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Genotyping of *Helicobacter pylori* virulence-associated genes shows high diversity of strains infecting patients in western Venezuela

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SUMMARY

Background: *Helicobacter pylori* is a major cause of chronic gastritis and an established risk factor for gastric adenocarcinoma. This bacterium also exhibits an extraordinarily high genetic diversity.
Methods: The genetic diversity of *H. pylori* strains from Venezuelan patients with chronic gastritis was evaluated by PCR-typing of *vacA*, *cagA*, *iceA*, and *babA2* virulence-associated genes using DNA extracted directly from biopsies. The nucleotide sequence and prevalence of size variants of *iceA1*, *iceA2*, and *babA2* PCR products were introduced in this analysis.
Results: The frequency of *vacA* s1 was associated ($p < 0.01$) with moderate/severe grades of atrophic gastritis. The *cagA*, *iceA1*, *iceA2*, and *babA2* genotypes were found in 70.6%, 66.4%, 33.6%, and 92.3% of strains, respectively. The frequency of *iceA2* and its subtype *iceA2*_D were higher ($p < 0.015$) in cases with moderate/severe granulocytic inflammation. The most prevalent combined genotypes were *vacA* s1m1/*cagA*/*iceA1*/*babA2* (26.3%), *vacA* s2m2/*iceA1*/*babA2* (19.5%), and *vacA* s1m1/*cagA*/*iceA2*/*babA2* (18.8%). Sequence analysis of *iceA1*, *iceA2*, and *babA2* PCR-amplified fragments allowed us to define allelic variants and to increase the number of genotypes detected (from 19 to 62). A phylogenetic tree made with *iceA1* sequences showed that the *H. pylori* strains analyzed here were grouped with those of Western origin.
Conclusions: Our results show that patients from the western region of Venezuela have an elevated prevalence of *infection with H. pylori* strains carrying known virulence genotypes with high genetic diversity. This highlights the importance of identifying gene variants for an early detection of virulent genotypes.

1. Introduction

Helicobacter pylori is a Gram-negative spiral-shaped bacterium that persistently colonizes the human gastric mucosa. The presence of the bacterium causes chronic inflammation that may lead to non-cardia gastric adenocarcinoma, gastric lymphoma, and peptic ulceration.[1] Although *H. pylori* infects half of the world's population, only a fraction of colonized individuals develop peptic ulcer and gastric cancer.[2,3] This fact could be associated either with the genetic diversity of *H. pylori*, or with the particular host genetic background, and/or specific interactions between a particular strain and its host.[4]

The genetic diversity in *H. pylori* is greater than that exhibited by most bacteria, with exceptionally high rates of DNA point mutations and intra- and inter-genomic recombination that allow its adaptation to changing environments.[5] The most significant consequences of these genomic changes are antibiotic resistance and differences in the prevalence or expression of bacterial virulence factors that may determine the clinical outcome of gastric diseases.

Several genes, such as *vacA*, *cagA*, *iceA*, and *babA2*, have been identified as markers for enhanced pathogenicity of *H. pylori*. The vacuolating cytotoxin gene (*vacA*) is present in all *H. pylori* strains.[6] Variations in VacA structure at its signal (s) and mid (m) regions result in different levels of cytotoxicity.[6] Specifically, s region variations are associated with the vacuolating activity of VacA, whereas variations in the m region determine the cell specificity of vacuolation, by affecting the binding of the toxin to the host cells. Toxin production is highest in s1/m1 strains, moderate in s1/m2 strains, and scarce or null in *H. pylori* expressing s2/m2 forms.[6,7]

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Table 1
PCR primers for amplification of *vacA*, *cagA*, *iceA1*, *iceA2*, and *babA2* sequences

