vacA genotypes and EPIYA motifs of Helicobacter pylori in patients with atrophic and non-atrophic gastritis

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Santiago Escalante
Pontificia Universidad Catolica del Ecuador
lescalante@puce.edu.ec Corresponding Author

Cesar Navarrete
Pontificia Universidad Catolica del Ecuador

Daniela Nuñez
Pontificia Universidad Catolica del Ecuador

Jorge Reyes
Pontificia Universidad Catolica del Ecuador

Andres Zabala
Pontificia Universidad Catolica del Ecuador

Galo Pazmiño
Pontificia Universidad Catolica del Ecuador

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Abstract

Summary

Background

Helicobacter pylori is the main microorganism causing gastrointestinal diseases, such as chronic gastritis, peptic ulcer, MALT lymphoma, among others. The presence of the s1/m1 genotype of the vacA gene and EPIYA phosphorylation motifs of the cagA gene have been linked to the production of prolonged gastric inflammation. This study determines the presence of these virulence genotypes and their relationship with atrophic gastritis.

Methods

We included 231 patients with a history of dyspepsia undergoing upper gastrointestinal endoscopy. Samples of gastric tissue were taken to establish, through molecular techniques, the presence of H. pylori by amplifying the ureA and flaA2 housekeeping genes; in addition, the alleles of signal (s) and of the middle region (m) present in the vacA gene were amplified; and by sequencing the repeating patterns of the tyrosine phosphorylation motifs within the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs of the cagA gene were also amplified. A chi-square test was performed in order to establish the relationship between the virulence genes and the degrees of gastric injury.

Results

A total of (91/231) samples were positive for H. pylori, of which (57/91) amplified the cagA gene and (66/91) the vacA gene. 81.8% (54/66) of the positive samples for the vacA gene showed the combination of the s1/m1 alleles, associated mostly with atrophic gastritis (AG). The most frequent EPIYA motifs were ABC and ABCC, with 54.4% (31/57) and 40.4% (23/57) respectively. A relation of the genes with AG and its injury severity with a p>0.05 value was observed. The cagA+/vacA s1/m1+/EPIYA ABC pattern is found in most samples. A p=0.02 relationship was found between the presence of the vacA gene and the cagA gene.

Conclusions

The results show a higher proportion of gastric atrophy in patients infected with H. pylori. The sum of the pathogenicity factors such as the cagA+/vacA s1/m1+/EPIYA ABCC genotype increases the
virulence potential of the microorganism, suggesting that the coexistence of these genes could result in an increase in the severity of the progression of inflammation that leads to precancerous lesions.

Introduction

*Helicobacter pylori* is a Gram negative microaerophilic bacillus existing in between 35 and 70% of the population, depending on the geographical area (1); its ability to colonize in the acid environment of the gastrointestinal epithelium has allowed it to associate with different gastric and duodenal pathologies (2) that can range from gastric or duodenal peptic ulcer, to more severe cases where the infection contributes to the development of dysplasia, adenocarcinoma and lymphoma of lymphoid tissue associated with MALT mucosa (3).

Atrophic gastritis (AG) is a pre-neoplastic pathology; it usually starts with multifocal gastric atrophy (MGA), followed by type I or complete intestinal metaplasia, type II or incomplete intestinal metaplasia, dysplasia, and finally, the development of carcinoma (4), with a relative risk between 2.7 and 7 if they are associated with infection by *H. pylori* (5).

VacA vacuolizing cytotoxin, encoded by the gene of the same name - vacA, is a virulence factor present in almost all circulating strains of *H. pylori* (6), related to induced apoptosis, immunomodulation and vacuolization in gastric epithelial cells (7, 8). Its toxicity is associated with variation in its variable regions of signal (s-region), with its two variations, s1 and s2, in the middle one (m-region) with its variations, m1 and m2; and in the intermediate one (i-region) (9). The different combinations between variations have shown that the s1/m1 genetic variant is associated with diseases such as peptic ulcer; in addition, the prolonged inflammation produced by this cytotoxin leads to the development of gastric atrophy (10, 11).

