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***Helicobacter pylori* Genotyping and Sequencing Using Paraffin-Embedded Biopsies from Residents of Colombian Areas with Contrasting Gastric Cancer Risks**

Liviu A. Sicinschi^{*}, Pelayo Correa^{*}, Richard M. Peek Jr^{*}, M. Constanza Camargo^{*}, Alberto Delgado^{*}, M. Blanca Piazuolo^{*}, Judith Romero-Gallo^{*}, Luis E. Bravo[†], and Barbara G. Schneider^{*}

^{*} Division of Gastroenterology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

[†] Department of Pathology, School of Medicine, Universidad del Valle, Cali, Colombia

Abstract

Background—*cagA*-positive and *vacA s1* and *m1* genotypes of *Helicobacter pylori* are associated with an elevated risk of gastric cancer (GC). We determined these genotypes using paraffin-embedded gastric biopsy specimens harvested from infected individuals and compared genotype distributions in two Colombian populations residing in geographic regions with a high and low incidence of GC.

Methods—DNA from paraffin-embedded gastric biopsies from 107 adults was amplified using primers specific for *cagA*, for the *cag* ‘empty site’, for the *s* and *m* alleles of *vacA*, and for *H. pylori* 16S rRNA.

Results—*H. pylori* infection was detected by molecular assays in 97 (90.7%) biopsies. Complete genotyping of *cagA* and *vacA* was achieved in 94 (96.9%) cases. The presence of *cagA* was detected in 78 of 97 cases (80.4%); when considered separately, *cagA* and *vacA s* regions were not significantly associated with a particular geographic area. The *vacA m1* allele and *s1m1* genotypes were more common in the area of high risk for GC ($p = .037$ and $p = .044$, respectively), while the *vacA m2* allele and *s2m2* genotypes were more prevalent in the low-risk area. The prevalence of the combination of *cagA*-positive, *vacA s1m1* genotypes was 84.3% and 60.5% for high and low risk areas, respectively ($p = .011$).

Conclusions—*H. pylori cagA* and *vacA* genotyping from paraffin-embedded gastric biopsies permitted reliable typability and discrimination. The more virulent *cagA*-positive *s1m1* strains, as well as *vacA m1* genotype, were more prevalent in high risk than in low risk areas, which may contribute to the difference in GC risk between those two regions.

Keywords

H. pylori; *cagA*; *s* and *m vacA* alleles; 16S rRNA; paraffin-embedded biopsies; PCR; gastric cancer; vacuolating cytotoxin; sequencing; synonymous and missense mutations

Helicobacter pylori is a spiral-shaped, microaerophilic bacterium that colonizes the mucous layer of the human stomach. Once established, the pathogen may reside in the majority of

carriers for years or decades in the absence of symptoms [1–5]. However, in a small proportion of hosts, the organism can induce severe gastrointestinal diseases such as gastric cancer (GC) [6–8]. The clinical outcome of the infection may be related to host immunologic defense mechanisms, environmental factors, and/or the virulence capacity of the bacteria [9,10]. Several genes, including *cagA* and *vacA*, have been identified that likely play a role in the pathogenesis of *H. pylori* infection.

The *cagA* gene is present in more than 50% of *H. pylori* strains and encodes the CagA protein [11]. *cagA* is a constituent of a pathogenicity island (*cagPAI*) [12], a 40-kbp DNA insertion containing genes that encode a type IV secretion system, and this gene is associated with gastric mucosal atrophy, duodenal ulcer, and gastric carcinoma [13–15].

The *vacA* gene is present in all *H. pylori* strains and encodes an 88-kDa vacuolating toxin (VacA) that affects epithelial cells and which is important in the pathogenesis of peptic ulcer and gastric adenocarcinoma [16–19]. Within the *vacA* gene, two regions of marked sequence diversity can be distinguished. The s-region (encoding the signal peptide) is present as either an *s1* or an *s2* allele, while the m-region (the middle region of the toxin) can be either *m1* or *m2* [16,20]. The mosaic combination of s- and m-region alleles determines the production of the vacuolating cytotoxin and is associated with the pathogenicity of the bacterium. *vacA s1/m1* strains produce large amounts of vacuolating toxin, *s1/m2* strains produce moderate amounts, and *s2/m2* strains produce very little or no active toxin [16]. The *vacA s2/m1* genotype has been reported in Mexican and South African populations [21,22], but those strains are rare. The *vacA s1* and *m1* bearing strains have been associated with increased virulence and greater gastric epithelial damage and ulceration than *s2* and *m2* strains [16,23]. Within type *s1* strains, several subtypes (*s1a*, *s1b*, and *s1c*) have been distinguished [24], the *s1a* being associated with strains producing most actively the vacuolating cytotoxin [16].

