

Original Article

Detection of *vacA* and *cagA* genes in *Helicobacter pylori* strains in Salvadoran patients

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Abstract

Introduction. *Helicobacter pylori* infection has become an prevalent disease and an economic and public health problem. The main concern is that some strains are associated with gastric cancer, especially the *vacA* and *cagA* genes. **Objective.** Determine the prevalent *Helicobacter pylori* genotypes in patients who attended the Specialty Clinic of the Salvadoran Social Security Institute. **Methodology.** A quantitative, descriptive, cross-sectional study was conducted. The unit of analysis was endoscopic gastric samples. A consecutive non-probabilistic sampling was performed. The selection of patients was carried out by a gastroenterologist. The real-time polymerase chain reaction technique was applied to identify the virulence genes, *cagA* and *vacA* of cultures positive for *Helicobacter pylori*, using standardized methods. **Results.** Ninety-seven participants were obtained, from whom a biopsy sample was acquired and cultured; however, the recovery rate of *Helicobacter pylori* in culture was 26 %. All samples were positive for the 16S rRNA ribosomal virulence genes and the *cagA* and *vacA* oncogenes. The infection was predominant in females, with 76 %, and the average age was 55 years. It was found that 64 % of positive participants had a previous diagnosis of infection with *Helicobacter pylori*. **Conclusion.** The virulence genes *cagA* and *vacA* were found in all samples positive for *Helicobacter pylori*.

Keywords

Helicobacter pylori, Polymerase Chain Reaction, Oncogenes, Stomach Neoplasms, Virulence.

Resumen

Introducción. La infección por *Helicobacter pylori* se ha vuelto un problema económico y de salud pública, convirtiéndose en una enfermedad prevalente. La principal preocupación es que algunas cepas están asociadas con el cáncer gástrico, especialmente los genes *vacA* y *cagA*. **Objetivo.** Identificar la presencia de los genes *vacA* y *cagA* en cepas de *Helicobacter pylori* aisladas a partir de muestras de biopsias gástricas. **Metodología.** Se realizó un estudio transversal descriptivo. La unidad de análisis fueron las muestras gástricas endoscópicas. La selección de los pacientes la realizó un gastroenterólogo. Se aplicó la técnica de Reacción en Cadena de la Polimerasa en tiempo real para la identificación de los genes de virulencia, *cagA* y *vacA* de los cultivos positivos al *Helicobacter pylori*. **Resultados.** Se obtuvieron 97 participantes; sin embargo, se obtuvo un porcentaje de recuperación de *Helicobacter pylori* en cultivo de 26 %. Todas las muestras fueron positivas para el gen ribosomal rRNA16s y los genes de virulencia *cagA* y *vacA*. La infección predominó en el sexo femenino con un 76 %, la edad promedio fue de 55 años. Se encontró que el 64 % de participantes positivos tenían diagnóstico previo de infección por *Helicobacter pylori*. **Conclusión.** Los genes de virulencia *cagA* y *vacA*, se encontraron todas las muestras positivas a *Helicobacter pylori*.

Palabras clave

Helicobacter pylori, Reacción en Cadena de la Polimerasa, Oncogenes, Neoplasias Gástricas, Virulencia.

Introduction

The high incidence of *Helicobacter pylori* (*Hp*) infection probably contributes to the fact that gastric cancer mortality ranks second among cancer deaths, worldwide.ⁱ⁻ⁱⁱⁱ Due to the causal relationship between *Hp* and gastric tumors, the International Agency for Research on Cancer (IARC) recognized *Hp* as a Group 1 carcinogen, indicating that it is a definite carcinogen.ⁱ⁻ⁱⁱⁱ Currently, *Hp* is

the only bacterium that has achieved this distinction, classified as dangerous in terms of oncogenicity. According to this information, gastric colonization by this bacterium increases up to six times the risk of gastric cancer up to six times, compared to people who do not present colonization, so it has been the only bacterium with a significant association with gastric adenocarcinoma; in addition, it has been related to one of the main causes of cancer deaths worldwide.ⁱ⁻ⁱⁱⁱ

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Detección de genes *vacA* y *cagA* en cepas de *Helicobacter pylori* en pacientes salvadoreños

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No conflicts of interest.