Gene	Primer sequence (5′ → 3′)		PCR conditions	Reference
vacA				
s1/s2	VAI-F	ATGGAAATACAACAAACACAC	94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35 cycles)	Chattopadhyay et al. (2004)
	VAI-R	CTGCTTGAATGCGCCAAAC		
m1/m2	VAG-F	CAATCTGTCCAATCAAGCGAG	94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35 cycles)	Chattopadhyay et al. (2004)
	VAG-R	GCGTCAAAATAATTCCAAGG		
cag-PAI empty site	Luni 1	ACATTTTGGCTAAATAAACGCTG	94 °C, 1 min; 56 °C, 30 s; 72 °C, 45 s (35 cycles)	Rahman et al. (2003)
	R5280	GGTTGCACGCATTTTCCCTTAATC		
iceA1	iceA1F	TATTTCTGGAACTTGCGCAACCTGAT	94 °C, 1 min; 55 °C, 45 s; 72 °C, 45 s (35 cycles)	Rahman et al. (2003)
	M.Hpy1R	GGCCTACAACCGCATGGATAT		
cagA	cag5c-F	GTTGATAACGCTGTCGCTTC	94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min (35 cycles)	Chattopadhyay et al. (2004)
	cag3c-R	GGGTTGTATGATATTTTCCATAA		
iceA2	cycSf	CGGCTGTAGGCACTAAAGCTA	94 °C, 1 min; 55 °C, 45 s; 72 °C, 45 s (35 cycles)	Rahman et al. (2003)
	IceA2R	TCAATCCTATGTGAAACAATGATCGTT		
babA2	bab7-F	CCAAACGAAACAAAAAGCGT	94 °C, 1 min; 57 °C, 45 s; 72 °C, 30 s (35 cycles)	Sheu et al. (2003)
	bab7-R	GCTTGTGTAAAAGCCGTCGT		

The *cagA* gene is a marker for the presence of the *cag* pathogenicity island (*cag*-PAI) of approximately 40 kb, whose presence is associated with the more severe clinical outcomes.[8,9] A type IV secretion system translocates CagA protein into gastric epithelial cells, where it is phosphorylated. When this modification occurs, CagA affects various cellular processes and signal transduction pathways, such as disruption of tight and adherent junctions that lead to proinflammatory and mitogenic responses–effects.[9,10] Another putative virulence factor is IceA, whose gene has two main allelic variants, *iceA1* and *iceA2*. The expression of *iceA1* is up-regulated on contact of *H. pylori* with human epithelial cells and may be linked with peptic ulcer disease.[11,12] The blood group antigen binding adhesin (BabA), a 78-kDa outer membrane protein encoded by the *babA2* gene, binds to Lewis b antigens and ABO antigen.[13,14] Although three *bab* alleles have been identified (*babA1*, *babA2*, and *babB*), only the *babA2* gene product is functionally active.[15] Studies in Western populations have associated the presence of the *babA2* gene with gastric cancer.[14,16]

H. pylori infection is very common in Venezuela and gastric cancer is an important cause of cancer-related death in this country. Although our group and others have recently reported the prevalence of *H. pylori* and host genetic factors associated with gastric diseases,[17–22] few studies have investigated the genetic diversity of this pathogenic bacterium in Venezuela. The aim of this work was to assess the genetic diversity of *H. pylori* strains infecting patients with chronic gastritis in the western region of Venezuela through the evaluation of the prevalence of several genes coding for virulence factors.

2. Methods

2.1. Patient samples

A total of 148 *H. pylori*-positive patients (52 men and 96 women) diagnosed with chronic gastritis by histopathological analysis were included in this study. Their mean age was 44 years (range 18–88 years). Patients with criteria indicating an endoscopy were referred to the Gastroenterology Service of the Hospital Antonio María Pineda (HAMP), Barquisimeto, Venezuela. Exclusion criteria were: age under 18 years, previous gastric surgery and *H. pylori* eradication treatment, history of bleeding and coagulation disorders, consumption of antibiotics during the 3 months preceding endoscopy, and consumption of bismuth salts, proton pump inhibitors, or sulcrafate in the previous month. Six biopsies were obtained from each patient: three from the antrum and three from the gastric corpus.

Two antral and two corpus sections of each patient were stained with hematoxylin–eosin and evaluated by two independent expert pathologists from the Department of Pathology (HAMP-UCLA). The evaluation was performed in accordance with the Sydney classification system with regard to the presence and degree of atrophic gastritis (N/M: no/mild; M/S: moderate/severe), granulocytic infiltration (G0/G1: absence/mild; G2/G3: moderate/severe), and lymphocytic infiltration (L0/L1: absence/mild; L2/L3: moderate/severe). The remaining biopsies were used for DNA isolation by Wizard Genomic DNA Purification Kit (Promega), in accordance with the manufacturer's instructions. For the PCR reactions we used 1–2 μl of genomic DNA.

2.2. H. pylori *status and genotyping*

Identification of the *cagA*, *vacA* s1/s2, *vacA* m1/m2, *iceA1*/*A2*, and *babA2* genotypes was carried out using primer sets selected from previous published works (Table 1).[23–25] Specific PCR reactions were conducted in a final volume of 25 μl containing 0.4–0.8 μM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 1.25 U of GoTaq DNA Polymerase (Promega), under the cycling conditions shown in Table 1. Samples confirmed as *H. pylori*-positive by PCR detection of the *vacA* gene were included in the study.

