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Virulence characterization of *Helicobacter pylori* in Ecuadorian patients with gastric infection and gastric cancer

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RESUMEN

Helicobacter pylori es un espirilo Gram negativo y microaerófilo que coloniza el epitelio gástrico. Su colonización induce inflamación gástrica crónica en todas las personas infectadas. Sin embargo, solo entre el 10 y 20% de estas infecciones provocan enfermedades asociadas a *H. pylori*. La interacción entre la diversidad de genes de virulencia presentes en las cepas de *H. pylori*, la susceptibilidad genética del huésped y los estilos de vida u otros cofactores ambientales causan el desarrollo de enfermedades asociadas a *H. pylori*. El objetivo de este estudio fue caracterizar siete genes de virulencia de *H. pylori* en un total de 141 ecuatorianos infectados asintomáticos o con un estado clínico particular (síntomas dispépticos y cáncer) (65 hombres, 71 mujeres, edad media 49.4 ± 17.1 años, rango 18- 85 años) y su relación con la aparición de estos. La prevalencia de los genes de virulencia *cagA, vacA, iceA1, dupA, babA2, sabA* y *jhp0947* fue del 13,6%, 54,6%, 17,7%, 51,1%, 34,1%, 72,9% y 85,1% respectivamente. Solo los genes *sabA* (OR: 2.94, IC 95% 1.0-8.8) y *dupA* (OR: 2.2, IC 95% 1.0-4.9) se asociaron estadísticamente con síntomas dispépticos (valor *p:* 0.024 y valor *p:* 0.034, respectivamente) y ninguno de los siete genes estuvo relacionado con el cáncer gástrico. La asociación de la presencia de *H. pylori* y sus genes de virulencia con relación al cáncer gástrico debe estudiarse más a fondo en la población ecuatoriana con un mayor número de pacientes.

ABSTRACT

Helicobacter pylori is a Gram-negative and microaerophilic spirillum that is known to colonize the gastric epithelium. Its colonization induces chronic gastric inflammation in all infected people. However, only 10 to 20% of these infections generate diseases associated with *H. pylori*. The interaction between the diversity of virulence genes present in strains of *H. pylori*, the genetic susceptibility of the host and lifestyles or other environmental cofactors cause the development of diseases associated with *H. pylori*. The objective of this study was to characterize seven *H. pylori* virulence genes in 141 infected Ecuadorians with or without symptoms (dyspeptic symptoms and cancer) (65 men, 71 women, mean age 49.4 ± 17.1 years, range 18-85 years) and its relationship with these symptoms. The prevalence of the virulence genes *cagA, vacA, iceA1, dupA, babA2, sabA* and *jhp0947* was 13.6%, 54.6%, 17.7%, 51.1%, 34.1%, 72.9% and 85.1%, respectively. Only the *sabA* (OR: 2.94, 95% CI 1.0-8.8) and *dupA* (OR: 2.2, 95% CI 1.0-4.9) genes were statistically associated with dyspeptic symptoms (p-value: 0.024 and p-value: 0.034, respectively) and none of the seven genes was related to gastric cancer. The association of the presence of *H. pylori* and its virulence genes in relation to gastric cancer should be studied further in the Ecuadorian population with a greater number of patients.

CONTENT

TABLES

PART I

GENERAL INTRODUCTION

Virulence characterization of *Helicobacter pylori* in Ecuadorian patients with gastric infection and cancer gastric

1. STATE OF THE ART

Helicobacter pylori is a Gram-negative and microaerophilic spirillum that it is known to colonize the gastric epithelium (Malnick, Melzer, Attali, Duek, & Yahav, 2014). It is postulated that at least two-thirds of the world population is colonized by this bacterium, showing a higher proportion in inhabitants of developing countries (Ahmed & Sechi, 2005). *H. pylori* colonization in the gastric epithelium induces chronic gastric inflammation in all infected individuals (Uemura et al., 2001). Nonetheless, only 10 to 20% of these infections result in *H. pylori*-associated diseases, usually conditioned to the geographical area, as African and South Asia countries; where there is a high prevalence of *H. pylori* infection and low rates of gastric diseases such gastric cancer (Blaser & Atherton, 2004; Cervantes-García, 2016; Nguyen, Barkun, & Fallone, 1999; Shmuely et al., 2003).

The interaction among the diversity of virulence genes present in *H. pylori* strains, host genetic susceptibility, and lifestyles or other environmental cofactors remains unknown. However, its interaction of certain factors causes the development of *H. pylori*-associated diseases (Amieva & El-Omar, 2008; Atherton, 2006), such as gastritis, peptic ulcer, gastric atrophy, gastric adenocarcinoma and lymphoma mucosa-associated lymphoid tissue (MALT) (Chomvarin et al., 2008; Yoshio Yamaoka, 2010). These diseases produce a complex set of dyspeptic symptoms centered in the upper abdominal area, such as pain and heartburn, nausea, heaviness, and flatulence (Shmuely et al., 2003).

Several *in vivo* studies have shown that the toxic action of the *H. pylori* virulence gene products can alter the molecular composition of gastric epithelial cells, being able to induce several damage such as mutations of genes that regulate the cell cycle, deficiencies in the mechanisms

of DNA repair, loss of adhesive properties of the cell, and leading to cellular malignant transformation (Bartchewsky et al., 2009; Ishaq & Nunn, 2015).

2. GASTRIC CANCER

Gastric cancer is the main concern from all health problems produced by *H. pylori*, being the fifth most common cancer worldwide and the third cause of death related to cancer in both sexes (data available from the International Agency for Research on Cancer; GLOBOCAN2018, http://globocan.iarc.fr/). In 1994, the World Health Organization (WHO) characterized this bacterium as a class I carcinogen due to its association with the etiological role of gastric cancer (Ishaq & Nunn, 2015).

However, several studies showed epidemiological data that generate contradictions in some regions about its association with gastric cancer. A low incidence of gastric cancer has been reported in countries of Africa and South Asia, countries in which the prevalence of *H. pylori* is extremely high in the population (Nguyen et al., 1999). Therefore, it is postulated that cancer development is dependent on certain components of the *H. pylori* strains among other factors (Wang, Liu, & Gao, 2015).