This disease has become a public health problem due to its high prevalence; in addition, prevention measures have not been clearly identified, which makes it necessary to have local data on the pathogenicity of *Hp* strains, through the detection of *cagA* and *vacA* genes, due to the consequences that this infection and, specifically, these oncogenes entail.^{iv,v}

Real-time polymerase chain reaction (RT-PCR) allows the identification of genes relevant to the diagnosis of various diseases, including the detection of *Hp* virulence factors. Endoscopic tests in patients with *Hp* infection and dyspepsia often reveal diagnoses such as chronic antral gastritis, erosive antral gastritis, and nodular antral gastritis, which have been associated with the presence of the *Hp* virulence genes *cagA* and *vacA*. In addition, the most common premalignant gastric lesions, such as gastric atrophy, intestinal metaplasia, and low-grade dysplasia, are also associated with these same *Hp* virulence factors.^{vi}

The most common endoscopic diagnoses in patients with dyspepsia are chronic antral gastritis, erosive antral gastritis, and nodular antral gastritis, as well as the presence of the *cagA* and *vacA* genes in isolated strains. The most frequent premalignant gastric lesions are gastric atrophy, intestinal metaplasia, and low-grade dysplasia.^{vii}

The prevalence of the *cagA* oncogene is 50 %, while that of the *vacA* oncogene is 87.5 %.^{viii} These data are essential for the development of public health strategies aimed at detecting and effectively managing the infection.

The *cagA* and *vacA* proteins are considered important virulence markers in *Hp*. These markers can be identified more easily through the genes that encode them, called virulence-associated genes. Furthermore, the simultaneous presence of *Hp* has been detected in the oral cavity and gastric mucosa, with variations in prevalence depending on the population analyzed, the sampling method, and the techniques used for detecting the bacterium.^{ix}

The presence of *Hp* in the oral mucosa of patients with dyspepsia leads to dissemination, with the potential for reinfection after treatment for *Hp* eradication, so ideally, treatment for the oral cavity should be applied.^x

It is essential to identify strains with carcinogenic potential in the general population, especially those with histopathological features that increase their risk for *Hp* infection. In cases of gastric cancer, these strains are usually highly virulent, and it has been documented that, in some individuals, multiple oncogenic strains can coexist in

the gastric mucosa. Therefore, it is essential to further study the virulence genes associated with oncogenicity in *Hp*, as well as to strengthen strategies for eradicating the bacterium.^{xi,xii}

In El Salvador, there is no study on the genetics of *Hp*, and the country lacks a diagnostic service for the general population; therefore, this research is relevant. The objective of the study was to identify the presence of the *vacA* and *cagA* genes in *Helicobacter pylori* strains isolated from gastric biopsy samples.

Methodology

The research was of a descriptive cross-sectional type. It was conducted at the Santa Ana Regional Hospital and the Specialty office of the Salvadoran Social Security Institute (ISSS), from October 2021 to March 2022.

The unit of analysis was endoscopic samples from patients diagnosed with gastric disease. All samples from patients who attended in the period stipulated for data collection were included, and those who met the inclusion criteria, which were: patients over 18 years of age and who consulted in the gastroenterology department at Santa Ana Regional Hospital or Specialty Office in the department of San Salvador. Likewise, patients with or without previous treatments for *Hp* eradication or patients who had received treatment with amoxicillin, clarithromycin, or metronidazole in the last month for another reason, even if not for *Hp* eradication, were considered; and the last criterion was patients who agreed to participate in the study.

The selection of patients was made by a gastroenterologist, based on the aforementioned inclusion criteria, who indicated to the researchers the patient who was a candidate for inclusion in the study.

Regarding the variables and indicators investigated, general information was obtained from the participants, such as: age, sex, history of infection, endoscopic diagnosis, and the detection of *Hp* genotypes, specifically *VacA* and *CagA*.

The data collection instrument was a physical form. The information on the variables of interest was collected in two moments, the first was when the endoscopic sample was taken from the patient's file and the second when the results of the sample processing were obtained at the microbiology laboratory of the Evangelical University of El Salvador (UEES); The samples were transported in test tubes with distilled water and in a cooler to perform the bacterial culture and subsequent PCR-RT tech-

nique to identify the *VacA*, *cagA* and 16S rRNA genotypes of the *Hp*-positive cultures.