2.3. DNA sequencing and analysis

Several *iceA1*, *iceA2*, and *babA2* PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega). DNA sequencing was performed using an ABI-PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequencing was performed at least twice using the same primers (forward and reverse) as in the PCR assays. DNA sequence editing and analysis were performed with DNA for Windows v. 2.2 and DNAMAN (Lynnon Corporation). All the sequences were compared with GenBank nucleotide and protein databases using Blastn and Blastx algorithms, respectively (http://www.ncbi.nlm.nih.gov/blast).

2.4. Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are **JQ808066** and **JQ808067** for the *iceA1* gene, **JQ808069** to **JQ808071** for the *iceA2* gene, and **JQ808072** to **JQ808079** for the *babA2* gene.

2.5. Phylogenetic analysis

iceA1 sequences from 34 strains reported in different parts of the world (available in public databases: GenBank, EMBL, and DDB) and two *iceA1* sequences produced in the present work were used to construct a phylogenetic tree. For this analysis we selected

Table 2
Distribution of *Helicobacter pylori vacA* and *cagA* status according to histological changes in chronic gastritis patients

	Atrophic gastritis				Granulocytic infiltration				Lymphocytic infiltration				Total ($n=143$)	
	N/M ($n=126$)		M/S ($n=17$)		G0/G1 ($n=114$)		G2/G3 ($n=29$)		L0/L1 ($n=99$)		L2/L3 ($n=44$)			
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Genotype														
vacA s1	77	(61.1)	16	(94.1)[a]	71	(62.3)	22	(75.9)	65	(65.7)	28	(63.6)	93	(65.0)
vacA s2	49	(39.5)[a]	1	(6.3)	43	(38.4)	7	(25.0)	34	(35.1)	16	(37.2)	50	(35.7)
vacA m1	65	(51.6)	11	(64.7)	57	(50.0)	19	(65.5)	54	(54.5)	22	(50.0)	76	(53.1)
vacA m2	61	(48.4)	6	(35.3)	57	(50.0)	10	(34.5)	45	(45.5)	22	(50.0)	67	(46.9)
vacA s1m1	65	(51.6)	11	(64.7)	57	(50.0)	19	(65.5)	54	(54.5)	22	(50.0)	76	(53.1)
vacA s1m2	12	(9.5)	5	(29.4)[b]	14	(12.3)	3	(10.3)	11	(11.1)	6	(13.6)	17	(11.9)
vacA s2m2	49	(39.5)[a]	1	(6,3)	43	(38.4)	7	(25.0)	34	(35.1)	16	(37.2)	50	(35.7)
cagA	86	(68.3)	15	(88.2)	79	(69.3)	22	(75.9)	70	(70.7)	31	(70.5)	101	(70.6)
vacA s1m1/ *cagA*	64	(50.8)	11	(64.7)	56	(49.1)	19	(65.5)	53	(53.5)	22	(50.0)	75	(52.4)
vacA s1m2/ *cagA*	9	(7.1)	5	(29.4)[b]	12	(10.5)	2	(6.9)	9	(9.1)	5	(11.4)	14	(9.8)
vacA s2m2/ *cagA*	12	(9.5)	0	(0.0)	11	(9.6)	1	(3.4)	8	(8.1)	4	(9.1)	12	(8.4)

N/M, no or mild; M/S, moderate/severe; G0/G1, absence/mild; G2/G3, moderate/severe; L0/L1, absence/mild; L2/L3, moderate/severe.
[a] $p<0.01$.
[b] $p<0.03$.

sequences that showed query coverage of more than 95% with a 381-nt region of our sequences (corresponding to a hypothetical open reading frame (ORF)). Multiple nucleotide alignments were carried out using ClustalW. A neighbor-joining tree was constructed with MEGA 5 software using the bootstrap method at 1000 replications with the two-parameter model of Kimura.

2.6. Statistical analysis

The Chi-square or Fisher's exact test was used to compare the differences among the various groups. Logistic regression analysis was used to calculate the odd ratios (ORs) for the dependent variable. A *p*-value of <0.05 was considered statistically significant. These estimations were performed with SSPS 11.0 software.