H. pylori is a genetically heterogeneous species [25,26] and, in addition to being associated with specific diseases, certain genotypes are more frequent in certain ethnicities or geographic regions of the world [26–29]. For example, *cagA* positivity has been detected in almost all *H. pylori* strains isolated from infected individuals in Asian countries [27,28]. Conversely, in Western Europe, *cagA*-positive strains are less prevalent and more frequently found in ulcer or GC patients [29]. In East Asian countries, the predominant *vacA* genotype is *s1m1*, irrespective of disease outcome [28,30], except in Taiwan, where the predominant genotype is *s1m2* [31]. A previous study performed in Colombia in 2002 by our research team [32] examined the distribution of *cagA* and *vacA* genotypes in frozen gastric biopsies from two populations with contrasting GC risks. One group comprised individuals living at high altitude in the Andes Mountains in the towns of Pasto and Tuquerres, where rates of gastric premalignant lesions and GC were extremely high [33]. The low-risk group comprised individuals from the Pacific coast town of Tumaco, where the incidence of gastric premalignant lesions and GC is low [34,35]. The results of that study showed significantly higher frequencies of *cagA*-positive and *vacA s1* and *m1* genotypes in the area of high risk for GC, compared with the population from the low risk area, suggesting that there may be an association between *H. pylori* infection and GC incidence in these areas. However, whether the high incidence of GC in the high-risk area is associated with specific *H. pylori* genotypes remains undefined.

The aim of this study was to genotype *H. pylori* strains from paraffin-embedded gastric biopsies collected from *H. pylori* infected individuals and compare the distribution and variability of genotypes in those two well-defined Colombian geographic regions with a high and low incidence of GC.

Materials and Methods

Subjects

Participants were consecutively recruited from two areas with contrasting GC risks in the State of Nariño, Colombia. The subjects were volunteers with dyspeptic symptoms from the general population, responding to an open invitation to the community, between November 1998 and September 2003. All participants were divided into two groups (Table 1). One group consisted of individuals living at high altitude in the mountains in the towns of Pasto and Tuquerres (56.1%), where the rates of GC are very high. Another group consisted of participants from the Pacific coast town of Tumaco (43.9%), where the incidence of GC is low. From the volunteers, all males between 40 and 59 years old meeting inclusion criteria ($n = 107$) were included in this study. From previous experience, we expected this age and sex group to have higher prevalence of preneoplastic lesions and of *H. pylori* infections. Exclusion criteria were previous gastrectomy and concomitant major chronic diseases. The endoscopic procedure was performed by either of two experienced gastroenterologists. During endoscopy of the upper gastrointestinal tract, gastric mucosal biopsies were obtained from the antrum, incisura angularis, and corpus of the stomach. The ethics committee of all three hospitals and the ethics committee of Universidad del Valle in Cali, Colombia, approved this study. All 107 selected participants provided informed consent to participate in this study.

Clinical Specimens

Paraffin-embedded gastric biopsies from 107 subjects with *H. pylori* infection and/or varying gastric symptoms were used to determine the presence and the genotypes of *H. pylori*. None of the samples overlapped with specimens included in any previous study. The paraffin blocks (4–9 years old) were cut on a standard microtome using the sandwich method: sections of 4 μm for hematoxylin and eosin staining and modified Steiner silver stain were cut before and after sections of a total of 30 and 60 μm for polymerase chain reaction (PCR). To increase the amount of bacterial DNA for analysis, sections from the antrum and incisura angularis, and from the corpus of the stomach were combined in a single specimen for DNA preparation, for each subject.

Histopathology

Histopathologic diagnosis for each subject was independently assessed by two experienced pathologists (P.C. and M.B.P.) following published guidelines [36,37]. Diagnoses included normal, non-atrophic gastritis (NAG), multifocal atrophic gastritis without intestinal metaplasia (MAG), intestinal metaplasia (IM), and dysplasia (DYS). Cases with discordant diagnoses were reviewed in a double-head microscope and a consensus was reached. The presence of *H. pylori* was assessed by using Steiner silver stain [38]. The pathologists were blinded to the site of residence of the patients and to the *H. pylori* genotyping results.

Bacterial Cultures

Eight biopsies, frozen in thioglycolate, were obtained from subjects from high and low risk areas and were used in this study as controls for sequencing. The biopsies were homogenized under sterile conditions in 100 μL of sterile phosphate-buffered saline (PBS, pH 7.4) using a homogenizer (Kimble-Kontes, Vineland, NJ, USA). The homogenate was plated onto selective trypticase soy agar (TSA) with 5% sheep blood containing vancomycin (20 $\mu\text{g}/\text{mL}$), bacitracin (200 $\mu\text{g}/\text{mL}$), nalidixic acid (10 $\mu\text{g}/\text{mL}$), and amphotericin B (2 $\mu\text{g}/\text{mL}$) (Sigma, St Louis, MO, USA), and 1 : 10 dilution was plated on TSA plate (BBL) with no antibiotics. Agar plates were placed in microaerobic conditions (7% O_2 , 5% CO_2 , and 88% N_2 , using Campy Pak Plus envelope, BBL) at 37 °C and incubated for 4–8 days until small, gray, translucent colonies were obtained. Bacteria morphology was examined following Gram staining. After

biochemical analyses were performed for oxidase and urease activity, swabs and single colonies were frozen for storage and used for DNA extraction.