The strains of *H. pylori* that have a cagA gene, translate a protein of the same name- CagA; this is translocated inside the gastric epithelial cells thanks to a Type IV secretion system, inducing a cascade of tyrosine phosphorylation dependent on Src and Abl kinases. The carboxy-terminal region known as tyrosine phosphorylation motifs contains amino acid sequences Glu-Pro-Ile-Tyr-Ala (EPIYA). The binding of different proteins to these EPIYA phosphorylation points allow the activation and inactivation of different signaling pathways that promote dynamic reorganization of the cytoskeleton.
and the activation of signaling pathways for cell proliferation (14, 15).

To this date, four types of EPIYA motifs have been described and have been named according to the sequence of amino acids close to the motif: EPIYA-A, EPIYA-B, EPIYA-C and EPIYA-D (16). These four motifs can be combined and, depending on these combinations, their virulent potential is known; the strains of *H. pylori* isolated in Western countries consist mainly of EPIYA-A, B, and one or more repetitions of segment C; whereas the East Asian strains mainly have a combination of EPIYA-A, B and D motifs (17,18). In the West, strains with multiple repetitions of the EPIYA-C motif have been associated with the formation of gastric adenocarcinoma.

In Ecuador, despite the fact that gastric cancer is the second in lethality in men and the third in women, information about factors that predispose the development of this pathology is scarce. The evidence presented that associates *H. pylori* as a carcinogenic agent (19) is clear; in addition, given that the prevalence is very high, this study identified the variants of the vacA gene and the EPIYA motifs, with the objective of expanding the genotyping data of *H. pylori*. These data allow expanding the knowledge of circulating strains and their relationship with precancerous pathologies such as atrophic gastritis.

**Materials And Methods**

**Patients**

A total of 231 persons over 18 years diagnosed with dyspepsia were involved in the study. Upper gastrointestinal endoscopies were performed in the Gastroenterology Department of a hospital in the city of Quito, from September 2016 to February 2017. Two samples of gastric biopsy were taken under the Department protocol for histopathological and molecular analysis. No patient ingested antibiotics or inhibitors of the proton pump, at least two weeks before the sampling.

**Histopathological diagnosis**

A biopsy sample was submitted to the Hospital's Pathology laboratory. Histological characteristics were determined by using the Sydney criteria (20).

**DNA Extraction**

The second biopsy was transported in saline at a temperature of 2-8°C (36-46°F) in order to be
processed in the laboratory. DNA extraction began using about 14 mg of tissue. The QIAamp DNA Mini® kit was used, following the manufacturer’s instructions. The sample was previously fragmented to obtain better results. The DNA concentration was determined by spectrophotometry using a NanoDrop® (Thermo Scientific).

Detection of *H. pylori* and characterization of *cagA* and *vacA* genes

The *ureA* and *flaA2* genes were used for the identification of *H. pylori* according to Smith et al (21), and Madhi et al (22). The protocol and primers used to determine the variants of the *vacA* and *EPIYA* motifs of the *cagA* were described in previous studies (21-24). In a final volume of 25µl, 0.2µM of the first forward and 0.4µM of the first reverse, 1X of GoTaq® Green Master Mix, 1µl of DNA with a concentration of approximately 50ng/µl were added. The PCR products were visualized on a 2.5% agarose gel with migration for 90 minutes at 90 volts, stained with GelStar™ Nucleic Acid Stain Lonza®, and comparing them with a molecular weight marker Promega® DNA Ladder of 100bp. The size of the PCR products were: *flaA2* (504bp), *ureA* (411bp), s1 variant (259bp), s2 variant (286bp), m1 variant (567bp) and m2 variant (642bp). In order to determine the EPIYA motifs the PCR product was sequenced by Macrogen Inc. Korea® and its analysis was carried out in the bioinformatics program Mega v.5.0®, using *cagA* of *H. pylori* (Access number NC_000915.1) as a reference.