3. Results

3.1. Studied sample

All 148 samples included in this study were successfully typed for *cagA*, *vacA* s1/s2, *vacA* m1/m2, *iceA1*/*A2*, and *babA2* genes from DNA extracted from the gastric biopsies. Patients who were positive for the presence of more than one allele per gene were excluded from the analysis of association with histological changes in the gastric mucosa.

3.2. vacA *and* cagA *genotypes*

The most virulent *vacA* s1 allele was predominantly (65.0%) present in chronic gastritis samples (Table 2). The middle region *vacA* m1 allele was found in 53.1%. There was a statistical association ($p<0.01$) between *vacA* s1 and M/S atrophic gastritis. *vacA* s1m1, *vacA* s1m2, and *vacA* s2m2 genotypes were observed in 53.1%, 11.9%, and 35.7%, respectively, and although the frequency of *vacA* s1m2 was lower, this genotype showed a significant association with M/S atrophic gastritis ($p<0.03$).

The *cagA* gene was detected in 70.6% of the *H. pylori*-infected patients (Table 2). The combination *vacA* s1m1/*cagA*$^+$ was the most frequent among *cagA*-positive samples (52.4%), moreover, these 'double-positive' strains represented 98.6% (75/76) of the *vacA* s1m1 samples. The *cagA*-negative status, in samples that did not yield a *cagA* gene-specific product with cag5c-F/cag3c-R primers,

Table 3
Distribution of *Helicobacter pylori iceA* and *babA2* status according to histological changes in chronic gastritis patients

	Atrophic gastritis				Granulocytic infiltration				Lymphocytic infiltration				Total ($n=143$)	
	N/M ($n=126$)		M/S ($n=17$)		G0/G1 ($n=114$)		G2/G3 ($n=29$)		L0/L1 ($n=99$)		L2/L3 ($n=44$)			
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)	*n*	(%)
Genotype														
iceA1	85	(67.5)	10	(58.8)	82	(71.9)	13	(44.8)	70	(70.7)	25	(56.8)	95	(66.4)
A (740 bp)	20	(15.9)	4	(23.5)	21	(18.4)	3	(10.3)	20	(20.2)	4	(9.1)	24	(16.8)
B (758 bp)	65	(51.6)	6	(35.3)	61	(53.5)	10	(34.5)	50	(50.5)	21	(47.7)	71	(49.7)
iceA2	41	(32.5)	7	(41.2)	32	(28.1)	16	(55.2)[a]	29	(29.3)	19	(43.2)	48	(33.6)
A (546 bp)[b]	3	(2.4)	1	(6.3)	4	(3.5)	0	(0.0)	3	(3.0)	1	(2.3)	4	(2.8)
B (651 bp)[b]	25	(19.8)	2	(12.5)	19	(16.7)	8	(28.6)	16	(16.2)	11	(25.6)	27	(19.0)
D (756 bp)[b]	13	(10.3)	3	(18.8)	9	(7.9)	7	(25.0)[a]	10	(10.1)	6	(14.0)	16	(11.3)
babA2	116	(92.1)	16	(94.1)	106	(93.0)	26	(89.7)	90	(90.9)	42	(95.5)	132	(92.3)
A (210 ± 1 bp)[b]	11	(8.9)	0	(0.0)	11	(9.8)	0	(0.0)	9	(9.3)	2	(4.5)	11	(7.8)
B (234 bp)[b]	34	(27.4)	5	(29.4)	28	(25.0)	11	(37.9)	28	(28.9)	11	(25.0)	39	(27.7)
C (250 ± 2 bp)[b]	41	(33.1)	6	(35.3)	40	(35.7)	7	(24.1)	29	(29.9)	18	(40.9)	47	(33.3)
D (269 bp)[b]	28	(22.6)	5	(29.4)	25	(22.3)	8	(27.6)	22	(22.7)	11	(25.0)	33	(23.4)

N/M, no or mild; M/S, moderate/severe; G0/G1, absence/mild; G2/G3, moderate/severe; L0/L1, absence/mild; L2/L3, moderate/severe.
[a] $p<0.015$.
[b] Because of the detection of more than one subtype of *iceA2* and *babA2* in some samples, frequencies for these subvariants were determined considering a sample size of 142 and 141, respectively.