Preparation of Genomic DNA from Bacterial Strains

DNA was extracted from three reference strains and from eight single colonies for this study. *H. pylori* strain J99, genotype *cagA*+, *vacA* *s1/m1*, and *H. pylori* strain SS1, genotype *cagA*+, *vacA* *s2/m2*, were used as controls for *vacA* genotypes. The same *H. pylori* strain SS1 was used as a control for the presence of the *cagA* gene, while the *H. pylori* strain Tx30a (ATCC 51932), with genotype *cagA*-, *vacA* *s2/m2*, was used as a positive control for the empty site. *H. pylori* strain J99 was also used as a positive control and *H. felis* was used as a negative control for the presence of *H. pylori* in gastric biopsy samples in the 16S rRNA analysis. *Campylobacter jejuni* NCTC 11168, which is closely related to *H. pylori*, and *H. canis* strain 98-715 also served as negative controls. DNA from all negative control strains was extracted using QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA extracted from single colonies cultured from frozen biopsies obtained from individuals from the same regions of high and low risk was used for PCR amplification of *m1* and *m2* *vacA* fragments and sequencing.

Preparation of Genomic DNA from Biopsy Specimens

DNA was extracted from paraffin sections as previously described [39] with minor modifications. Briefly, sections of a total of 30–60 µm thickness from paraffin-embedded gastric biopsies from each patient were collected in sterile microfuge tubes for DNA extraction. To prevent cross-contamination, disposable blades were used and the microtome was thoroughly cleaned with 10% bleach, followed by water before and after processing of each tissue block, and all sections were handled with disposable toothpicks. For deparaffinization, sections were treated twice for 1–2 minutes with octane at room temperature, and sections were then suspended in 100% ethanol. After complete evaporation of ethanol, tissue samples were digested in 50 µL of proteinase K [1 mg/mL in 50 mmol/L Tris/HCl buffer, pH 8.0, with 1 mmol/L EDTA (ethylene diamine tetraacetate)] and 0.45% Tween 20 at 52 °C overnight. Digested material was heated at 95 °C for 15 minutes to denature the proteinase K, and this preparation was used as a template for PCR without additional purification. Quantitation of DNA from positive and negative control strains was determined spectrophotometrically.

PCR Amplification and Analysis of Amplified DNA Products

To ensure optimal sensitivity in paraffin-embedded materials, separate PCRs were performed for *vacA-s*, *vacA-m*, *cagA*, *cag* empty site (ES), and 16S rRNA. Five sets of primers were initially used in this study (Table 2), all generating amplicons not exceeding 350 bp. To amplify *vacA s* regions, primers VA1F and VA1R were selected, resulting in generation of fragments of 259 bp for the type *s1* variants or fragments of 286 bp for type *s2* variants [16]. For the detection of the *m* region of the *vacA* gene, a mixture of four forward primers, MF1.1, MF1.2, MF1.3, and MF1.4, and one reverse primer MR1, was used in a simplex PCR, resulting in amplification of fragments of 107 bp for *m1* or 182 bp for *m2* strains [24]. The *cagA* gene, the *cag* empty site and the 16S rRNA gene were detected with primers yielding amplicons of 183, 106, and 109 bp, respectively [39–42] (Table 2).

Because of the low frequency of successful amplification of the primer set for the *m vacA* region used in simplex PCR, an additional set of primers was employed that allowed the simultaneous analysis of the *m vacA* alleles by multiplex PCR, as reported by Koehler et al. [43] (Table 2). This primer set generated amplicons of 340/301 bp for *vacA m1* and 169/101 bp for *vacA m2*. Larger *vacA m1* and *m2* amplicons for sequencing were obtained using a primer set HPMG-F and HPMG-R [24] (Table 2). All PCR mixtures consisted of 10 mmol/L Tris-HCl (pH 8.3), mol/L of each 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 300 µM dNTP, 0.3 µmol

of each forward and reverse primer, and 0.6 U of HotStar Taq DNA Polymerase (Qiagen) in a final volume of 20 μ L. Three microliters of DNA extracted from gastric biopsies was added to each reaction mixture. The mixture was covered with mineral oil to prevent evaporation.