Statistical analysis

Descriptive statistical analyzes were carried out. Pearson's chi-square test was used to analyze the statistical relationship between the severity of gastric injury, the *vacA* gene variants and EPIYA phosphorylation motifs of the *cagA* gene. A confidence level of 95% with p< 0.05 value was considered statistically significant.

Results

From the 231 patients with dyspeptic symptoms, 132 (57.1%) were men and 99 (42.9%) were women ranging in age from 18 to 85 years old; patients between 53 to 69 years old were the most frequent ones (44.1%). The histopathological study identified 113 patients (48.9%) with non-atrophic gastritis (NAG) and 118 patients (51.1%) with atrophic gastritis (AG); of these, 53 presented multifocal gastric atrophy (MGA) and 65 with intestinal metaplasia (IM).
The presence of *H. pylori* was detected in 39.4% (n = 91/231) samples, of which 50 corresponded to AG, and 41 to NAG. Figure 1A. The *vacA* gene was identified in 72.5% (66/91) of all biopsies for positive *H. pylori*; of these, 29 were related to NAG and 37 to AG, with a p-value > 0.05. The *s1/m1* genotype (Figures 1C and 1D) was more prevalent in AG, 17 cases in MGA and 14 in IM.

The *cagA* gene was detected in 62.6% (57/91) of all samples (Figure 1B). The EPIYA motifs were found in patients with both AG and NAG, the most frequent being the ABC motif with 54.4% (31/57) - Figure 2A (Access Number: MK558085); followed by ABCC with 40.4% (23/57) - Figure 2B (Access Numbers: MK558086, MK558083). Also: AABC - Figure 2C (Access Number: MK570156); ABA - Figure 2D (Access Number: MK558082); ABBCC - Figure 2E (Access Number: MK573557). The EPIYA motif with the highest number of repetitions is EPIYA-C, which was the most frequent in MGA (Table 2). However, it could not be concluded that there is a significant statistical relationship between the EPIYA motifs and the severity of gastric injury. It was observed a variation of A by T in the EPIYT-B motif (44%) - Figure 2F.

The combination of genotypes was found more frequent for *cagA+ vacA+ s1/m1+/EPIYA-ABC* with 26.4% (24/91), of which 6 are in patients with MGA, 7 in patients with IM and 11 in patients with NAG. The second most common genotypic combination was *cagA+/vacA+ s1/m1+/EPIYA-ABCC* present in 18.6% (17/91), of which 8 cases occurred in patients with MGA, 3 in patients with IM and 6 in patients with NAG; its relationship with the injury severity is p > 0.05 - Table 3.

**Figure 1**

**Figure 2. Alignment of the EPIYA motifs using the strain of Helicobacter pylori 26695 as a reference.**

A) Motifs EPIYA-ABC, B) Motifs EPIYA-ABCC, C) Motifs EPIYA-ABBCC, D) Motifs EPIYA-ABA, E) Motifs EPIYA -AABC, F) Mutations found in the EPIYA-A motif (red box indicates change from K to Q) and EPIYA-B (red box indicates change from A to T).

**Discussion**

Although the association between *H. pylori* and diseases such as chronic atrophic gastritis, peptic ulcer, or gastric cancer are not completely clear, there is strong evidence that the presence of the *vacA* and *cagA* virulence genes or its variants increase this relationship (25).
The general prevalence of *H. pylori* was of 39.4% in dyspeptic patients diagnosed with AG and NAG. Similar prevalence have been reported in Mexico (26) with 30.5%; and Brazil (2) with 57%. In Ecuador (Reyes et al.), with a similar population, 45% was reported by using conventional culture techniques to identify them (27). Although there are reports that in Latin America the prevalence is higher (70 and 80%) (28) due to sociodemographic factors. Burucoa et al suggests that the current prevalence has decreased due to health improvements (29).

The association between AG and the development of gastric cancer has been widely described (30). About two thirds of patients with AG have infection due to *H. pylori*; in many cases its progression towards a carcinogenic disease is irreversible (31). In our study it was found that *H. pylori* was present in 42.4% (50/118) of patients with atrophic gastritis; results similar to those reported by Vilar and Silva who reported 50.5% (32). In Asian countries such as Japan, this relationship is increasing; it has been reported between 54% and 70%, possibly influenced by its high population density (33, 34).