Table 4
Frequency distribution of *Helicobacter pylori* combined genotypes in chronic gastritis patients

No.	Genotype	*n*	(%)	Number of genotypes taking into consideration subtypes of *iceA1*, *iceA2*, and *babA2* genes
1	*vacA* s1m1/*cagA*/*iceA1*/*babA2*	39	(26.3)	10
2	*vacA* s1m1/*cagA*/*iceA2*/*babA2*	28	(18.8)	12
3	*vacA* s2m2/*cagA*/*iceA1*/*babA2*	8	(5.4)	4
4	*vacA* s2m2/*cagA*/*iceA2*/*babA2*	4	(2.7)	4
5	*vacA* s1m2/*cagA*/*iceA1*/*babA2*	10	(6.8)	7
6	*vacA* s1m2/*cagA*/*iceA2*/*babA2*	4	(2.7)	1
7	*vacA* s1m1/*cagA*/*iceA1*	4	(2.7)	2
8	*vacA* s1m1/*cagA*/*iceA2*	4	(2.7)	2
9	*vacA* s2m2/*iceA1*/*babA2*	29	(19.5)	7
10	*vacA* s2m2/*iceA2*/*babA2*	7	(4.7)	4
11	*vacA* s1m1/*iceA1*/*babA2*	1	(0.70)	1
12	*vacA* s1m2/*iceA1*/*babA2*	1	(0.70)	1
13	*vacA* s1m2/*iceA2*/*babA2*	1	(0.70)	1
14	*vacA* s2m2/*iceA1*	2	(1.4)	1
15	*vacA* s1m2/*iceA1*	1	(0.70)	1
16	*vacA* s1s2 m1m2/*cagA*/*iceA1*/*babA2*	2	(1.4)	1
17	*vacA* s1m1 m2/*cagA*/*iceA1*/*babA2*	1	(0.70)	1
18	*vacA* s1m1 m2/*cagA*/*iceA2*/*babA2*	1	(0.70)	1
19	*vacA* s1m1/*cagA*/*iceA1 iceA2*/*babA2*	1	(0.70)	1
	Total	148		62

was confirmed by obtaining an empty *cag*-PAI site-specific 550-bp amplicon (Table 1).

3.3. Prevalence of iceA and babA2 genotypes and variants of PCR-amplified fragments

The *iceA* gene was detected in all samples (Table 3); 66.4% (95/143) were positive for *iceA1* and 33.6% (48/143) for *iceA2* alleles. On amplification of the *iceA1* gene with primers iceA1F and M.Hpy1R,[23] we noticed that the size of the PCR products was slightly different among samples, and after sequence analysis, we defined two subtypes of *iceA1* fragments (*iceA1*_A: 740 bp, and *iceA1*_B: 758 bp) that differed by the presence or absence of 16 bp at the 5′ end of the *iceA1* gene (Table 3; see also **Supplementary Material** Figure S1). Out of the 95 patients positive for *iceA1* strains, 24 (25.3%) were infected with the subtype *iceA1*_A and 71 (74.3%) harbored the i*ceA1*_B allele (Table 3). The mentioned variation in *iceA1* gene nucleotide sequence can be observed in *H. pylori* reference strains from different continents.

The prevalence of the *iceA2* gene was higher in patients with severe histopathological changes (Table 3): N/M atrophic gastritis, 32.5% vs. M/S atrophic gastritis, 41.2%; G0/G1, 28.1% vs. G2/G3, 55.2% ($p < 0.015$); and L0/L1, 29.3% vs. L2/L3, 43.2%. The *iceA2*-specific PCR performed in this work yielded fragments of 546 bp ($n = 4$), 651 bp ($n = 27$), and 756 bp ($n = 16$) (Table 3), which according to DNA sequence analysis corresponded to the *iceA2*_A, B, and D variants described by Figueiredo et al.[26] More specifically, we found that the *iceA2*_D subtype was predominant in G2/G3 cases ($p < 0.015$ vs. G0/G1). One sample, not included in the association analysis, showed a simultaneous amplification of *iceA2*_B and D fragments.

The *babA2* gene was detected in 92.3% of patients (Table 3) and no statistically significant differences were found between its distribution and histological changes. Variations in the 5′ end of the *babA2* gene amplified by primers bab7-F/R resulted in fragments of four different sizes. These *babA2* subtypes were confirmed by sequence: *babA2*_A (210 ± 1 bp), *babA2*_B (234 bp), *babA2*_C (250 ± 2 bp), and *babA2*_D (269 bp) (see **Supplementary Material** Figure S2), representing 8.5%, 29.5%, 36.4%, and 25.6% of the *babA2*-positive samples. Only two samples were observed with coexistence of two *babA2* subtypes (not included in the above analysis). Analysis of some *babA2* nucleotide sequences showed CT repeats at the 5′ end of the gene (**Supplementary Material** Figure S2); these repeats have been associated with translocation processes that originate chimeric *babB*/*babA* genes.