The PCR programs for *cagA* and *vacA m* amplification comprised 9 minutes at 94 °C, followed by 60 cycles of 30 seconds at 94 °C, 45 seconds at 50 °C, 45 seconds at 72 °C, and a final incubation at 72 °C for 5 minutes. The amplification programs for *vacA s*, *cag* empty site, and 16S rRNA consisted of 15 minutes at 95 °C, followed by 60 cycles of 1 minute at 94 °C, 1 minute at 55 °C for *vacA s* fragment (at 54 °C for ES, at 52 °C for 16S rRNA, at 58 °C for multiplex PCR), 1 minute at 72 °C, and a final incubation at 72 °C for 5 minutes. PCR products (10 μ L of each sample) in all cases were electrophoresed in 2% agarose gels stained with ethidium bromide for 2 hours at 60 Volts. Positive and negative reagent control reactions, including a no-template control, were included in each experiment.

Sequencing

The diversity of the *vacA m* region was analyzed by sequencing PCR products amplified from nine DNA samples from paraffin-embedded biopsies and from eight DNA samples isolated from single colonies of *H. pylori* strains from both high and low risk areas. Sequences of 301, 169, and 101 bp were amplified from the end of the 501–696 region of the *vacA*, determined by Skibinski et al. as required for full toxicity in HeLa cells [44]. Additionally, PCR products from amplification of larger *m1* and *m2* amplicons (401 and 476 bp, respectively [24]) from three DNA samples from paraffin-embedded biopsies were sequenced. These fragments were amplified from the beginning of the 501–696 region of *vacA* [44]. Sequencing was performed using BigDye terminator chemistry with an ABI 3730xl DNA Analyzer. All sequences of the *vacA m* fragments were compared with corresponding sequences for reference strains: *H. pylori* J99 (GenBank accession no. NC000921), *H. pylori* 60190 (ATCC 49503, GenBank accession no. U05676), and *H. pylori* Tx30a (GenBank accession no. U29401). Sequence construction and alignment was performed using GENEIOUS software, version 2.5.3 (Biomatters, Auckland, New Zealand). Persons performing all genotyping and sequencing reactions were blinded to the location of residence of the persons whose samples were being analyzed.

Statistical Analysis

For continuous and categorical variables, respectively, *t*-test and χ^2 tests were used to determine the statistical significance of differences in the distribution of age, histopathologic diagnosis, and *H. pylori* genotypes between areas. Fisher's exact test was used in some comparisons as appropriate. Analyses were performed using STATA 9.0 (Stata Corporation, College Station, TX, USA).

Results

Detection of *H. pylori* Infection by PCR

Of 107 DNA samples isolated from gastric biopsies, 93 (86.9%) were positive for *H. pylori* as determined by PCR for the 16S rRNA region of *H. pylori*. In 14 samples (13.1%), *H. pylori* DNA was not detected by the 16S rRNA assay. In 10 of those 14 samples, *cagA* and/or *vacA* amplifications were also negative, and consequently those 10 subjects were considered to be uninfected. For the other four cases, the 16S rRNA assay showed false negative results (3.7%), as assessed by multiple positive signals for *cagA* and/or *vacA* genotypes. As a result, 97 individuals (90.6%) were considered to be infected with *H. pylori*.

Detection of *H. pylori* Infection by Histopathology

Histologic examination of the Steiner-stained sections of gastric biopsies showed the presence of typical spiral-shaped *H. pylori* in 95 (88.8%) of the 107 subsets. Designation of biopsies as positive or negative for *H. pylori* was concordant by 16S rRNA PCR and histopathology in 97 of 107 biopsies (90.6%).

Genotyping of *H. pylori* Based on Virulence-Associated Genes

Analysis was considered complete if the *vacA* *s*- and *m*-regions as well as *cagA* could be detected. Complete genotyping was achieved in 94 of 97 DNA samples (96.9%) containing *H. pylori* DNA. In three DNA samples (3.1%), only *cagA* or only *m* or *s vacA* regions could be genotyped.

Detection of *cagA* and the *cag* Empty Site—The amplification of *cagA* generated 183-bp fragments from paraffin-embedded gastric biopsies from 78 of 97 *H. pylori*-positive subjects (80.4%; Table 3). The ES amplification produced 106-bp fragments from 33 of the paraffin-embedded gastric biopsies from the same *H. pylori*-positive subjects (34.0%; Table 3). Twenty-three DNA samples generated signals for both the *cagA* gene and for the ES (23.7%), suggesting the presence of mixed infections. Twenty-two of those 23 DNA samples also showed signals for different *s*- and *m-vacA* fragments, consistent with the diagnosis of mixed infections. The prevalence of *cagA*-positive strains was higher in patients from the high risk area (87.0%), as compared to those from the low-risk region (72.1%), however, this association was not statistically significant ($p = .076$; Table 3).