In our study, 72.5% of positive samples for *H. pylori* presented the *vacA* gene; of these, 81.8% corresponded to the s1/m1 alleles, being the most frequent genotype (54/66) associated to patients with gastric atrophy and intestinal metaplasia. In order to ensure its permanence in the gastric mucosa, the genetic information contained in the *vacA* gene allows *H. pylori* to evade the immune response. Its immunomodulatory role (35) prevents the maturation of phagosomes or prevents interaction with T lymphocytes; additionally, they have direct cytopathic effects on gastric cells, inducing cytotoxic vacuolization (36), causing gastric inflammation and consequently atrophy or intestinal metaplasia (37). However, the activation of oncogenic pathways is the most worrisome event; the evidence suggests that the presence of the s1/m1 genotype corresponds to the most virulent ones and is associated with the development of AG and later that of gastric cancer (38, 39), thus becoming risk markers (6, 40).

The *cagA* gene is one of the virulence factors with a prevalence between 60 and 70% of circulating strains (41); in our study it was 62.6%. The presence of EPIYA ABC and ABCC motifs associated with the *vacA* s1m1 genotype was found in patients with precancerous histopathological alterations such as multifocal gastric atrophy (14 patients) and intestinal metaplasia (11 patients). Gao suggests that
these two virulence mechanisms are associated (42) and therefore their virulent genotypes too.

The EPIYA motifs activate multiple signaling pathways in the host cell, depending on phosphorylation, such as: a) the family of Src kinases (SFK) that control the processes of mobility, differentiation and cell proliferation; they are also key players in the genesis of tumors; b) the family of Abl, c-Abl and Arg kinases that can directly phosphorylate EPIYA CagA motifs in late infection (10, 15, 43, 44). It also inhibits the pathways of the family of PAR1/MARK kinases that, together with apoptosis-stimulating protein p53-2 (ASPP2), prevents cellular apoptosis (45).

Apparently, the EPIYA motifs are the most important factors associated with the development of malignant neoplasms (16), showing that the higher the number of EPIYA-C repeats, the higher the probability of gastric atrophy, intestinal metaplasia and gastric cancer; thus, EPIYA-ABCCC has the capacity to activate in a higher proportion the growth factors, phosphorylation pathways, and inflammatory processes that contribute to intestinal metaplasia (46, 49). EPIYA-A, B and D have been described with higher prevalence in Asian regions (Japan, Korea and China), while EPIYA-A, B, C and their several repetitions are in the western region (Europe, North America, Latin America and Australia) (44, 47). In our study only EPIYA-C motifs were identified, corroborating the close relationship between the EPIYA motifs and the geographical area.

It is interesting to note that we identified the EPIYT-B motif, a mutation that suggests an EPIYA/EPIYT change, being the only mutation present in our study, with 44% of the analyzed samples. Zhang et al. (48) found EPIYT-B with 32.9%, proposing that this mutation has the ability to regulate the activity of CagA, thus interfering with the signaling pathways of the host related to the sequential process of cancer; therefore, it was found less associated with gastric pathologies such as cancer.

Despite the wide variability in the distribution of \textit{H. pylori} in the gastric mucosa, the use of molecular tests allows us to make a more accurate identification of the pathogenicity factors, by means of molecular sequencing techniques. Karlsson et al, for example, showed that the culture introduces a bias in the number of EPIYA motifs versus molecular determination directly done from gastric biopsy. (49).

Conclusion
This study suggests that the sum of the pathogenicity factors such as the cagA+/vacA s1/m1+/EPIYA ABCC genotype, increases the virulence potential of *H. pylori*, suggesting that the coexistence of these genes increases the severity of inflammation progression, which leads to pre-cancerous lesions such as intestinal metaplasia, which is a point of no return in carcinogenic progression. The findings from the Ecuadorian population present the first studies of the presence of virulent genotypes of *H. pylori*, evidencing a statistically significant relationship, $p=0.02$, between the presence of the cagA and vacA genes, suggesting that their virulence mechanisms do not act independently.