In Ecuador, the age-standardized incidence rate (ASR) of gastric cancer has been reported to be around 13.8/100.000, which is one of the highest incidence rates in the Latin-American Andean region (Ecuador, Colombia, Peru, and Chile). Gastric cancer possesses the highest mortality in Ecuador, followed by cancer of the cervix in women and prostate cancer in men (data available from the International Agency for Research on Cancer; GLOBOCAN2018, http://globocan.iarc.fr/). In 2015, several public hospitals across the country reported a prevalence of *H. pylori* ranging 45 to 47% in the Andean region and 85% in the coastal region of Ecuador (World Health Organization, 2015). These results were obtained through immunochromatographic and histopathological tests. In a recent molecular studyconducted by

our research group was determined that this prevalence is found in 56.9% of patients from the three Ecuadorian regions (Machado & Valenzuela, 2016).

However, it is still known very little about the association between the prevalence of *H. pylori* and its virulence genes and how this relation is involved with the incidence of gastric cancer in Ecuador, therefore, further studies are necessary to evaluate this relation.

3. *H. pylori* VIRULENCE FACTORS

Bacterial virulence factors are usually defined as a set of molecular mechanisms that allow a microorganism to infect a host, reproduce, invade tissues eluding the host's defenses and cause disease (Cross, 2008). Furthermore, in 2008, Yamaoka (2008) has proposed that virulence factors in *H. pylori* must obey the following criteria: 1) to have an *in vivo* correlation with any disease; 2) be epidemiologically consistent between populations and regions; 3) be biologically plausible (when deleting genes the virulence effect should be eliminated or attenuated). Nowadays, the main virulence gene factors already studied are genes related to their adhesion ability to the gastric epithelium (*babA*2 and *sabA* genes); the region of plasticity (such as *jhp0947* and *dupA* genes); and cell signaling and tissue damage (*iceA*1, *vacA* (s1) and *cagA* genes).

3.1. ADHERENCE TO GASTRIC EPITHELIUM

The survival of *H. pylori* in the human gastric epithelium depends on its adaptation to different biological stages, ranging from colonization and persistence due to the evasion of the immune system to its subsequent transmission to another host (Proenca-Modena, Acrani, & Brocchi, 2009). The outer membrane proteins (OMP) help to overcome some of these stages interacting with the hostile environment surrounding the bacteria, mainly in the early stages of infection (Odenbreit, 2005). The adhesins facilitate interaction with cellular receptors (Evans & Evans, 2000). Among them, the products of *babA* and *sabA* genes and have been recognized to effectively bond against cell receptors (Yoshio Yamaoka, 2008b).

3.1.1. *babA* gene

In 1993, Boren and his colleagues found that Lewis b blood group antigen (Le^b) acts as a receptor for *H. pylori*-specific adhesin and controls its binding to the gastric cavity and superficial mucosal cells (Pride, Meinersmann, & Blaser, 2001). This antigen showed to be a dominant antigen in the gastric mucosa of secretor-positive individuals and also expressed in erythrocytes and human epithelial cells (Agudo, 2010; Yoshio Yamaoka, 2008b). In 1998, the 78 K adhesin recognized by the Le^b antigen was detected on the outer bacterial membrane and designated as blood antigen-binding adhesin (BabA)(Ilver et al., 1998). Subsequent studies revealed that the protein with antigenic binding activity is encoded by the *babA* gene. Three *bab* alleles have been recognized: *babA*1, *babA*2, and *babB*; of which *babA*2 is the most common and functional allele (Colbeck, Hansen, Fong & Solnick, 2006).

BabA protein plays an important role in the adherence and adaptation of *H. pylori*, as well as in its persistence on the gastric epithelium (Olfat et al., 2005). Recently, a research group showed that the binding of BabA to the gastric receptor Le^b is sensitive to acid, showing an adhesion reduction to low pH; which could be restored by acid neutralization (Bugaytsova et al., 2017).

Additionally, BabA protein appeared to be involved in the delivery of other virulence toxins (CagA and VacA), which could contribute to increased damage to the gastric tissue. Several studies have shown that the presence of his adhesin is closely associated with the most severe disease outcomes, such as duodenal ulcer and adenocarcinoma (Olfat et al., 2005; Proenca-Modena et al., 2009).

3.1.2. *sabA* gene

A high degree of inflammation of the gastric epithelium is caused by the colonization of *H. pylori*. This inflammation promotes the replacement of Lewis b blood group antigen (Le^b receptor) by

sialyl-Lewis x/a antigens (sLe^x and sLe^a). SabA adhesin has been grouped into OMPs, showing the ability to bind to these sLe^x and sLe^a antigens (de Jonge, Pot, et al., 2004).

In healthy individuals, sialylated glycoconjugates are scarce in the gastric mucosa; however, persistent colonization of the human stomach with *H. pylori* leads to severe inflammation of the gastric mucosa and the replacement of Lewis antigens naturally produced by sialylated glucans(Yoshio Yamaoka, 2008a). Unlike Le^b receptors, which bind strongly to BabA adhesin, sLe^x antigen protrude less at the cell surface so binding to SabA is weaker and closer, therefore it can benefit *H. pylori* by allowing escape from the sites where the bactericidal host defense responses are most vigorous(Mahdavi et al., 2002).

Additionally, microarray studies showed that the expression of SabA is dependent on the intragastric pH gradient, showing increased expression in gastric cancer patients and a reduced expression in patients with duodenal ulcer (Merrell, Goodrich, Otto, Tompkins & Falkow, 2003; Yamaoka, 2008a). These microarray studies showed a drastic decrease of *SabA* expression under hyperacidity conditions.