Detection of *s* and *m* Alleles of *vacA*—Amplification of *vacA* *s* and *m* alleles allowed concomitant discrimination of *s1* and *s2* alleles as signals of 259 bp and 286 bp, respectively, and of *m1* and *m2* alleles as 107 bp and 182 bp fragments, respectively. Multiplex PCR amplification of the *m* fragments allowed simultaneous discrimination of *m1* as 340 bp and/or 301 bp and of *m2* as 169 and/or 101 bp fragments, respectively. Results from the *m-vacA* genotyping using both PCR assays are shown in Table 4.

Genotyping for the *vacA m* fragment using a set of primers for simplex PCR allowed detection of 15 *m1* signals (14.6%), two *m2* signals (1.9%), but no mixed *m1/m2* signals in a total of only 17 cases (15.8%) of 107 tested DNAs. Ninety of the cases (84.1%) were untypable by this assay after repeated attempts. Genotyping for the same *vacA* fragment using a primer set for multiplex PCR allowed detection of nine *m1* signals (8.4%), nine *m2* signals (8.4%), and seven mixed *m1/m2* signals in a total of 25 cases (23.4%). Genotyping with both primer sets, for simplex and multiplex PCRs, allowed detection of a total of 55 cases: 43 (40.2%) *m1*, nine (8.4%) *m2*, and three (2.8%) *m1/m2* cases. Combining results from simplex PCR only, from multiplex PCR only, and from those positive in both PCRs, the *H. pylori* strains from all 97 individuals were genotyped for the *vacA m* region (Table 4). As a result, use of the second set of primers in the genotyping of *m vacA* from paraffin-embedded gastric biopsies significantly increased the efficiency of the *vacA m* genotyping compared to the use of any single set alone ($p < .0001$ in either comparison).

Distribution of Virulence-Associated Genotypes in Areas of Low and High Risk for GC—The distribution of *cagA* genotypes in the two GC risk areas is shown in Table 3. Among the 97 *H. pylori*-infected subjects, the *vacA s1* genotype was predominant (93.6%) over the *s2* types (6.4%). Five samples contained both *s1* and *s2* types. An *s1:s2* ratio of 24 : 1 was present in the high-risk area and 10 : 1 was present in the low-risk area. No association of *vacA s* fragment distribution by area was found. The *vacA m1* to *m2* ratios were 7 : 1 in the high risk and 2 : 1 in the low risk area. The *m1* and *m2* alleles were found significantly more frequently in the high- and low-risk areas, respectively ($p = .037$). The *s1m1* genotypes

consisting of *s1* alone, *m1* alone, and *s1/s2*, *m1/m2* genotypes were more common in the high risk area (88.2% vs. 67.4%); the *s2m2* genotypes, including *s2* alone, *m2* alone, and *s1/s2*, *m1/m2* genotypes were more common in the low-risk area (16.3% vs. 3.9%, $p = .044$). The prevalence of *s1m1* genotypes and *cagA* positivity was 84.3% and 60.5% for high and low risk areas, respectively ($p = .011$). The comparative distribution of *vacA* *s* and *m* genotypes as well as of combinations of different groups of *s* and *m* genotypes is shown in Table 3.

The frequency of multiple genotypes containing *cagA* and multiple *vacA* genotypes in a single DNA extract was 31.5% and 37.2% in high and low GC risk areas, respectively. This difference was not significant (Table 1).

Histopathology Results

The distribution of histopathologic diagnoses is shown in Table 1. The histopathologic diagnoses and *H. pylori* genotypes stratified by area are shown in Table 5. All subjects with MAG, IM, and DYS from the high-risk area were infected with *cagA*+, *vacA s1m1 H. pylori* strains. In the low risk area, the progression of lesions correlated with the prevalence of virulent genotypes.

Sequencing Results

Sequences of four *vacA m1* DNA fragments (bases 2039–2335 with reference to *H. pylori* strain J99) amplified from strains from the high-risk area showed three variations at positions 2066, 2067, and 2188, which were assessed as missense mutations; the other eight variations were synonymous mutations. In other *m1* fragments from single colonies of *H. pylori* strains from low and high risk areas, these and additional variations were found (Fig. 1).

Sequences of four *m2* fragments from paraffin-embedded biopsies and from cultured single colonies from high and low risk areas (bases 2281–2449 with reference to strain Tx30a) showed one missense mutation and seven synonymous mutations (data not shown).

Sequences of two *m1* DNA fragments amplified from paraffin-embedded biopsies from residents of the high-risk area (equivalent to bases 1446–1846 with *H. pylori* reference strain 60190) showed 17 variations, of which four for the first fragment and seven for the second fragment were assessed as missense mutations generating different amino acids upon translation (Fig. 2). In the sequence of one *vacA m2* fragment from the low risk area (equivalent to bases 1764–2239 compared to *H. pylori* reference strain Tx30a), 23 base variations were found, of which six were non-synonymous (data not shown).