3.4. Genotype combinations

The frequency distributions of the combined genotypes of *H. pylori* in the 148 samples included in the study are presented in Table 4. We registered a total of 19 different genotype combinations, out of which the four more abundant were: *vacA* s1m1/*cagA*/*iceA1*/*babA2* (26.3%), *vacA* s2m2/*iceA1*/*babA2* (19.5%), *vacA* s1m1/*cagA*/*iceA2*/*babA2* (18.8%), and *vacA* s1m2/*cagA*/*iceA1*/*babA2* (6.8%). When we took into consideration the subtypes of *iceA1*, *iceA2*, and *babA2* found in this work, the number of different genotypes increased to 62 (Table 4). In this manner, the most prevalent genotypes *vacA* s1m1/*cagA*/*iceA1*/*babA2* and *vacA* s2m2/*iceA1*/*babA2*, included 10 and 12 subtypes or subgenotypes, respectively.

3.5. Phylogenetic analysis

To assess the phylogenetic relationship between Venezuelan *H. pylori* strains and those from other geographic regions, we used a region of 381 bp of the *iceA1* PCR-amplified fragments, corresponding to an ORF from a second putative ATG translation initiation site. For this analysis we also included the sequences of *iceA1*_A (V132) and *iceA1*_B (V133) subtypes (Figure 1). Sequences obtained from Venezuelan samples were closely related to those from Western strains (excluding Alaska). Interestingly, reference strains of *H. pylori* obtained from Amerindians of South America (PeCan4, Puno135, SJM180, and v225d) clustered in a group separated from the Venezuelan sequences analyzed here, being more closely related to East Asian strains.

4. Discussion

In this work we proposed a strategy of detection by PCR of allelic variants of several genotypic markers associated with an increased risk of disease, to evaluate the prevalence and genetic diversity of circulating *H. pylori* strains. In accordance with previous studies in Venezuelan populations,[17,19,27] we found a

