

Helicobacter pylori cagA and vacA genotypes in Cuban and Venezuelan populations

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The aim of this study was to determine the presence of Helicobacter pylori cytotoxin-associated gene (cagA)/vacuolating cytotoxin gene (vacA) among patients with chronic gastritis in Cuba and Venezuela. Gastric antrum biopsies were taken for culture, DNA extraction and PCR analysis. Amplification of vacA and cagA segments was performed using two regions of cagA: 349 bp were amplified with the F1/B1 primers and the remaining 335 bp were amplified with the B7629/B7628 primers. The VAI-F/VAI-R set of primers was used to amplify the 259-bp (s1) or 286-bp (s2) product and the VAG-R/VAG-F set of primers was used to amplify the 567-bp (m1) or 642-bp (m2) regions of vacA. cagA was detected in 87% of the antral samples from Cuban patients and 80.3% of those from Venezuelan patients. All possible combinations of vacA regions were found, with the exception of s2/m1. The predominant combination found in both countries was s1/m1. The percentage of cagA⁺ strains was increased by the use of a second set of primers and a greater number of strains was amplified with the B7629/B7628 primers in the Cuban patients (p = 0.0001). There was no significant difference between the presence of the allelic variants of vacA and cagA in both populations. The predominant genotype was cagA⁺/s1m1 in both countries. The results support the necessary investigation of isolates circulating among the human population in each region.

Key words: *Helicobacter pylori* - cagA - vacA - chronic gastritis

Helicobacter pylori is a microaerophilic bacterium that has the ability to establish an infection in human stomachs that can last for years or even decades, despite immune and inflammatory responses and the normal turnover of the gastric epithelium in which it resides (Kersulyte et al. 2000). Infection with this microorganism is not only the major cause of chronic active gastritis and most peptic ulcer diseases, but it is also associated with gastric cancers (Blaser & Berg 2001). Among the different genetic determinants involved in *H. pylori* virulence are the cytotoxin-associated gene (*cagA*) and the vacuolating cytotoxin gene (*vacA*). Various studies have demonstrated the importance of the presence of these genes in the strain affecting a patient and in the development of gastric disease (Faundez et al. 2002, Chen et al. 2005) *vacA* encodes a vacuolating toxin that is released by *H. pylori* and that injures epithelial cells via its pore-forming ability. *vacA* is present in all *H. pylori* strains, but

it is not similarly expressed in all strains. It includes two variable regions, denoted s and M. The s region (encoding the signal peptide) is located at the 5' end of the gene and exists as an s1 or s2 allele. Within type s1, two subtypes (s1a and s1b) can be distinguished. The m region (middle region) occurs as an m1 or m2 allele. All possible combinations of these *vacA* regions have been identified, with the exception of s2/m1. The mosaic combination of the s and m region allelic types correlates with the production of the cytotoxin and is thereby associated with the virulence of the strain (Atherton et al. 1995).

The cytotoxin-associated protein is a high molecular mass immunodominant protein encoded by *cagA* that is part of the *cag* pathogenicity island (Tummuru et al. 1993) and is also a disease-associated virulence factor. Several reports have evidenced geographical differences in the prevalence of *vacA* alleles and *cagA* status among *H. pylori* isolates (Kersulyte et al. 2000, Catalano et al. 2001, Valmaseda-Perez et al. 2001); however, there is limited information about *H. pylori* infection in the countries of the Caribbean basin. The aim of this study was to determine the frequency of different *H. pylori* *cagA/vacA* genotypes among patients with chronic gastritis in Cuba and Venezuela.

PATIENTS, MATERIALS AND METHODS

Subjects - Sixty-one patients from Venezuela, aged between 25-76 years (average 49 ± 14 years; 34 patients

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from the José Maria Vargas Hospital in Caracas, 17 from the Gastric Cancer Control Center in San Cristobal, Táchira and 10 from the University Hospital in Caracas) and 71 patients, aged between 23-75 years (average 50 ± 12 years), all from the Institute of Gastroenterology in La Havana, Cuba, all of which were referred for upper gastrointestinal endoscopy after presenting dyspeptic symptoms and participated in this study. Both groups were balanced regarding sex and socioeconomic conditions, with Venezuelan patients being scored socioeconomically according to the method of Graffar modified for Venezuela (Méndez 1986).