3.2. CELL SIGNALING AND TISSUE DAMAGE

3.2.1. *cagA* gene

The *cagA* gene codes for the most studied *H. pylori* virulence factor. This gene is generally used as a virulence marker for the island of pathogenicity cag (cagPAI) presence in *H. pylori* strains. This pathogenicity island contains about 30 genes that, together, encode for proteins that form a secretory system type IV (T4SS). This T4SS is used to export bacterial products to the cytoplasm of host cells, such as the CagA protein (Backert & Selbach, 2008).

CagA protein has been associated with severe diseases such as gastritis, peptic ulcer, atrophic gastritis, and gastric cancer, especially in Western countries. Therefore, clinical isolates of *H.*

pylori have been classified according to CagA status, more exactly, in CagA-producing strains (*cagA*-positive) and CagA-non-producing strains (*cagA*-negative). *In vitro* and *in vivo* studies showed its oncogenic activity, which positively influences the appearance of gastric cancer (especially in Western countries). However, in East Asia, the presence of the *cagA* gene in *H. pylori* isolates of patients without relation to any specific disease. This divergence between Western and East Asian countries could be explained by the fact that CagA activates the family of Src-family kinases and Alb kinases present in epithelial cells, which phosphorylate CagA at the C-terminal sites called EPIYA, by the certain amino acid sequence that it possesses (Glu-Pro-Ile - Tyr-Ala)(Segal, Cha, Lo, Falkow, & Tompkins, 1999). Variants have been detected in these EPIYA motifs according to the amino acids that flank them, classifying them into EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D. In Western *H. pylori* strains, these motifs generally have repeats of the EPIYA-A and EPIYA-B variants followed by EPIYA-C repeats, whereas in *H. pylori cagA* positive isolates from Asian patients it is more common to find EPIYA-D repeats after EPIYA-A and EPIYA-B (Yoshio Yamaoka, 2010).

The most studied molecular target of phosphorylated CagA is SHP-2, a tyrosine phosphatase, which possesses specific binding sites to phosphorylated tyrosines (Higashi et al., 2002). In the gene encoding SHP-2, mutations and polymorphisms have been found, which are related to gastric carcinogenesis (Higashi et al., 2002). This CagA-SHP-2 interaction triggers the activation of different cascades of altered signals which could contribute to abnormal cell development, promoted by cell cytoskeleton rearrangement, excessive cell proliferation, elongation and motility of epithelial cells and provoke apoptosis (Cervantes-garcía, 2016; Higashi et al., 2002). When not phosphorylated, CagA forms interact with several molecules, such as the adapter protein Grb2 and the PAR1 kinase, altering cell polarity and promoting cell proliferation and inflammatory response (Akopyants & al., 1998).

3.2.2. *vacA* gene

VacA toxin is the second most extensively studied *H. pylori* virulence factor worldwide. The main function of this toxin is to induce vacuolization in the epithelial cells, allowing several virulence mechanisms such as the release of hydrolases and cytochrome *c*, degradation of exogenous ligands, alteration of cell polarization, and induction or inhibition of apoptosis (Cover & Blanke, 2005; Willhite & Blanke, 2004). All these cellular effects trigger an acute inflammatory response and interact with cellular lineages of the immune system, inhibiting T cell activation and proliferation (Boncristiano et al., 2003).

The *vacA* is a well-known polymorphic gene and its protein is composed of two subunits (p33 and p55). Apparently, the *vacA* gene is present in all strains of *H. pylori* and the level of toxicity of VacA varies due to the allelic combination between the signal sequence (s1 or s2) and middle region (m1 or m2) and intermediate (i1 or i2) region(Atherton et al., 1995; Rhead et al., 2007). Several studies have shown that the s1/m1 genotype is more toxic than s1/m2, whereas the s2/m2 strains lack cytotoxic activity and the s2/m1 genotype rarely occurs(Yoshio Yamaoka, 2010). In addition, some countries of Western, Latin American, Middle Eastern and African have reported that patients infected with s1 genotype have a higher risk of peptic ulcer or gastric cancer, whereas a short N-terminal peptide extension in the mature protein of s2, blocks the biological activities of VacA (Letley et al., 2009; Van Doorn et al., 1998).

3.2.3. *iceA* gene

The epithelial contact-induced gene (*iceA*) was firstly described by Peek et al. (2000), showing the existence of two allelic variants *iceA*1 and *iceA*2. The allelic variants *iceA1* and *iceA2* are flanked upstream by the *cysE* gene encoding a protein homologous to a serine acetyltransferase

and downstream by the *hpy1M,* a CTAG-specific methyltransferase gene in *H. pylori*(Peek et al., 1998).

Unlike *iceA2, iceA1* have high homology (66%) with a *Neisseria lactamica* type II restriction endonuclease (*nlaIII*R)(Kidd et al., 2001) and increased expression of *hpy1M*, which suggests that the association *iceA1-hpy1M* form a system of a restriction-modification unit (RM)(Xu et al., 2002). These systems are traditionally composed of a methyltransferase that catalyzes the methylation of DNA and protects it from cleavage by the restriction enzyme. Thus, RM units serve as barriers against gene mobilization and phage infections, since foreign DNA entering the bacterium cannot be methylated and thus it is recognized and cleaved (Proenca-Modena et al., 2009; Xu et al., 2002). However, several deletions and insertions were reported in *iceA1* gene in *H. pylori* strains produce truncated proteins that do not have endonuclease activity but act as regulators of the RM system (Figueiredo et al., 2000). Thus, *iceA1* is nowadays considered a virulence factor due to it is induced after the contact of *H. pylori* with the gastric epithelium (Kidd et al., 2001). In addition, several epidemiological studies demonstrated an association between the presence of *iceA* and the development of gastric and duodenal ulcers (Kidd et al., 2001). In contrast, it is suggested that *iceA* shows a geographical variability but not a relationship with a specific gastric disease (Caner et al., 2007; Donahue et al., 2000; Podzorski, Podzorski, Wuerth, & Tolia, 2003; Yoshio Yamaoka et al., 1999).