Sequences of *vacA m1* and *m2* DNA fragments from paraffin-embedded biopsies from residents of the high- and low risk area aligned with reference strains, but showed 5.9% nucleotide diversity. Sequencing of 9 *m1* and 8 *m2* strains from paraffin-embedded biopsies at a length of 301/401 and 169/476 bp, respectively, revealed up to 23 base variations, up to seven of them resulting in missense mutations. No association was found between the number and the frequency of base variations in sequences and GC risk areas.

Discussion

The presence of regions within the same country, which differ widely in gastric adenocarcinoma incidence, provides a useful opportunity to investigate the mechanisms by which one population may be protected from this disease, while the other may be inherently susceptible. The reasons for these differences in cancer incidence are unknown. One possible explanation is differing rates of infection with *H. pylori* [45]. However, our data indicate that the prevalence of *H. pylori* infection is high in both regions. Using histopathology, Bravo et al. [32] found similarly high infection rates among residents of regions of both high and low

risk for GC: 73.8% in Pasto and Tuquerres, versus 79.5% in Tumaco. Using the 16S rRNA assay, we found even higher infection rates, likely due to the higher sensitivity of the techniques employed, as well as to the age and sex of patients included in this study. However, the rates of *H. pylori* infection were nearly identical in the high and low risk regions (90.0% vs. 91.5%). Therefore, differences in exposure to the major environmental risk factor *H. pylori* do not explain the difference in incidence rates for GC in the populations we studied.

The central finding of this study, resulting from *H. pylori* genotype analyses from paraffin-embedded biopsies, is that the high incidence of GC in the high-risk areas is associated with an increased incidence of specific *H. pylori* genotypes: the more virulent *cagA*-positive *s1m1* strains were more prevalent in the area of high risk than in the area of low risk. Considering the *vacA s1m1* genotypes separately, *s1m1* strains were more common in the high-risk area, while *s2m2* genotypes were more common in the low-risk area.

Few studies have compared *H. pylori* virulence-associated genes in high and low risk regions within the same country. A previous study on Colombian subjects [32] based on genotype analyses from frozen biopsies, reported significantly higher percentages of *cagA*-positive and *vacA s1* and *m1* genotypes in populations of Pasto and Tuquerres with high risk for GC, as compared to those of the population from the low risk area. A recent study from China [46] reported that the prevalence of *vacA s1* or *s1m1b* *H. pylori* strains was significantly higher in patients from the high risk area for GC, as compared to those from the area of low risk for GC. That study found no difference in the distribution of *cagA*-positive, *vacA m* alleles, and *iceA1* and *iceA2* genotypes in the two regions. Although there may be many differences in the development of GC between Colombia and China, it is important to note that the finding of the elevated incidence of *H. pylori* strains bearing *s1m1* in the higher risk regions is similar between the two populations.

In our study, we found a trend ($p = .076$) for a higher incidence of *cagA* positivity in strains from subjects living in the area of high risk, compared to those from the low risk region. The prevalence of *cagA* we detected is greater than that reported in studies on *H. pylori* strains from populations in Europe and the USA, where only one-half to two-thirds of strains carry the *cagA* gene [47–50]. In our study, all *cagA* negative strains were confirmed by the positive empty site assay.

Because we sampled entire biopsies, and not isolated single colonies, our analyses could easily detect the presence of mixed infections. Detection of mixed infections was enhanced by addition of the ES assay to produce a positive signal for *cagA*-negative strains and by addition of the multiplex PCR for the *vacA m* genotypes, which permitted higher detection and discrimination of *m1* and *m2* genotypes. The presence of mixed infections was not associated with risk for GC; therefore our data do not support variation in percentages of mixed infections as contributing to an elevated incidence of GC.

Mutations generated in certain regions of the *vacA* gene indicate that the vacuolating and other functions of those regions can be ablated following alterations of individual amino acid residues [51,52]. Ji et al. [53] determined that differences in target cell specificity between the *m1* and *m2* forms of the VacA protein were determined by the first 148 amino acids of the mid-region. Furthermore, within this region, the first 35 amino acids were found to be essential for toxicity in HeLa cells [53]. By analyzing chimeric strains created within the region bounded by amino acids 535–695, Skibinski et al. [44] determined that higher cytotoxicity was found in strains with an *m1* segment longer than 35 amino acids and established that the entire N-terminal 148 amino acids of the midregion was required for full toxicity in HeLa cells. Based on these observations, we sequenced portions of the *vacA m1* and *m2* regions located at the beginning and at the end of the region Skibinski identified (amino acids 501–695). We found greater

sequence conservation of the 3' end of this fragment, in bases 2039–2335 encoding amino acids 679–778 compared to the beginning of this region (bases 1446–1846 encoding amino acids 482–615). As recently shown in the case of a eukaryotic gene, naturally occurring variations in synonymous codons (silent mutations) can give rise to a protein product with the same amino acid sequence but different structural and different functional features due to altered protein folding [54]. We speculate that it is possible that the variability we found in the *m* region of the *vacA* gene of Colombian *H. pylori* strains may influence the structure of translated and secreted vacuolating toxin.