Abbreviations
DNA: Deoxyribonucleic acid; MGA: Multifocal Gastric Atrophy; Ala: Alanine amino acid; AG: Atrophic Gastritis; Glu: Glutamate amino acid; NAG: Non-atrophic Gastritis; Ile: Isoleucine amino acid; MALT: Mucosa Associated Lymphoid Tissue; IM: Intestinal metaplasia; PCR: Polymerase chain reaction; Pro: Proline amino acid; SHP2: Src homology region 2-containing phosphatase; Tyr: Tyrosine amino acid.

Declarations
Ethical approval and consent to participate

This study was approved by the ethics committee of the Pontifical Catholic University of Ecuador through official letter CEISH-191-2016; and by the ethics committee of the Specialties Hospital of the Armed Forces No. 1 through official letter No. 16-210-HE -1-5.

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Availability of data and materials
The set of generated and/or analyzed data during the study are not available to the public, because it contains patient identification data, but information can be requested from the corresponding author.
upon reasonable request.

Contribution of authors

NC - Collection of information and samples; analysis and interpretation of results; review and drafting of manuscript.

ND - Collection of information and samples; analysis and interpretation of results; review and drafting of manuscript

RJ - Design and conception; review and drafting of manuscript

ZA - Samples analysis, manuscript review

PG - Collection of information and samples, manuscript review

ES - Conception and design, manuscript review

All authors have read and approved the final manuscript

Conflict of interests

The authors declare no conflicts of interest in relevant competences for this study

Consent for publication

Does not apply

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Tables

| Table 1. Sequence of primers to identify H. pylori and amplify cagA and vacA genes |
|----------------------------------------|--------|--------|--------|
| Gen         | Primer sequence | Amplicon size | Tm°C | Reference |
| Urea        | F 5´GCCAATGGTAAATTAGTT3´ R 5´CTCCTTAATGTTTTTAC3´ | 411pb | 45°C | (21) |
|            | F 5´AGGGATTGCGTGTTAGC3´ R 5´AAATTCACCCTGGTTTCTG3´ | 504 pb | 55°C | (22) |
| cagA        | F 5´TGCTAAATTAGACAACTTGAGGCA3´ R 5´AATAATCAACACAAATCAGGCCAT3´ | 290 pb | 57°C | (23) |
| vacA s1/s2  | F 5´ATGGAAATACAACAACACAC3´ R 5´CTGCTTTAATGCGC3´ | 259/286 pb | 55°C | |
| vacA m1/m2  | F 5´CAAATCTGTCCACACACACACG3´ R 5´GGTCTAATACATTCCAAG3´ | 567/642 pb | 56.5°C | (24) |
| EPIYA       | F 5´GGAAACCTAGTCGTAAATG3´ R 5´ATCTTTGAGGCTTCTATCG3´ | 500-700pb | |
Table 2. Variants of the virulence genes of *Helicobacter pylori* in gastric biopsies of patients with gastritis

|              | GA n=118 | GNA= 113 | Total n=231 |
|--------------|----------|----------|-------------|
|              | AGM n = 53 | MI n=65 |             |
| Positive     | 28 | 22 | 41 | 91 |
| Negative     | 25 | 43 | 72 | 140 |

**vacA**

|              | GA n=50 | GNA= 41 | Total n=91 |
|--------------|----------|----------|-------------|
|              | AGM n = 28 | MI n=22 |             |
| Positive     | 20 | 17 | 29 | 66 |
| Negative     | 8 | 5 | 12 | 25 |

**vacA genotypes**

|              | GA n=37 | GNA= 29 | Total n=66 |
|--------------|----------|----------|-------------|
|              | AGM n = 20 | MI n=17 |             |
| s1/m1        | 17 | 14 | 23 | 54 |
| s2/m2        | 3 | 2 | 3 | 8 |
| s1/m2        | - | - | 3 | 3 |
| s2/m1        | - | 1 | - | 1 |