3.3. PLASTICITY REGIONS

In the *H. pylori* genome, there are several regions with low C-G content, when compared to the remaining genome, suggesting horizontal gene transfer from other bacterial species (Mitsushige Sugimoto, Watada, Jung, Graham, & Yamaoka, 2012). These regions are called "regions of plasticity", which are present in half of the genes from sequenced *H. pylori*-specific strains (Mitsushige Sugimoto et al., 2012). The *jhp0947* and *dupA* genes are present in these highly

variable genomic regions and have been associated with gastric cancer and duodenal ulcer respectively (Santos et al., 2003; Ganguly et al., 2016).

3.3.1. *Jhp0947* gene

The *jhp0947* gene has been frequently reported as a potential disease marker due to its detection in *H. pylori* strains from patients with gastric cancer (Occhialini et al., 2000). Although its function is still unknown, the 5 'region of *jh0947* is homologous to the *jhp0477* gene, which is part of the cag pathogenicity island (PAI), and works like a structural component of the *H. pylori* T4SS (de Jonge, Kuipers, et al., 2004). Also, the *jhp0947* gene has been associated with gastric cancer and duodenal ulcer in Western countries but it is still not related in East Asian countries (de Jonge, Kuipers, et al., 2004; Mitsushige Sugimoto et al., 2012). This contradiction analysis could suggest that the presence of this gene in *H. pylori* strains may vary on the geographical region and population (Proenca-Modena et al., 2009). In addition, it has been reported that *jhp0947* together with jhp0940, jhp0945 and jhp0949 in *H. pylori* strains in western countries could be associated with an increase in interleukin IL-8, IL-12, tumor necrosis factor alpha (TNF-α), and gamma interferon (INF-γ); triggering a strong inflammatory response in the gastric epithelium (Tobnagh, Bakhti, Navid, Zahri, & Bakhti, 2017)(de Jonge, Kuipers, et al., 2004).

3.3.2. *dupA* gene

This gene is composed of two open reading frames (*jhp0917* and *jhp0918*) and it had been shown to be homologous to the *virB4* gene of *Agrobacterium tumefaciens*, a gene encoding a component protein of the type IV secretion system in this bacterium (Lu, Hsu, Graham, & Yamaoka, 2005). Therefore, it is possible that another T4SS system exists outside the cag

pathogenicity island; however, more studies are needed to understand the role of this gene in duodenal ulcer or another clinical outcome (Lu et al., 2005; Shiota, Matsunari, Watada, Hanada, & Yamaoka, 2010).

The dupA is recognized as the first virulence factor of *H. pylori*-associated with differential risk for duodenal ulcer and gastric cancer (Shiota et al., 2010). A high prevalence of the *dupA* gene was detected in patients with duodenal ulcer while a low prevalence of the gene was found in patients with gastric cancer (Shiota et al., 2010). However, this relationship is only found in Asian and some of the Latin American countries (Colombia and Brazil) (Shiota, Suzuki, & Yamaoka, 2013).

PART II

SCIENTIFIC PAPER

I. INTRODUCTION

Helicobacter pylori is a Gram-negative and microaerophilic spirillum that it is known to colonize the gastric epithelium (Malnick et al., 2014). It is postulated that at least two-thirds of the world population is colonized by this bacterium, showing a higher proportion in inhabitants of developing countries (Ahmed & Sechi, 2005). *H. pylori* colonization of the gastric epithelium induces chronic gastric inflammation in all infected individuals (Uemura et al., 2001); however, only 10 and 20% of these infections result in *H. pylori*-associated diseases, also conditioned to the geographical area, as evidenced in African and South Asia countries where there is a high prevalence of *H. pylori* infection and moderate to low rates of gastrointestinal diseases (Blaser & Atherton, 2004; Nguyen et al., 1999; Shmuely et al., 2003).

The interaction between the diversity of virulence genes present in *H. pylori* strains, host genetic susceptibility, lifestyles or others environmental cofactors, cause the development of *H. pylori*-associated diseases (Amieva & El-Omar, 2008; Atherton, 2006), such as gastritis, peptic ulcer, gastric atrophy, gastric adenocarcinoma and lymphoma (Chomvarin et al., 2008; Yoshio Yamaoka, 2010). These diseases produce a complex set of dyspeptic symptoms centered in the upper abdominal area, like pain and heartburn, nausea, heaviness, and flatulence (Shmuely et al., 2003).

Gastric cancer is the most studied of the disorders produced by *H. pylori* due to it is the fifth most common cancer worldwide and the third cause of death related to cancer in both sexes data available from the International Agency for Research on Cancer; GLOBOCAN2018, http://globocan.iarc.fr/). In 1994, the WHO characterized the bacterium as a class I carcinogen because its association with the etiological role of gastric cancer (Ishaq & Nunn, 2015).

Several *in vivo* studies have shown that the toxic action of the *H. pylori* virulence gene products can alter the molecular composition of gastric epithelial cells, being able to induce serious damage, such as: mutations of genes that regulate the cell cycle; deficiencies in the mechanisms of DNA repair; loss of adhesive properties of the cell; and leading to cellular malignant transformation (Bartchewsky et al., 2009; Ishaq & Nunn, 2015). The main virulence gene factors already studied nowadays are genes related to their adhesion ability to gastric epithelium (*babA*2 and *sabA* genes), the region of plasticity (*jhp0947* and *dupA* genes), and cell signaling and tissue damage (*iceA*1, *vacA* (s1) and *cagA* genes)(Proenca-Modena et al., 2009).

The aim of this study is to characterize the mentioned virulence genes of clinical *H. pylori* strains in Ecuadorian patients and their relationship with the appearance of dyspeptic symptoms and gastric cancer.

II. MATERIAL AND METHODS

1. Patient data

From June 2015 to June 2016, a total of 246 asymptomatic or with particular clinical status (dyspeptic symptoms and cancer) patients (111 men, 130 women, mean age 49.4 ± 17.1 years, range 18-85 years) were submitted to gastric biopsies extraction at different health centers of the country (Quito: Eugenio Espejo Hospital, Sociedad de Lucha contra el Cáncer-SOLCA-Quito, particular health centers; Guayaquil: SOLCA-Guayaquil; Cuenca: Hospital José Carrasco Arteaga). Each subject was informed about the procedure, answered an epidemiological survey (personal, socioeconomic, nutritional and clinical data) and signed the informed consent 2014-130M approved by the Institutional Review Board of the Universidad San Francisco de Quito (USFQ). Underage patients and those who underwent antimicrobial treatments in the past three months were excluded from this study.