Each patient underwent upper gastroendoscopy for both endoscopic observation and biopsy collection for histological study, culture and PCR analysis. Endoscopic observation and histological confirmation were used to determine patient pathologies according to the updated Sydney system (Dixon et al. 1996) and patients with chronic active gastritis were selected for this study. All patients with chronic active gastritis were positive for *H. pylori*. In biopsies from Venezuelan patients, positivity was confirmed by the rapid urease test, histological study and PCR for *glmM* (Kamali-Sarvestani 2006). In Cuban patients, strains were isolated from gastric biopsies using the conditions described below.

Informed consent was obtained from all study subjects. The procedures used were in accordance with

ethical standards and the protocol was approved by the ethical committee.

Sample collection - Two antral biopsy specimens were taken from each dyspeptic patient: one for culture and one for PCR.

Culture of *H. pylori* from biopsies - Gastric biopsies were collected from the transport medium and cells were plated onto blood agar and chocolate agar plates. Plates were incubated for seven days at 37°C in a microaerobic atmosphere (7% CO₂). Isolates of *H. pylori* were identified by Gram staining and catalase, oxidase and urease testing.

DNA extraction - In Venezuela, DNA was obtained from the biopsies of the 61 patients using a proteinase K protocol. Biopsies were incubated overnight at 50°C in lysis buffer (10 mM Tris-HCl, 0.1% saccharose, pH 8.0). Samples were then treated with an isoamyl chloroform-phenol mixture. DNA was precipitated with ammonium acetate and 70% ethanol and then resuspended in 50 µL of DNase-free water.

In Cuba, DNA was extracted from the strains isolated from the 71 patients by harvesting cells from plates and suspending them in a sterile saline solution. Following centrifugation, the pellets were resuspended in STE buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl) and lysozyme (50 mg/mL on 50% glycerol) and treated with 10% SDS

TABLE I

Primers used for cytotoxin-associated gene (*cagA*) and the vacuolating cytotoxin gene (*vacA*) typing of *Helicobacter pylori*

Allele	Primer designation	Product size	Reference
<i>cagA</i>	B7629/B7628	335 bp	Gonzalez-Valencia et al. (2000)
<i>cagA</i>	F1/B1	349 bp	Tummuru et al. (1993)
<i>vacA</i> m1/m2	VAG-R/VAG-F	567bp (m1) 642bp (m2)	Atherton et al. (1995)
<i>vacA</i> s1/s2	VA1-F/VA1-R	259bp (s1) 286bp (s2)	Atherton et al. (1995)

TABLE II

Prevalence of cytotoxin-associated gene (*cagA*) and allelic variants of vacuolating cytotoxin gene (*vacA*) in *Helicobacter pylori* strains isolated from Cuban and Venezuelan patients

	<i>cagA</i>		<i>vacA</i>			
	<i>cagA</i> ⁺ n (%)	<i>cagA</i> ⁻ n (%)	s1 n (%)	s2 n (%)	m1 n (%)	m2 n (%)
Cuba ^a	62 (87)	9 (13)	44 (70)	19 (30)	31 (49)	32 (51)
Venezuela ^b	49 (80)	12 (20)	47 (77)	14 (23)	38 (62)	23 (38)

a: isolates from Cuban patients (n = 71) without co-infection (n = 63); b: isolates from Venezuelan patients (n = 61).

and proteinase K (1000 µg/mL). Samples were treated with an isoamlic chloroform-phenol mixture and DNA was precipitated with ammonium acetate and 70% ethanol. DNA was suspended in 50 µL of DNase-free water.