The current study demonstrated high typability of the *H. pylori* 16S rRNA and *cagA* genes, and discrimination of the genotypes of the *vacA* gene directly in paraffin-embedded gastric biopsies. The isolation of DNA from routinely formalin-fixed and paraffin-embedded tissue samples permitted the use of archival materials for molecular analysis. A major problem using these specimens is fragmentation of nucleic acids, which could lead to false negative results. Consequently, careful DNA extraction [55] is a critical step in the procedures preceding the PCR reactions. With attention to DNA preparation and amplicon size, archival materials can successfully be used for *H. pylori* genotyping.

The overall positive PCR results in our study were largely concordant with histology results. The differences between histology and PCR results could be due to the patchy distribution of the *H. pylori* in the stomach. However, because of higher PCR sensitivity, more individuals were found to be infected with *H. pylori* as assessed by PCR, compared with histopathology. In our study, the results of genotyping of paraffin-embedded gastric biopsies showed higher typability compared to those reported by Nimri et al. (44%) [56] and Scholte et al. (93% and 90%) [57,58].

In conclusion, the present study permitted high typability of the *H. pylori* 16S rRNA and genotyping of *vacA* and *cagA* genes from archival materials. Although we found a high prevalence of *H. pylori* infection in Colombian areas of both high and low risk for GC, infections with strains of *s1m1* and *cagA*-positive genotypes, as well as *s1m1* genotypes separately, were found more frequently in residents of areas of high risk, whereas infection with *vacA* *s2m2* strains was more frequent in the population at low GC risk. Mixed infections were found with the same frequency in both populations.

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Figure 1.

Alignment of six representative *vacA m1* sequences from six Colombian *H. pylori* strains from high and low risk areas with a homologous sequence fragment of the *H. pylori* reference strain J99. Variations at 2066, 2067, and 2188 were assessed as missense mutations. The other eight variations were synonymous mutations. No differences were found in the frequency of variations in sequences from high and low risk areas. HR – high risk for GC; LR – low risk for GC.

Figure 2.

Alignment of protein sequences of two representative *vacA m1* Colombian strains (PC118 and PC210) from a high-risk area with sequence of *H. pylori* reference strain 60190. A total of four and seven different amino acids were found in protein sequences of PC118 and PC 210 compared with protein sequence of *H. pylori* reference strain 60190.

Table 1Demographic distribution, status of *H. pylori* infection and histopathologic diagnoses in Colombian subjects

Variables	Low risk n = 47	High risk n = 60	Two-sided <i>p</i> value
Age, mean (SD)	48.7 (5.4)	47.2 (5.7)	0.168 ^a
Infected subjects, by PCR, n (%)	43 (91.5)	54 (90.0)	1.00 ^b
Subjects with multiple infection, n (%)	16 (37.2)	17 (31.5)	0.554 ^c
Histopathologic diagnosis ^d , n (%)			
Normal/Non-atrophic gastritis	19 (42.2)	27 (47.4)	0.171 ^c
Multifocal atrophic gastritis	12 (26.7)	7 (12.3)	
Intestinal metaplasia/Dysplasia	14 (31.1)	23 (40.3)	

^a *p* value for difference using a *t*-test.^b *p* value for difference using Fisher's exact test.^c *p* value for difference using χ^2 test.^d Five cases classified as gastritis NOS (atrophy could not be assessed) were not included in this comparison.

SD, standard deviation.

Table 2

PCR primers used in this study

Amplified gene and region	Primer designation	Primer sequence (5'-3')	Reference
<i>cagA</i>	cagAF	TTG ACC AAC AAC CAC AAA CCG AAG	[40]
	cagAR	CTT CCC TTA ATT GCG AGA TTC C	
<i>cag</i> empty site	ES-F	AAG AGA GAG TGG TGC GAT	[39]
	Rnew-1R	TCA TGC GAG CGG CGA TGT G	[41]
<i>vacA</i>	VA1F	ATG GAA ATA CAA CAA ACA CAC	[16]
<i>s1, s2</i>	VA1R	CTG CTT GAA TGC GCC AAA C	
<i>vacA</i> simplex PCR	MF1.1F	GTG GAT GCC CAT ACG GCT AA	[24]
	MF1.2F	GTG GAT GCT CAT ACA GCT (AT)A	
<i>m1, m2</i>	MF1.3F	GTG GAT GCC CAT ACG ATC AA	
	MF1.4F	GCG AGC GCT CAT ACG GTC AA	
	MR1-R	(AG)TG AGC TTG TTG ATA TTG AC	
<i>vacA</i> , multiplex PCR	M1F1	CAA CAA TCA AGG CAC TAT CAA (C/T)TA	[43]
	M1R1	CCG CAT GCT TTA ATG TCA TCA G	
	M1F2	TGG TCC GAG GCG GG(A/C) AAG T	
	M1R2	TCA TCA GTA TTT CGC ACC ACA C	
	M2F1	TTT GGA GC(C/T) CCA GGA AAC ATT G	
<i>m1, m2</i>	M2F1	C(C/T)A CAC GCC CAT CTT GGA CAA	
	M2F2	ACC CTA AA(C/T) AGC AAC GCA AGC	
	M2R2	GAC AAA AAG ATT CAT CGT GCC TT	
16S rRNA	Hp1	CTG GAG AGA CTA AGC CCT CC	[42]
	Hp2	ATT ACT GAC GCT GAT TGT GC	
<i>vacA</i>	HPMG-F	CACAGCCACTTTCAATAACGA	[24]
<i>m1, m2</i>	HPMG-R	CGTCAAAATAATTCCAAGGG	