2. Primer design

The *in-silico* primer design for each gene analyzed (*cagA, vacA* (s1), *iceA1, dupA, babA2, sabA* and *jhp0947*) was based on the alignment of genomic sequences obtained at NCBI (National Center for Biotechnology Information) using the CLUSTAL W2 version 2.0 (Larkin et al., 2007), which demonstrated common conserved regions that served as targets for primer design in Primer3 software version 0.4.0. (Untergasser et al., 2012). The main selection criteria for each pair of primers were: high complementarity and specificity with the target sequence, low complementarity with non-target sequences, melting temperature between primers (Forward/Reverse), absence of self-complementarity to avoid the formation of dimers, or sub-products Unwanted (Machado et al., 2013). These criteria were analyzed using the software mFold Web Server(Zuker, 2003), nBLAST (Basic Local Alignment Search Tool, NCBI, 2015) and Probe Match(Kim et al., 2009). The primers used in this study were synthesized by Invitrogen (USA) and listed in Table 1.

3. Gastric Biopsies collection

A gastroenterologist through a gastric endoscopy in clinical control consultations extracted stomach biopsies. From each individual, a sample of the stomach of antrum and corpus (Lee & Kim, 2015) was taken and separately placed in 10 mL plastic tubes with methanol and immediately stored at -20 °C until further DNA extraction.

4. DNA Extraction

DNA extraction was adapted from a previously published method (Mejía, Muñoz, Trueba, Tinico, Zapata, 2015). Briefly, gastric biopsy specimens immersed in methanol were centrifuged for 5 min at 766 relative centrifugal force (RCF) obtain the cell pellet; the procedure was performed in duplicate, removing the supernatant after each centrifugation. Subsequently, additional washes were performed in triplicate by adding 500 µl of 1X PBS (137mM NaCl, 2.7mM KCl,

4.3mM Na 2 HPO 4 .7H 2 O, 1.4mM KH 2 PO 4, pH 7). To lyse the cells, the cell pellet was suspended in 700 µl of CTAB (cetyl trimethyl ammonium bromide) solution (2% CTAB w / v, 1.4 mM NaCl, 20 mM EDTA pH 8, 100 mM HCl pH 8) and incubated for 2 hours at 65°C with shaking (Gramley, Asghar, Frierson, & Powell, 1999). Then, 20 µl of proteinase K was added to digest the sample. In order to separate DNA from cellular products and proteins, 700 µL of chloroform: isoamyl alcohol (24: 1) was added, mixing strongly to produce an emulsion, which was centrifuged for 5 minutes at 14385 RCF. 500 µl the aqueous supernatant phase was transferred to a new 1 mL plastic tube and precipitated with 1000 µL of 100% ethanol, mixing gently. The solution was stored for 24 hours at -20 ° C. To obtain the DNA pellet, the solution was centrifuged for 15 minutes at 14,385 RCF, washed with 70% ethanol and again centrifuged for 15 minutes at 14,385 RCF. The supernatant was discarded and the DNA pellet suspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8, 0.1 mM EDTA) and then stored at -20 ° C. The DNA concentration was quantified and the purity of extraction sample was evaluated (phenolic contamination: 260/230; protein contamination: 260/280nm) by using the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) according to the manufacturer's instructions.

5. Detection of Virulence Genes by PCR

A total of 141 from 246 samples were identified as *H. pylori*-positive in a preliminary analysis How? Histopathology? From this sample set, the *cagA, vacA-s1, iceA1, dupA, babA2, sabA* and *jhp0947* virulence genes of the *H. pylori*-positive samples were amplified by the polymerase chain reaction (PCR) in a final volume of 20 µL, containing 20 ng/ µL of DNA template, 1X PCR buffer, 2.5 µM of MgCl2 (2 µM for *cagA* and *babA*2, 2,15 µM for *jhp0947*), 0.25 mM of dNTPs mix, 0.05 U/µL of GoTaq DNA polymerase (Promega, USA) and 0.4 µM of each forward and reverse primer, except for the *iceA1* gene, for which a concentration of 0.2 µM of each primer was used. The amplification was carried out in a T100™ Thermal Cycler (Bio-Rad, USA) according to the following program: an initial denaturation step at 94 °C for 2 min, followed by 40 cycles

(37 cycles for *iceA* and *babA*) of denaturation at 94 °C for 30 s, annealing at primer specific temperature (shown in Table 1) for 30 s and elongation at 72°C for 1 min; followed by a final extension step for 5 min at 72 °C. Amplified PCR products were resolved on 1% agarose gel using 1X TBE and 0.5 µL/mL of ethidium bromide and were revealed in Gel Doc™ XR System (Bio-Rad, USA) (Essawi Tamer, Hammoudeh Wail, Sabri Israr, Sweidan Walid, 2013). To determinate the size of the bands, molecular size ladder of 100 bp (Promega, USA), positive controls were obtained from colleagues of University of Minho from Portugal and negative controls were PCR water.

III. STATISTICAL ANALYSIS

The presence or absence of each clinical picture was evaluated with respect to the presence of virulence factors in *H. pylori* infective strain, using the chi-square (x2) statistical test. The univariate logistic regression analysis was used to determine the risk (OR) of carrying such factors and their different combinations (genotypes) in relation to the clinical picture. A *p*-value <0.05 was considered statistically significant. These estimations were performed with STATA 14.0 version software (Chiurillo et al., 2013; Godoy et al., 2003). Microsoft Office Professional Plus Excel 2010 was used to calculate the frequency distribution of combinations of *H. pylori* genotypes (Vasco et al., 2014).