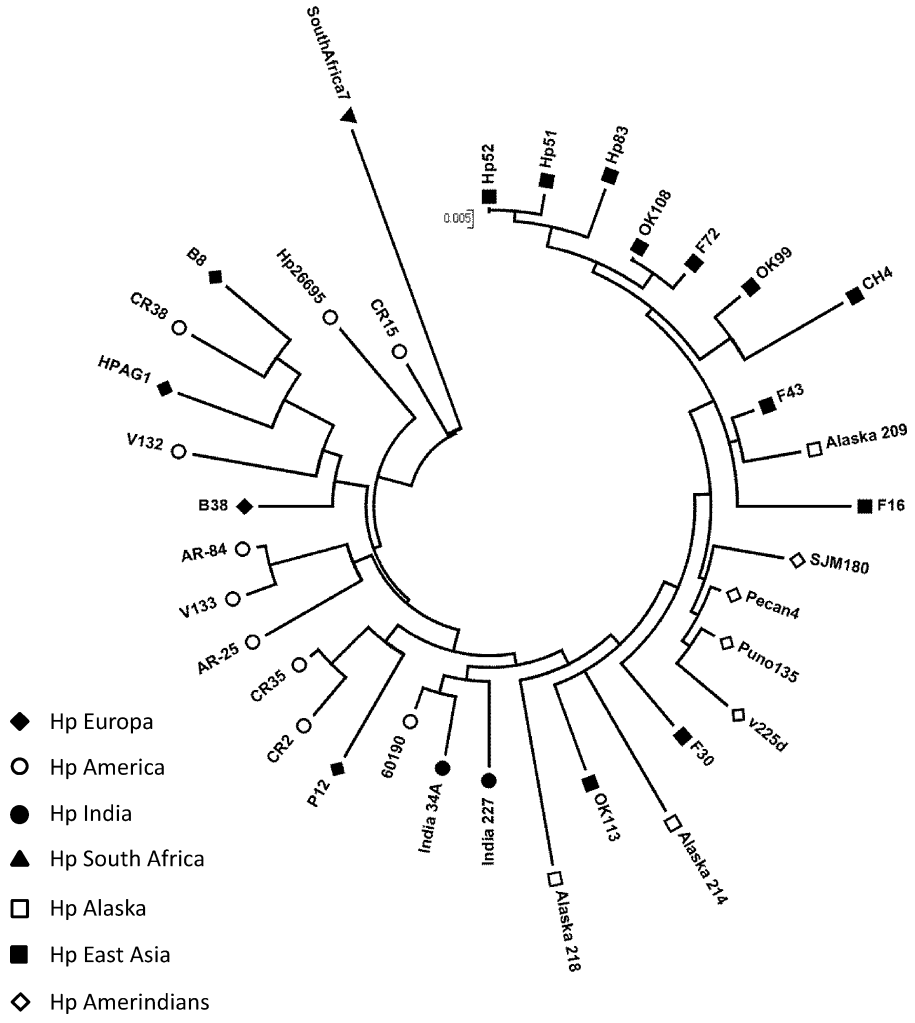

Figure 1. Phylogenetic tree based on the 381-bp sequence of the *iceA1* gene of *Helicobacter pylori* strains from Venezuela (this work) and sequences of strains reported from different parts of the world (available in public databases). The neighbor-joining tree was generated using MEGA 5 software and the bootstrap method at 1000 replications and the Kimura two-parameter method. The strains used are as follows (GenBank accession numbers are in parenthesis): Alaska 214 (**AF001538**), Alaska 218 (**AF001539**), Alaska 209 (**AF001537**), AR-25 (**AY185132**), AR-84 (**AY185134**), B8 (**FN598874**), CH4 (**AF459446**), CR2 (**AF326599**), CR15 (**AF326603**), CR35 (**AF326606**), CR38 (**AF326607**), F16 (**AF157529**), F30 (**AP011941**), F43 (**AF157533**), F72 (**AF157535**), Hp51 (**CP000012**), Hp52 (**CP001680**), Hp83 (**CP002605**), HPAG1 (**CP000241**), India34A (**AF239992**), India227 (**AF239991**), OK99 (**AF157547**), OK108 (**AF157549**), OK113 (**AF157550**), PeCan4 (**CP002074**), Puno135 (**CP002982**), P12 (**CP001217**), SouthAfrica7 (**CP002336**), SJM180 (**CP002073**), v225d (**CP001582**), V132 (**JQ808066**), V133 (**JQ808066**), 26695 (**AE000511**), 60190 (**U43917**). Geometric shapes mark the geographic origins of the bacterial isolates.

high frequency of *vacA* s1 and m1 alleles, *cagA* gene, as well as the *vacA* s1m1 genotype. The *vacA* s1 allele was observed to be associated with more severe atrophic gastritis, and although *vacA* s1m2 and *vacA* s1m2/*cagA* genotypes were not the most prevalent in chronic gastritis samples, their frequencies were also higher in M/S atrophic gastritis ($p < 0.01$) because of the strong correlation existing between *vacA* m2 and *cagA*-positive alleles with *vacA* s1.

In a previous work we found no association between *cagA*-positive strains and histological changes in chronic gastritis.[21] It is known that approximately half of the *H. pylori* strains isolated in Western countries do not carry the *cag*-PAI (considered as *cagA*-negative), and although it is known that in Western populations *cagA*-positive strains are commonly associated with peptic ulceration, atrophic gastritis, and gastric adenocarcinoma, the levels of inflammation and atrophy are significantly higher in patients infected with East Asian *cagA*-positive strains than in patients infected with Western *cagA*-positive strains.[10,28]

Additionally, although the *vacA* s2m2 genotype is often linked with the absence of the *cag*-PAI,[6] 24% (12/50) of analyzed samples harboring the *vacA* s2m2 genotype also contained the *cagA* gene. Moreover, a recent study showed that 66% of *H. pylori* strains were *vacA* s2m2/*cagA*$^+$ (predominantly non-Asian *cagA*-positive strains), almost all of them isolated from Alaskan native individuals.[29]

As was shown in our recent work with a lower number of chronic gastritis samples,[22] *iceA1* was the more frequently found allele of this locus; nonetheless, *iceA2* prevalence was higher in samples with atrophic gastritis and more severe grades of granulocytic and lymphocytic infiltration. According to the present work, this trend, and the association with G2/G3, is mainly due to the presence of *iceA2*_D subtype. Although the function of the *iceA2* product remains unclear, Kidd et al.[30] were able to discriminate a potential association between *iceA2*_D subtype and peptic ulcer disease.