**cagA**

|              | GA n=50 | GNA= 41 | Total n=91 |
|--------------|----------|----------|-------------|
|              | AGM n = 28 | MI n=22 |             |
| Positive     | 17 | 12 | 28 | 57 |
| Negative     | 11 | 10 | 13 | 34 |

**EPIYA Motifs**

|              | GA n=29 | GNA= 28 | Total n=57 |
|--------------|----------|----------|-------------|
|              | AGM n = 17 | MI n=12 |             |
| ABC          | 7  | 7  | 17 | 31 |
| ABCC         | 10 | 4  | 9  | 23 |
| *AABC        | - | 1  | - | 1 |
| *ABB         | - | - | 1 | 1 |
| *ABBCC       | - | - | 1 | 1 |

* EPIYA motifs with unconventional repetition patterns, GA: atrophic gastritis, GNA: non-atrophic gastritis, AGM: multifocal gastric atrophy, MI: intestinal metaplasia
Table 3. Frequency of genotypes cagA region (EPIYA) and variants s/m of the vacA gen present in the biopsies with the of gastric lesion.

| Combination of genotypes                  | HISTOLOGICAL DIAGNOSIS |       |       |
|-------------------------------------------|------------------------|-------|-------|
|                                           | GA n=26                | AGM n=14(%) | MI n=12(%) |
| cagA+/vacA s1/m1+/EPIYA ABC               | 6(43)                  | 7 (58) | 11 (55) |
| cagA+/vacA s1/m1+/EPIYA ABCC             | 8 (57)                 | 3 (25) | 6 (30)  |
| cagA+/vacA s1/m1 +/EPIYA AABC            | -                      | 1 (8)  | -      |
| cagA+/vacA s1/m1+ EPIYA ABA              | -                      | -      | 1 (5)  |
| cagA+/vacA s1/m1+/ EPIYA ABCC            | -                      | -      | 1(5)   |
| cagA+/vacA s1/m2+/EPIYA ABCC             | -                      | -      | 1(5)   |
| cagA+/s2/m1+/EPIYA A ABCC                | -                      | 1(8)   | -      |
| Relación entre genes cagA y vacA          | *p=0.02                |       |       |
|                                           | GA n=11                | AGM n=6(%) | MI n=5(%) |
|                                           | cagA-/vacA s1/m1+      | 3 (50) | 3 (60) | 4 (44) |
|                                           | cagA-/vacA s2/m2+      | 3 (50) | 2 (40) | 3 (33) |
|                                           | cagA-/vacA s1/m2+      | -      | -      | -      | 2 (|
|                                           | GA n=3                 | AGM n=3(%) | MI n=0(%) |
|                                           | cagA+/vacA-/EPIYA ABC  | 1 (33) | -      | 6 (75) |
|                                           | cagA+/vacA-/EPIYA ABCC | 2 (67) | -      | 2 (25) |

*chi-square of Pearson, GA: atrophic gastritis, GNA: non-atrophic gastritis, AGM: multifocal gastric atrophy, MI: intestinal

Figures
Amplified genes of H. pylori present in gastric biopsy of patients with dyspepsia. A.

Identification genes: ureA with 411 bp of weight and flaA2 with 504bp of weight. B.

Pathogenicity gene: cagA with 289 bp of molecular weight. C. Pathogenicity gene: vacA, s1 allelic variant with 259bp of weight, and s2 with 286bp of weight, differentiated in the electrophoretic run D. Pathogenicity gene: vacA, m1 allelic variant with 567bp of weight, and m2 with 642bp. 2.0% agarose.
Alignment of the EPIYA motifs using the strain of Helicobacter pylori 26695 as a reference.

A) Motifs EPIYA-ABC, B) Motifs EPIYA-ABCC, C) Motifs EPIYA-ABBCC, D) Motifs EPIYA-ABA, E) Motifs EPIYA -AABC, F) Mutations found in the EPIYA-A motif (red box indicates change from K to Q) and EPIYA-B (red box indicates change from A to T).