IV. RESULTS

1. *In silico* analysis of the primers for virulence genes

The evaluation of the design of primers through several computer tools, demonstrated a high *in silico* complementarity and specificity with the target sequence of each gene analyzed. The primer pair showed complementarity with more than a hundred sequences from each gene evaluated in BLAST. Likewise, the analysis performed in Probe Match did not show any compatibility with 16S-rRNA sequences loaded in this database. Additionally, the Gibbs energy values presented in the mFold software was positive and mostly greater than one,

demonstrating a low probability that the designed primers fold on themselves and forming undesired dimers or sub-products (Table 1).

Table 1 PCR primers for amplification of *cagA, vacA (s1), iceA1, dupA, babA2*, *sabA* and *jhp0947* sequences.

Genes		Sequence (5'-3')	Annealing (°C)	PCR product (pb)	Identification[a] (%)	ΔG[b] (kcal/mol)	16S-rRNA[c] compatibility
cagA	F	tcgctgacaaggatcccaat	63	215 bp	100%	0,88	None
	R	cgcattaatgagtgtggcca				1,65	
***vacA*(s1)**	F	cgcatcaacactaacgctga	64	206 bp	100%	0,6	None
	R	Ggcataaccgcccacttgatt				0,65	
***iceA*1**	F	ataagcggttggagtttgcg	63.1	220 bp	100%	0,5	None
	R	tattcctgcaccaactcccc				1,96	
dupA	F	tcacgcctaagacctcaaact	63	228 bp	100%	1,56	None
	R	ggatttaccgcttcctgtgc				0,24	
***babA*2**	F	aaagatgatcacagacgcgc	64	269 bp	100%	0,59	None
	R	ttgaggggttgttgcatgtg				1,56	
SabA	F	cagcgggccaatacaactac	60	297 bp	100%	1,47	None
	R	ctaacaaatcgctcccaccg				0,95	
jhp0947	F	ttaagcgtccccaatcccat	62	229 bp	100%	2,06	None
	R	tctttccccttgctcgtgaa				1,35	

[a] NCBI identification percentage parameter of the first 100 alignments

[b] mFold Gibbs energy

[c] Probe Match

2. Demographic data

From the group set of 141 patients *H. pylori*-positive (56.9% of the global sample of 246 patients), five patients did not participate in the collection of socioeconomic data. However, their biopsies were included in the analysis. The study population showed 71 of 136 patients (52.2%) were female and 65 of 136 patients (47.8%) were male, showing an age range of 18-85

years with an average of 49.4 ± 17.1 years. The age category with a greater number of patients was volunteers older than 49 years (49.3%), as shown in Table 2. Most of the individuals in this study were from the Andean region of Ecuador with 121 representatives (89%).

Table 2 Demographic data of patients with *H. pylori* infection.

GENDER	**N (%)**
Female	71 (52.2)
Male	65 (47.8)
AGE, AVERAGE, DS (range) [N=141]	49.4, 17.1, (20-84)
AGE CATEGORIES	
18-29 years	21 (15.4)
30-39 years	24 (17.6)
40-49 years	24 (17.6)
> 49 years	67 (49.3)
REGION OF ORIGIN	
Andes	121 (89)
Coast	15 (11)
Amazon	0

3. *H. pylori* infection and clinical status

From the initial study set of 241 patients (excluding 5 patients who did not respond to the epidemiological survey), we analyzed the association between *H. pylori*-positive biopsies (56.9%) and cancer or dyspeptic symptoms. We did not find a significant association between *H. pylori* infection and gastric cancer or dyspeptic symptoms, as shown in Table 3.

Table 3 Distribution of 241 patients with different clinical status, according to *H. pylori* infection.

***H. pylori* infection**	**Clinical status**			
	Gastric Cancer - N(%)		**Dyspeptic Symptoms - N(%)**	
	Negative	**Positive**	**Negative**	**Positive**
Negative	90 (42,45%)	15 (50%)	28 (37,84%)	77 (45,83%)
Positive	121 (57,55%)	15 (50%)	45 (61,64%)	91 (54,17%)
***p* value**	0.435		0.282	

4. Prevalence of *H. pylori* virulence genes

The prevalence of virulence genes *cagA, vacA, iceA1, dupA, jhp0947, babA2 and sabA* in the *H. pylori*-positive biopsies is summarized in Table 4.

Table 4 Frequency of virulence genes in positive H. pylori biopsies.

	Genes						
	N (%)						
	cagA[a]	*vacA*	*iceA*	*dupA*	*Jhp0947*	*babA2*[a]	*sabA*
Positive	19 (13.6)	77 (54.6)	25 (17.7)	72 (51.1)	48 (34.1)	102 (72.9)	120 (85.1)
Negative	121 (86.4)	64 (45.4)	116 (82.3)	69 (48.9)	93 (65.9)	38 (27.1)	21 (14.9)
Total	140 (99.3)	141 (100)	141 (100)	141 (100)	141 (100)	140 (99.3)	141 (100)

[a] Due to the depletion of one of the samples, this virulence factor was not tested.

5. Association between clinical status and *H. pylori* virulence genes

In relation to different clinical symptoms, there was not any statistical association between seven evaluated genes and gastric cancer (Table 5). However, the statistical association that appeared between gen *babA2* and cancer was a *p*-value of 0.08.

Concerning to the association between virulence genes and dyspeptic symptoms, the *sabA* and *dupA* genotypes were detected in 70.7% (OR: 2.94, 95% CI 1.0-8.8) and 75.4% (OR: 2.2, 95% CI 1.0-4.9) of patients with dyspeptic symptoms, respectively. Thus, these genotypes were statistically associated with this condition (*p*-value: 0.024 and *p*-value: 0.034, respectively).

However, the remain genes did not show any association with dyspeptic symptoms in patients (see Table 5/6).

Table 5 Association between cancer and *H. pylori* virulence genes.