A high frequency of the *babA2* allele is more common in East Asia, but it has also been reported in other Western countries when the primers described by Sheu et al.[25] were used, although with contradictory results with regard to its association with clinical outcomes.[17,31] These primers were designed to detect a 10 bp deletion in the signal region of the *babA* gene with the idea of establishing a firm association between *babA2*-positive strains and

the expression of a functional protein, thus avoiding the underestimation that occurs when using primers located in polymorphic zones of the gene.[16] However, the presence of CT repeats close to the *babA* start codon in some PCR products suggests the presence of chimeric *babA2/B* genes and that the protein production is subject to a phase variation through slipped-strand mispairing.[32] Therefore, as recommended by Yamaoka,[33] information obtained from currently used PCR-based methods to evaluate *babA* status must be interpreted with caution. In this work, taking advantage of the fact that the PCR reaction yielded products of different sizes, we defined four *babA2* subtypes that varied in the promoter region of the gene.

One striking characteristic of the pathogenic bacterium *H. pylori* is its remarkable allelic diversity and genetic variability, a fact that determines that every isolate from unrelated subjects appears to have a distinct fingerprint.[34] Also given the elevated mutation rate and frequent recombination of *H. pylori* during the course of chronic infection, strains may undergo genetic alterations in vivo.[18] Both housekeeping and virulence-associated genes are affected by genetic variability, and it is believed that this variability may play a role in human host adaptation. In this work we showed that the number of different genotype combinations increased from 19 to 62 when PCR-product size variants of *iceA1*, *iceaA2*, and *babA2* were considered. In other words, by PCR-typing polymorphic virulence genes, a different genotype was observed in every 2.4 analyzed individuals, a fact that highlights the elevated variability of the strains circulating in Venezuela.

The heterogeneity of *H. pylori* genotype observed can be considered on two levels: genotypic variation among strains and variations in *H. pylori* populations within an individual host. The first one can include, in our samples, the presence of non-conserved genes (e.g., *cagA*) and/or mosaic forms of gene elements (e.g., *iceA1*_A and B), while population differences involve the fact that the human can be simultaneously infected with more than one *H. pylori* strain, and that a single strain may represent a cluster of closely related organisms or quasispecies.[35] Although the aim of this work was not to study the prevalence of mixed infections, some of the obtained genotype combinations suggest the presence of co-infections with divergent *H. pylori* strains.

In Colombian populations, de Sablet et al.[36] showed that strains isolated from a high-risk region (Andes Mountains, Nariño) for gastric cancer were all of European origin, thus suggesting that the phylogeographic origin of *H. pylori* strains may explain geographic differences in gastric cancer prevalence. Analysis of *iceA1* sequences clearly showed that Venezuelan strains clustered closer to those of Western countries. But even though the number of *iceA1* sequences from Western strains available in the databases is limited, our phylogenetic tree was similar to that reported for other *H. pylori* virulence-associated and housekeeping genes.[37] Also, similar to what has been shown with other virulence-related gene sequences,[37–39] an *iceA1* sequence from a characterized Venezuelan Amerindian strain (v225d) grouped with 'Amerindian cluster' close to East Asian strains. Our results are consistent with the admixed ethnic origin of the population of the central–western region of Venezuela, and also with the hypothesis stating that similarities between the Amerindian and East Asian strains are due to the first colonizers of the New World who brought *H. pylori* with them.[40] Moreover, a predominance of *H. pylori* strains of European origin in 'Mestizo' populations of Colombia and Venezuela has been reported.[36,41]

In summary, we determined virulence-associated genotypes of *H. pylori* using known, and newly defined in this work, allelic variants, to demonstrate the high genetic variability of strains circulating in the central–western region of Venezuela, whose population exhibited a high prevalence of infection with *H. pylori* strains harboring genes and allelic forms associated with disease outcome. Even though it is unknown whether any association of the studied variants with BabA or IceA1 expression exists, we propose that they may be used for comparative studies of the genetic diversity of *H. pylori* strains. A more systematic analysis of the impact of this genetic heterogeneity in *H. pylori*-infected patients from Venezuela is required. Moreover, to our knowledge this is the first attempt to use *H. pylori iceA1* sequences to make a phylogenetic tree, which allowed us to group *H. pylori* strains from a Venezuelan urban/admixed population with Western strains.

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Ethical approval: This study was approved by the Bioethics Committee of the School of Health Sciences, Universidad Centroccidental Lisandro Alvarado (UCLA), and all patients gave their written informed consent to participate in the study.

Conflict of interest: No conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2013.03.004.

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