Each of the samples were double seeded (sample 30 was used to improve the percentage of bacterial recovery) on Columbia agar enriched with 5 % horse defibrinated blood and bacterial growth enzyme inhibitors and placed in a CO₂ incubator with the following conditions: 5-10 % O₂, 5-10 % CO₂, 80-90 % N₂, 35 - 37°C, 95 % humidity, until seven days before the culture was considered negative.^{xiii,xiv} For colonies suggestive of *Hp*, Gram staining was performed to search for curved Gram-negative bacilli before performing RT-PCR, which was carried out using standardized methods.^{xiii,xiv} Nucleic acid extraction was performed from gastric biopsies using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany), following the manufacturer's recommendations. Each kit allowed the purification of up to 50 samples. Each extraction yielded approximately 60 µL of genomic DNA, sufficient to perform the reactions in duplicate and ensure the reproducibility of the results.

For the amplification of the *vacA* and *cagA* genes, the iTaq™ Universal SYBR® Green Supermix enzyme was used, preparing the master mix according to the manufacturer's instructions. The reactions were performed in a final volume of 20 µL, including specific primers for the *vacA* and *cagA* genes. The primer sequences were taken from the study by Ranjbar *et al.*,^{xv} which demonstrated high sensitivity and specificity for these molecular targets.

Real-time PCR amplifications were performed on the MiniOpticon Real-Time PCR System MiniMyGo S® under previously validated thermocycling conditions for the detection of *Hp* genes. All procedures were performed in a sterile and controlled environment to avoid cross-contamination.^{xvi}

The data obtained were processed using Excel 2010 and presented in tabular form. Qualitative variables were represented in frequencies and proportions; quantitative variables were presented using measures of central tendency. The information was stored by the researchers and subsequently delivered to UEES for final storage. Averages, frequencies, and percentages were calculated to obtain the results.

Each of the participants was asked for informed consent upon verifying that they met the inclusion criteria, in which the importance of the study was explained to them, as well as the respect for ethical principles and confidentiality, that there was no remuneration, and that the data would be used in a group manner and for scientific

research purposes. The research protocol was submitted for evaluation by the UEES ethics committee and approved in minute 187, dated August 9, 2019. It was also submitted for evaluation by the ISSS ethics committee and was approved on June 29, 2020. It had the approval of the ISSS gastroenterologists, as well as the directors of the health centers where the research was carried out (Santa Ana Regional Hospital, Specialty Clinic of the ISSS).

Results

Information was collected from 97 participants, from whom a biopsy sample was obtained and submitted for culture. However, *Hp* recovery was achieved in 26 % of the cases, i.e. 25 samples were positive. The results obtained from the *Hp*-positive samples are presented below. According to the general data collected, the infection predominantly affected females, with a prevalence of 76 %. The average age was 55 years (standard deviation, 16.3), with a range from a minimum of 25 years to a maximum of 85 years. Regarding the history of *Hp* infection, it was found that 64 % of the positive participants had a previous diagnosis of infection, which indicates that they were probably reinfected patients or those in whom the bacteria could not be eradicated.

Within the endoscopic data collected (Table 1), the diagnosis of gastropathy/acute gastritis was the one that predominated in 60 % of the cases, followed by erosive gastropathy, with 20 %, in a smaller proportion, with 8 %, nodular gastritis, esophagitis, and gastric and duodenal ulcers in 4 %.

Table 2, shows that the two oncogenes, *vacA*, *cagA*, were detected in all the samples positive for *Hp* (n=25).

Discussion

The purpose of the study was to identify the genotypes of *Hp* strains, for which endoscopic gastric samples were collected to be cultured; however, a lower percentage of recovery was obtained compared to the number of samples collected. In a study conducted in Costa Rica, the viability of *Hp* bacteria culture was determined by obtaining gastric biopsies from 44 participants. *Hp* was recovered in 27 biopsies, with a recovery percentage of 61.4 %, ^{xvii} indicating that the percentage of recovery in culture was lower than the number of samples taken. This may be because the conditions of transport and growth of *Hp* are demanding; in addition, it requires a medium reduced in O₂ and loaded

with CO₂, contrary to the great majority of bacteria. In addition, as mentioned by Molina-Castro *et al.*, among some of the reasons that can influence a false negative result in the culture are excessive contamination, because it does not allow successful isolation of *Hp* colonies, an altered physiological state of the bacteria and a low bacterial load in the sample, since they produce an alteration in their environment and reduce their viability, hindering growth in culture media.^{xvii}