PCR amplification of the 349-bp region of *cagA* (Table I) - A 349-bp region of *cagA* was amplified by PCR using primers F1/B1 (Tummuru et al. 1993). Aliquots were taken from each DNA suspension of every patient and processed as follows: 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension at 72°C for 6 min.

PCR amplification of the 335-bp region of *cagA* - A 335-bp region of *cagA* was amplified by PCR using primers B7628/B7629 (Gonzalez-Valencia et al. 2000). Aliquots of each DNA suspension from every patient were obtained and processed as follows: 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension at 72°C for 6 min.

PCR amplification of the s1/s2 and m1/m2 alleles of *vacA* - We used the VA1-F/VA1-R (Atherton et al. 1995) set of primers to amplify the 259-bp (s1) or the 286-bp (s2) region of the conserved portion of *vacA*. The second set of primers, VAG-R/VAG-F (Atherton et al. 1995) was used to amplify the 567-bp (m1) or the 642-bp (m2) region of the conserved portion of *vacA*. Aliquots were taken from each DNA suspension of every patient and processed as follows: 35 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min and a final extension at 72°C for 6 min for s1/s2; and 35 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1.5 min and a final extension at 72°C for 5 min for m1/m2. All of the PCR products were visualized by electrophoresis in a 1% agarose gel stained with ethidium bromide. Electrophoresis was conducted at 80 V for approximately 2 h and gels were examined under UV light.

Statistical analysis - All statistical analyses were performed using GraphPad InStat. A chi-square test was used to compare the frequencies. A p value < 0.05 was considered as significant.

RESULTS

In this study, we amplified different alleles of *vacA* and *cagA* by PCR. A total of 61 strains from Venezuelan patients and 71 strains from Cuban patients were analyzed. Out of the 71 patients from Cuba, we found that eight had a coinfection with at least two different *H. pylori* isolates, because we detected the presence of both m1 and m2 alleles. Thus, the number of isolates analyzed for the prevalence of *cagA* and allelic variants of *vacA* was reduced to 63.

To type the *H. pylori* strains isolated from the patients examined in this study, we amplified by PCR different alleles of the genes of the two major virulence factors of this bacteria, *cagA* and *vacA*. The amplification results are shown in Table II. *cagA* was detected in 62 of the 71 Cuban patients (87%) and in 49 of the 61 Venezuelan patients (80%), with no significant differences detected between the two countries. Four different *vacA* alleles

were amplified from all isolates. We found all possible combinations of these *vacA* regions except s2/m1. The most prevalent combination of *vacA* alleles found in both countries was s1/m1, with no significant difference found between the two countries.

The results of the prevalence of *cagA* in relation to the different allelic combinations of *vacA* are represented in Table III. We observed that the predominant genotype was *cagA*⁺/s1m1 in both countries, with no significant difference found (Table III).

As previously mentioned, *cagA* was found in isolates from 62 of the 71 Cuban patients (87%) and 49 of the 61 Venezuelan patients, with no significant difference found between the two countries. However, we found a significant difference in the amplification efficiency of the two primers, as the 335-bp primers more effectively amplified this region of *cagA* in *H. pylori* isolates from Cuban patients.

The 349-bp region of *cagA* gene was amplified from 34 of the 61 Venezuelan patients (56%) and from 20 of the 71 Cuban patients (28%).

We used a different set of primers, which amplify a 335-bp region of the same gene, to confirm the detection of *cagA*. In both countries, the percentage of detected *cagA*⁺ strains was increased by the use of these primers, to 80% of the Venezuelan strains and 87% of the Cuban strains, with no significant difference found between the two countries. However, a significantly greater number of strains was amplified with the 335-bp primers from the Cuban patients (p = 0.0001) (Table IV).

Therefore, the distribution of *cagA*⁺/s1m1 strains, relative to the different sets of primers, also showed a significant difference in both countries. The *cagA*⁺335/s1m1 strains were more frequent in Cuban patients than in Venezuelan patients and consequently the *cagA*⁺349/s1m1 strains were more frequent in the Venezuelan patients than in the Cuban patients (p = 0.0001) (Figs 1, 2).

