacA *s1/m1/i1* genotype has a significant association with the presence of the cagA gene. In the case of vacA *s1/m2* strains, it has been observed that whether the prevalence of type *i1* is greater that the prevalence of *i2* strains and vice versa depends on the geographical region. (2, 26) A low prevalence of the vacA *s2/m1* combination was found in this study. This agrees with previous findings which suggests that the low prevalence of this genotype may be explained by negative selection of this subtype because it is unfavorable for microorganisms. (26)

In addition, this study describes intermediate region of the vacA gene strains in strains circulating in this Colombian population. Almost all of the vacA *s1/m1* strains (94.4%) were *i1* and almost all the vacA *s2/m2* strains were *i2*. This agrees with what was reported in the first description of the intermediate region. (2) The majority of strains analyzed were vacA *i2*, and only three patients were found to be infected by vacA *i3* strains defined as those with cluster B similar to *i1* and cluster C similar to type *i2*. (11) The *i3* variant could reflect a recombination process between type *i1* and *i2* strains. (6, 11) The clinical or pathological importance of this variant remains unknown, and our study found no significant associations with endoscopic findings. However, all vacA *i3* strains were cagA positive. A larger sample of patients infected with *i3* strains is needed to gain a better understanding of the nature of this genotype and the development of the disease in these patients.

In contrast to the significant associations between the vacA *s1/m1/i1*-cagA (+) genotype and some severe digestive diseases such as atrophic gastritis, ulcers and gastric adenocarcinoma observed in other studies, (33) no significant associations were found between the cagA-vacA status of the strains and the severity of the endoscopic findings in this study despite the fact that most of the strains isolated were vacA *s2/m2/i2*-cagA (-) from patients who mostly presented follicular gastritis (Figure 2). This could be the result of the small sample sizes of each of the categories of severity of endoscopic findings (mild or severe) and the high prevalence of type II strains in the patients. For this reason, the vacA genotype cannot be used to identify high-risk patients in the study population.

In conclusion, this study provides new information on the prevalence of cagA and vacA virulence factors, and their association with endoscopic findings. Studies conducted on the west coast and in the southern Colombian Andes differ from the findings of this investigation highlighting the need to increase the number of studies that compare different regions and Colombian populations. (34, 35) This is especially true since the data published in Colombia show great variations in the presence of genetic markers of pathogenicity and their correlations with the severity of gastric pathologies.

On the other hand, recent studies have indicated that H. pylori strains in the Colombian population originating in the European line (HpEurope) have evolved independently. (36) It is likely that molecular mechanisms typically associated with severe gastric diseases associated H. pylori behave differently in the Colombian population thanks to the different ancestries present. (37) If the bacterium-host relationship is considered, it is possible that the lack of correlations between cagA and vacA and the pathology of the infection relative to the findings of other studies could be thoroughly investigated only if the ancestral distributions in the various regions of Colombia.

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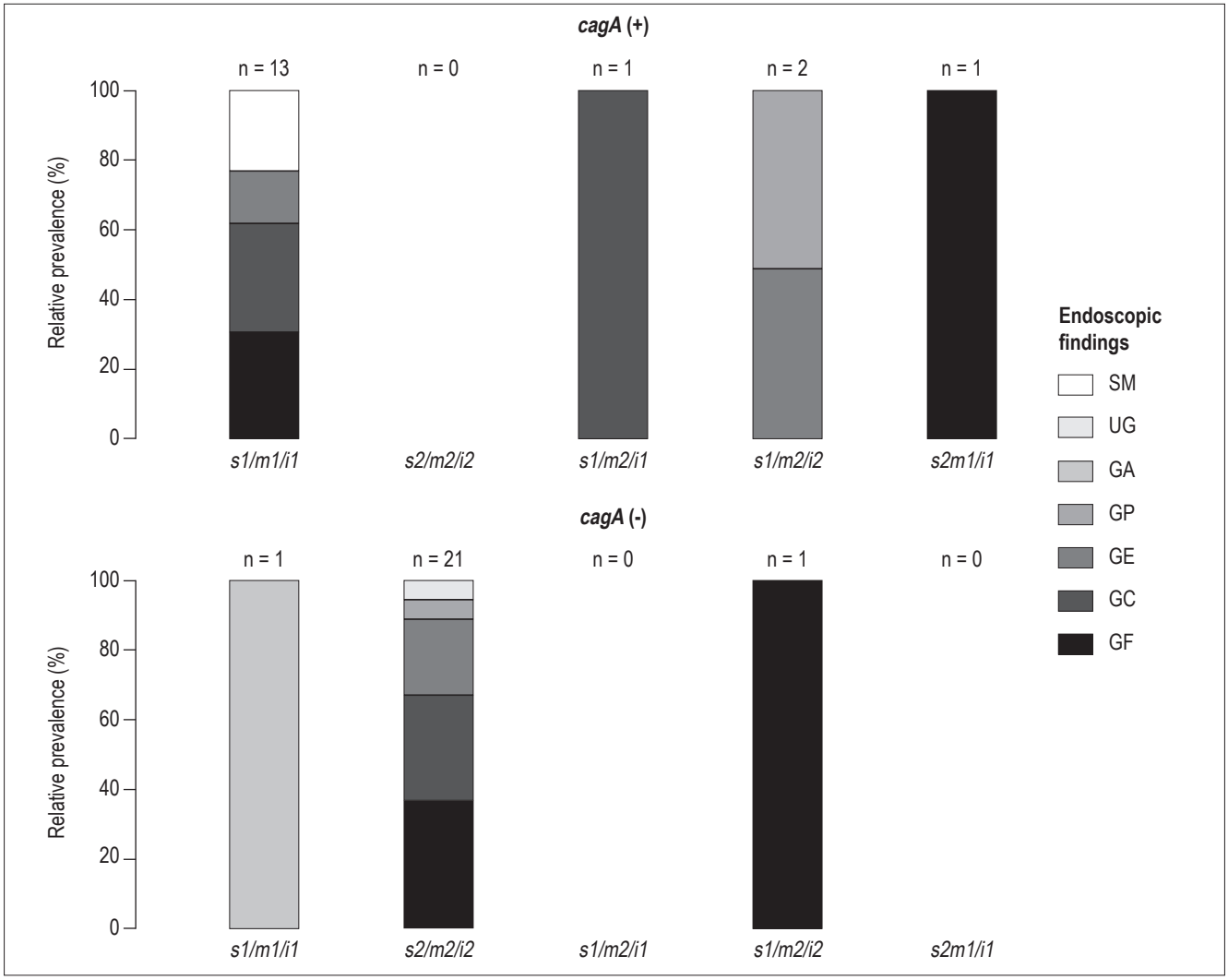


**Figure 2.** Relative prevalence of endoscopic findings according to the genotype of H. pylori strains isolated in these patients. 52.5% of patients were infected with H. pylori vacA *s2/m2/i2*-cagA negative strains which were mostly related to follicular gastritis. Patients who had different cagA or vacA genotypes in the antrum and corpus were excluded. Suspicion of metaplasia (SM), gastric ulcer (UG), atrophic gastritis (GA), petechial gastritis (GP), erosive gastritis (GE), chronic gastritis (GC) and follicular gastritis (GF).

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