Table 1. Endoscopic diagnosis

Diagnosis	Frequency	Percentage
Acute gastropathy/ gastritis	15	60 %
Nodular gastritis	2	8 %
Gastric and duodenal ulcer	1	4 %
Erosive gastropathy	5	20 %
Esophagitis	2	8 %
Total	25	100 %

Table 2. Positive samples for *Helicobacter pylori* genes using PCR-RT

Genotype	Frequency (n=25)	Percentage
VacA	25	100 %
CagA	25	100 %

Among the study participants, the female group was the predominant one; unlike a study about the prevalence of *Hp* in asymptomatic patients in Ecuador, where, it was found that *Hp* infection predominated in the male sex with 51.5 %; in addition, it is exposed that hygiene habits influence the significant increase of cases in the male sex.^{xviii} However, it should be noted that women are more likely to consult than men, which may increase the proportion of infected women.ⁱ

Some studies show an increase in the prevalence of *Hp* infection according to sex,^{xix-xx} others report that infection predominates in the female sex,^{xix-xxi} however, this difference is not so significant, therefore, it is said that the distribution of the disease is homogeneous, according to sex.^{xix-xxi} Models have even been created to predict the prevalence of *Hp* infection as a function of climatic conditions. In the end, it is concluded that in developing countries, the prevalence of this infection remains high,

apparently regardless of climatic conditions or the sex of the patients.^{xix}

Regarding the age most affected by *Hp* infection, a study from Cuba reported an average age of 69 years for infected patients, with the maximum age being between 60 and 69 years (57.1 % infected);^{xx} however, the average age varies according to the region,^{xxi} i.e., the prevalence will behave differently in each age group and region of the world. In developing countries, the prevalence rates are highest, ranging from 60 % to 80 % in the adult population. In contrast, countries with high socioeconomic development have an infection rate reduced to 30-50 % in the adult population.^{xxi} The highest prevalence has been found in Africa (79.1 %), followed by South America and the Caribbean (63.4 %), and finally North America (37.1 %) and Oceania (24.4 %).^{xxi} Variations in the region are due to differences in hygiene conditions, access to drinking water, overcrowding, food availability and climate; additionally, they depend on each country's guidelines for diagnosis and treatment.^{xviii-xxi}

The average age of this study is within the range reported by other researchers.^{xix-xxi} The recognition of the most affected age group is important because surveillance, detection, and treatment measures should be intensified in these groups.^{xix-xxi}

Regarding the participants who had a previous diagnosis of *Hp* infection, it can be observed that most patients already had this history. According to a study conducted in Mexico, the annual recurrence of *Hp* infection was 9.3 %, with an annual reinfection of 7 %. This data was lower compared to data reported for developing countries with a higher prevalence of *Hp*.^{xxii} It is also mentioned that developed countries tend to have a low prevalence of *Hp* infection.^{xxii} Regarding the type of strains found, *cagA* and *vacA* were isolated in reinfection. The reinfection rate for *cagA* was 10 % and 5.3 % for *vacA*.^{xxii} The high prevalence of *Hp* infection could explain why the patients in this study had a history of diagnosis of *Hp* infection and probable reinfection or recurrence.

The endoscopic diagnoses found in this study were gastropathy, acute gastritis, erosive gastropathy, gastric and duodenal ulcer, nodular gastritis, and esophagitis in patients reporting *Hp* infection. A study was carried out in Panama,^{xxiii} where endoscopic diagnoses of erosive gastritis (33.5 %), nodular gastritis (3.5 %), non-erosive gastropathy (48.6 %), gastric intestinal metaplasia (5.14 %), duodenal ulcer (5.14 %), duodenal ulcer and esophagitis were found, duodenal and gastric ulcer (4.2 %), in which an association was found between nodular gastritis, intestinal

metaplasia, duodenal ulcer, and gastric ulcer with *Hp* infection. Likewise, Duarte-Chang mentions that there are studies reporting the association between nodular gastritis, peptic ulcer disease, and *Hp* infection.^{xxiii}