	Gastric Cancer - N(%)		
	Positive	Negative	*p*-value
cagA			
Positive	1 (6.7)	18 (14.9)	0.387
Negative	14 (93.33)	103 (85.12)	
vacA (s1)			
Positive	6 (40)	69 (56.6)	0.224
Negative	9 (60)	53(43.44)	
iceA1			
Positive	1 (1.7)	24 (19.7)	0.218
Negative	14 (93.33)	98 (80.33)	
dupA			
Positive	8 (53.33)	62 (50.82)	0.854
Negative	7 (46.7)	60 (49.2)	
jhp0947			
Positive	4 (26.7)	43 (35.25)	0.509
Negative	11 (73.33)	79 (64.8)	
babA2			
Positive	8 (53.33)	90 (74.4)	0.087
Negative	7 (46.7)	31 (25.62)	
sabA			
Positive	12 (80)	105 (86.7)	0.530
Negative	3 (20)	17 (14)	

Table 6 Association between dyspeptic symptoms and *H. pylori* virulence genes.

	Dyspeptic Symptoms - N(%)		
	Positive	Negative	*p*-value
cagA			
Positive	11 (57.9)	79 (68.10)	0.382
Negative	8 (42.11)	37 (31.90)	
vacA (s1)			
Positive	53 (71.62)	38 (61.3)	0.202
Negative	21 (28.4)	24 (38.71)	
iceA1			
Positive	16 (64)	75 (67.6)	0.732
Negative	9 (36)	36 (32.43)	
dupA			
Positive	52 (75.36)	39 (58.21)	0.034
Negative	17 (24.64)	28 (41.8)	
jhp0947			
Positive	31 (66)	60 (67.42)	0.864

Negative	16 (34.04)	29 (32.6)	
babA2			
Positive	68 (70.10)	22 (57.9)	0.176
Negative	29 (29.90)	16 (42.11)	
sabA			
Positive	82 (70.7)	9 (45)	0.024
Negative	34 (29.31)	11 (55)	

6. Genotype combinations

The frequency distribution of the combination of *H. pylori* genotypes analyzed in the 141 samples included in this study resulted in 47 different combinations. However, the most recurrent genotypes in our group set were the following: (1) *dupA*$^{+}$*/sabA*$^{+}$*/vacA*$^{+}$*/iceA*$^{-}$*/jhp0947*$^{-}$*/babA2*$^{+}$*/cagA*$^{-}$ (12.1%); (2) *dupA*$^{+}$*/sabA*$^{+}$*/vacA*$^{+}$*/iceA*$^{-}$*/jhp0947*$^{+}$*/babA2*$^{+}$*/cagA*$^{-}$ (8.51%); (3) *dupA*$^{-}$*/sabA*$^{+}$*/vacA*$^{+}$*/iceA*$^{-}$*/jhp0947*$^{-}$*/babA2*$^{+}$*/cagA*$^{-}$ (5.7%); and (4) *dupA*$^{+}$*/sabA*$^{+}$*/vacA*$^{-}$*/iceA*$^{-}$*/jhp0947*$^{-}$*/babA2*$^{+}$*/cagA*$^{-}$ (5%). Furthermore, the analysis of these 4 genotypes with dyspeptic symptoms or cancer were realized but no statistically representative difference was found in our group set (p >0.05).

V. DISCUSSION

In this study, we analyze the prevalence of 7 virulence genes of *H. pylori* associated with the development of dyspeptic symptoms and gastric cancer. The gene most frequently found was *sabA* in 120 of the 141 patients (86%), showing similar frequencies in European countries (France: 86%, Holland: 93%)(de Jonge, Pot, et al., 2004) and certain Asia countries (Taiwan: 80%, Japan: 80%, Iran: 86.6%) (Pakbaz et al., 2013; Sheu et al., 2006; Yanai et al., 2007). Likewise, in American countries, the prevalence of *sabA* has been reported around 90% (Goudarzi, Rezaee, Rafizadeh, & Taghavi, 2012). Our data collaborated with these previous studies suggesting that this gene is commonly frequent in *H. pylori* strains worldwide. The *babA2* gene frequency in our study (73%) was similar to those previously reported in Brazil (69.3%), Germany (71.9%), Japan

(84.9%) and China (80%) (Erzin et al., 2006; Mattar et al., 2005; Mizushima et al., 2001; Olfat et al., 2005; Yu et al., 2002). In contrast, a frequency of 48% was previously reported in South Korea and this discrepancy was also detected in populations with a high risk of gastric cancer, where *babA* is usually more prevalent. (Ansari & Yamaoka, 2017). The prevalence of this gene together with a proven expression of it could partly clarify the so-called "Asian enigma" that arises when analyzing populations with high rates of *H. pylori* infection and low risk of gastric cancer (Miwa, Go, & Sato, 2002). This enigma is caused by the divergence between the risk of gastric cancer and the rate of infection by *H. pylori* among several populations (Reference). There is a strong link between *H. pylori* infection and gastric cancer in many countries, such as Japan. In contrast, the prevalence of *H. pylori* infection is high in some countries, including India and Bangladesh, but with reports of low rates of gastric cancer (Miwa et al., 2002). Factors that can influence the development of gastric cancer include the genetic diversity of *H. pylori* strains and genetic differences in the host in several ethnic groups, including gastric acid secretion and genetic polymorphisms in proinflammatory cytokines. These factors, together with environmental factors, such as personal hygiene and eating habits, reflect the multifactorial etiology of gastric cancer (Ansari & Yamaoka, 2017; Zaidi, 2016).