Table 3

Comparative distribution of *cagA* and *vacA* s and m genotypes in areas of Colombia with high and low risk for gastric cancer

Variables	Low risk	High risk	Two-sided <i>p</i> value ^a
<i>cagA</i> positive, n (%)	31 (72.1)	47 (87.0)	0.076
ES positive, n (%)	18 (41.9)	15 (27.8)	0.146
<i>m-vacA</i> region, n (%)	n = 43	n = 54	
<i>m1</i> + mixed forms	30 (69.8)	47 (87.0)	0.037 ^b
<i>m2</i>	13 (30.2)	7 (13.0)	
<i>s-vacA</i> region, n (%)	n = 43	n = 51	
<i>s1</i> + mixed forms	39 (90.7)	49 (96.1)	0.407
<i>s2</i>	4 (9.3)	2 (3.9)	
<i>s-</i> and <i>m-vacA</i> regions, n (%)	n = 43	n = 51	
<i>s1m1</i> ^c	29 (67.4)	45 (88.3)	0.044
<i>s2m2</i> ^d	7 (16.3)	2 (3.9)	
Other (<i>s1m2</i>)	7 (16.3)	4 (7.8)	
<i>cagA</i> and <i>vacA</i> genotyping, n (%)	n = 43	n = 51	
<i>cagA</i> positive and <i>vacA s1m1</i>	26 (60.5)	43 (84.3)	0.011
Others	17 (39.5)	8 (15.7)	

^a *p* value for difference using Fisher's exact test.

^b *p* value for difference using χ^2 test.

^c Combining *s1m1* + *s1* and *m1/m2* + *s1/s2* and *m1*.

^d Combining *s2m2* + *s2* and *m1/m2* + *s1/s2* and *m2*.

ES, empty site.

Table 4Results of the *vacA m* genotyping using two primer sets for simplex or multiplex PCR

<i>vacA</i> detected region	<u>Number of positive signals with primer sets for:</u>			Positive signals with all: simplex PCR, multiplex PCR, and with both: n (%)
	1. Simplex PCR only n (%)	2. Both simplex and multiplex PCR n (%)	3. multiplex PCR only n (%)	
<i>m1</i>	15 (14.6)	43 (40.2)	9 (8.4)	67 (62.6)
<i>m2</i>	2 (1.9)	9 (8.4)	9 (8.4)	20 (18.7)
<i>m1/m2</i>	0	3 (2.8)	7 (6.5)	10 (9.3)
Total detected <i>m</i>	17 (15.8)	55 (51.5)	25 (23.4)	97 (90.7)
Negative results:	90 (84.2)	52 (48.5)	82 (76.6)	10 (9.3)
<i>p</i>	$p_{1,2} < .0001$	$p_{1,3} = .23$	$p_{2,3} < .0001$	

Table 5

Comparative distribution of histopathologic diagnoses and *H. pylori* genotypes in Colombian areas of high and low risk for GC

Gastric cancer risk area							
Genotypes	Low risk				High risk		
	Normal/NAG n = 17	MAG n = 12	IM/DYS n = 13	Two-sided <i>p</i> value ^a	Normal/NAG n = 22	MAG n = 7	IM/DYS n = 19
							Two-sided <i>p</i> value ^d
<i>cagA</i> positive and <i>vacA sImI</i>	7 (41.2)	7 (58.3)	12 (92.3)	0.013	15 (68.2)	7 (100)	19 (100)
Others	10 (58.8)	5 (41.7)	1 (7.7)		7 (31.8)	0 (0)	0 (0)

^a *p* value for difference using Fisher's exact test.

NAG, non-atrophic gastritis; MAG, multifocal atrophic gastritis without metaplasia; IM, intestinal metaplasia; DYS, dysplasia.