According to a Guatemalan study on premalignant lesions, the found that among the cases studied, 83 % presented some premalignant lesion such as atrophy, metaplasia, or dysplasia, among which the most frequent was gastric atrophy (70 %), followed by gastric intestinal metaplasia (11 %) and dysplasia (2 %). When reviewing the presence of *Hp* in these endoscopic findings, it was found that in atrophy, 62 % had *Hp* infection, in intestinal metaplasia, 66 % and in gastric epithelial dysplasia, 67 %.^{xxiv}

Endoscopic findings are important in *Hp* infection, since they allow the detection of premalignant lesions; due to this, the Kyoto classification criteria have been created, which evaluate atrophy, intestinal metaplasia, thickened folds and nodularity; however, ideally, these patterns must have histological confirmation, which at the moment is still the gold standard for histopathological diagnosis. Nevertheless, optical endoscopic diagnosis is important, since it saves unnecessary biopsies in certain cases; there is even talk that the application of artificial intelligence in endoscopic diagnosis could improve the effectiveness of the diagnosis.^{xxv}

Regarding genotyping, it was found that the two oncogenes of interest for the study, *cagA*, *vacA*, and the rRNA 16s ribosomal gene, were detected in all *Hp*-positive participants. The prevalence of *cagA* and *vacA* strains is said to be variable, with data reported in Ghana of 74.8 %, Nigeria 90 %, South Africa 95 %, Japan 100 %, Brazil 47.8 %, in Colombia from 43 % to 90.5 %, among others,^{vii} data in the range of the proportions found in this study.

In a 2021 study conducted in El Salvador, the presence of *Hp* was detected by PCR in 20 % of the irrigation water used for food crops. Of these, 100 % of the isolated strains carried the *vacA* and *cagA* genes;^{xxvi} these findings coincide with the data found in the present study.

Amplification of the 16S rRNA gene was performed to confirm the identification of *Hp*. This ribosomal gene is widely used for the identification of *Hp* in stool, blood, and biopsy samples using different techniques (qRT-PCR, antibodies, antigens); in addition, although it is not directly involved in antibiotic resistance, it is used in studies together with other genes to track resistant strains.^{xxvii}

The Maastricht V/Florence Consensus Report takes up the topic of the importance of *Hp* eradication and mentions that it can

be successful in preventing progression to gastric cancer, because people who have strains with one of the "gastric cancer phenotypes" have an increased risk of cancer.^v

It also mentions that, in the case of infected patients with active chronic gastritis, eradication of *Hp* is the cure for this disease, improving the symptomatology and quality of life of affected individuals. Several meta-analyses show that the premalignant gastric atrophy lesion can be reversed to a certain extent, both in the antrum and in the gastric body.^v

Likewise, it takes up the clinical and economic benefits of *Hp* eradication. It refers that there are studies that have evaluated the cost-effectiveness of *Hp* screening and treatment policies for the prevention of gastric cancer, concluding that *Hp* screening and treatment is cost-effective and that effects can be seen in the short and medium term, and that the reduction of infection and complication costs are seen in the long term. This benefit may be most significant in communities with a high risk of gastric cancer, decreasing the severe burden of morbidity and mortality from this disease.^v

According to Maastricht V, countries with a significantly increased risk of gastric cancer, mainly due to the high prevalence of oncogenic strains, should offer endoscopic and/or serological screening and surveillance, targeting mainly people between 50-65 or 70 years of age.^v

One of the most important limitations of this study was its interruption due to the COVID-19 epidemic, from the purchase of supplies to the suspension of endoscopies. This caused a delay in the research execution, but the results were not affected.

According to the results of this research, the importance of the implementation of molecular techniques is notorious, for the detection of oncogenes that are available to the general population, in addition, the expansion of the search should not only be limited to oncogenes, but also to genes related to antimicrobial resistance in order to perform epidemiological surveillance for *Hp*.^{v,xxviii}

Conclusions

Most participants had a previous diagnosis of *Hp* infection, which is important because it implies the existence of reinfection, failure to eradicate, or recurrence of the bacterium.

The main endoscopic diagnoses were acute gastropathy/gastritis, followed by erosive gastropathy, nodular gastropathy, esophagitis, and gastric ulcer. Such findings could be produced by the presence of bacteria, and some of these endoscopic

diagnoses are recognized as premalignant lesions in other investigations.

In this study, the two virulence genes, *cagA* and *vacA*, were found to be present in all samples in which *Hp* was isolated; this data is relevant due to the risk of gastric cancer produced by these strains.

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