On the other hand, all of *H. pylori* strains showed the polymorphic gene *vacA*, which depending on its allelic combination, could produce a cytotoxin with different vacuolization activities (Proenca-Modena et al., 2009). It is well known that genotypes *vacA*s1 and *vacA*s1/m1 are found in individuals with ulcers and gastric cancer, especially in western countries(De Gusmão et al., 2000; Erzin et al., 2006). We found a *vacAs1* prevalence of 55%, in agreement with previous reports of 47% and 60% in Ecuadorian patients (Albán, 2016; Mora Echeverria, 2009). Similar values of *vacA*s1 were also detected in Chile (42.4%) and Venezuela (54%) (Garcia et al., 2006; Ghose, Perez-Perez, van Doorn, Domínguez-Bello, & Blaser, 2005). However, our results differed

markedly from previous studies in Brazil (83%), Colombia (76.8%) and Costa Rica (75.3%) (Arevalo-Galvis, Trespalacios-Rangell, Otero, Mercado-Reyes, & Poutou-Pinales, 2012; Ashour et al., 2002; Con et al., 2007). Also, in most Eastern and European countries, high prevalence of 75 to 100% is reported and showing a significant association with duodenal ulcer and gastric cancer (Hennig, Trzeciak, Regula, Butruk, & Ostrowski, 1999; Ozbey & Aygun, 2012; Rudi et al., 1999; van Doorn, Figueiredo, Rossau, et al., 1998; Yoshio Yamaoka et al., 1999).

The high diversity of genomic and allelic combinations of *H. pylori* is well known in the so-called "plasticity region"; in which the *dupA* and *jhp0947* genes are present (Pereira et al., 2014). In relation to the *dupA* gene, it was found in 72 of the 141 (51.1%) patients, agreeing with previous studies in the USA (43.5%), Brazil (41.5%), Belgium (43.7%) and Iran (49.7%) (Argent, Burette, Miendje Deyi, & Atherton, 2007; Douraghi et al., 2008; Pereira et al., 2014). However, our data differed from studies realized in Mexico (37.5%), Colombia (35.8%), Japan (21.3%), China (32.3%) and India (37.5.3%)(Arachchi et al., 2007; Argent et al., 2007; Gomes et al., 2008; Lu et al., 2005; Romo-Gonzalez et al., 2015) . This difference may be conditioned by the variation of the gastric environment that depends on the geographic location of the population. For the *jhp0947* gene, the prevalence of 34% was found in our study, in agreement with reports from USA (38%) and Netherlands (33%)(Mitsushige Sugimoto et al., 2012). However, it was higher than found in East Asian countries, such as South Korea (8.6%) and Japan (2.7%) (Mitsushige Sugimoto et al., 2012). Nonetheless, high values had been reported in others countries of America, such as Colombia(55.3%) (Mitsushige Sugimoto et al., 2012), Brazil (70%) (Santos et al., 2003) and Mexico (77.6%) (Romo-Gonzalez et al., 2015). This variation in frequencies, as with other genes, is probably conditioned by the gastric niche where *H. pylori* is found.

Meanwhile, the *iceA*1 gene prevalence was 18% in our study set, like Brazil (16%) and the USA (16%). On the other hand, high prevalence rates exceeding 50% have been reported worldwide, especially in East Asia and North Africa (Chomvarin et al., 2008; H Kadi, M Halawani, &

Abdelkader, 2014; Leanza et al., 2004; Nishiya et al., 2000; Ribeiro, Godoy, Benvengo, Mendonca, & Pedrazzoli, 2003; Yoshio Yamaoka et al., 1999). Despite this disparity, this gene could potentially be involved in the transcriptional regulation of genes and DNA methylation and thus lead to alteration in the expression of genes involved in virulence or pathogenesis (Donahue et al., 2000). Further studies should characterize its roll in *H. pylori* pathogenesis.

Finally, the *cagA* gene is one of the most influential markers of *H. pylori* virulence in the development of gastric diseases, including atrophic gastritis and gastric cancer, mainly in Western countries (Chomvarin et al., 2008; Proenca-Modena et al., 2009; van Doorn, Figueiredo, Sanna, et al., 1998). Its presence has been detected in approximately 60% of the world population (Cervantes-garcía, 2016). In our study, the *cagA* gene was only detected in 19 of the 141 (14%) patients, showing lower values from the other two studies from Chile (24.2 and 38%). These authors reported a persistent *cagA* negative *H. pylori* isolates in clinical patients (Garcia et al., 2006; Martinez et al., 2001). Another study performed in Panama population reported a similar frequency of 20% (Sasaki et al., 2009). The prevalence of *cagA* detected with primers designed by us is extremely low in comparison with other countries, so it is necessary to evaluate through sequencing the effectiveness of them or confirm these frequencies with more studies for the future. In summary, it was not possible to find an association between the *cagA* gene and gastric cancer and with dyspeptic symptoms as initially postulated in the present study.

The limitations of this study, precisely, are evidenced in the results found with the *cagA* gene due to the low prevalence found. Therefore, these results should be taken with caution not only due to memory bias and the selection of surveyed patients but also due to prior knowledge in the design of primers and that the design tools of primers generally include low sensitivity of detection of objectives, limited specificity stringency options and limited coverage of organisms in search databases (Ye et al., 2012).

Overall, the association between the clinical status and the presence of *H. pylori* was not significant (p >0.05) for our study set. However, *sabA* and *dupA* were significantly associated with the presence of dyspeptic symptoms (p-value: 0.024 and p-value: 0.034, respectively). Thus, the patients infected with *H. pylori sabA* positive showed three times more susceptible to evidence dyspeptic symptoms when compared to those patients infected with *H. pylori sabA* negative. These results illustrated an important association between *sabA* and the appearance of dyspeptic symptoms caused by gastric diseases. This association is reflected in countries like Colombia and the USA, where there is a high prevalence of *sabA* in diseases such as duodenal ulcer (44%), gastritis (66%) and gastric cancer (70%) (Y Yamaoka et al., 2006). However, our results did not show any association between the *sabA* gene and gastric cancer. Similarly, the risk of presenting dyspeptic symptoms among patients infected with *H. pylori dupA* positive is twice as high of the outcome. These symptoms could derive from a duodenal ulcer, which it has been related to the presence of this gene (Hussein, 2010).

VI. CONCLUSIONS

The virulence factors *sabA* and *dupA* were the only genes statistically related to the appearance of dyspeptic symptoms, demonstrating that these genes are probably involved in the development of diseases such as gastritis or peptic ulcer (gastric or duodenal). However, the same results were not found in patients with gastric cancer. The association of the presence of *H. pylori* and its virulence genes in relation to gastric cancer should be further studied in a population set with a greater number of patients.

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