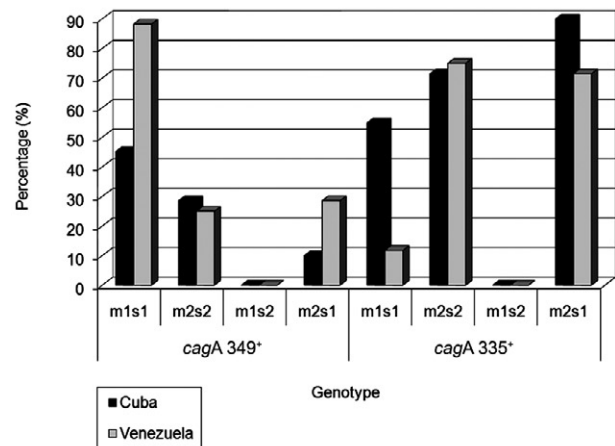


Fig. 1: genotype frequency of cytotoxin-associated gene (*cagA*) positive *Helicobacter pylori* strains isolated from patients in Cuba and Venezuela.

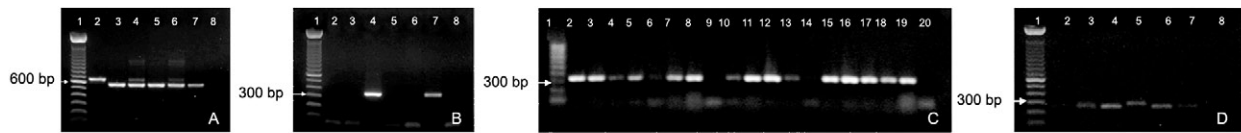


Fig. 2: electrophoresis photographs of all amplified genes. A: PCR amplification of m1/m2 region of the vacuolating cytotoxin gene (*vacA*) gene (Lane 1: ladder; 2-6: DNA samples from strains; 7: positive control; 8: negative control); B: PCR amplification of the 349bp region of the cytotoxin-associated gene (*cagA*) gene (Lane 1: ladder; 2-6: DNA samples from strains; 7: positive control; 8: negative control); C: PCR amplification of the 335bp region of the *cagA* gene (Lane 1: ladder; 2-18: DNA samples from strains; 19: positive control; 20: negative control); D: PCR amplification of s1/s2 region of the *vacA* gene (Lane 1: ladder; 2-6: DNA samples from strains; 7: positive control; 8: negative control).

TABLE III
Prevalence of cytotoxin-associated gene (*cagA*) related to the different allelic combinations of vacuolating cytotoxin gene (*vacA*) and to the country of origin

	<i>cagA</i> ⁺				<i>cagA</i> ⁻			
	s1m1 n (%)	s2m2 n (%)	s1m2 n (%)	s2m1 n (%)	s1m1 n (%)	s2m2 n (%)	s1m2 n (%)	s2m1 n (%)
Cuba	31 (49)	14 (22)	10 (16)	0 (0)	0 (0)	5 (8)	3 (5)	0 (0)
Venezuela	35 (57)	8 (13)	7 (11)	0 (0)	3 (5)	6 (10)	2 (3)	0 (0)

isolates from Cuban patients without co-infection (n = 63) and isolates from Venezuelan patients (n = 61).

TABLE IV
Frequency of positivity according to the DNA fragment of cytotoxin-associated gene (*cagA*) amplified in Cuba and Venezuela

	bp region 335 ⁺ n (%)	bp region 349 ⁺ n (%)	Total n
Cuba	42 (59)	20 (28)	62
Venezuela	15 (25)	34 (56)	49

DISCUSSION

Infection with *H. pylori* has been related to serious gastroduodenal diseases, such as gastric and duodenal ulcers and gastric cancers. This infection is a worldwide health problem and thus it is important to diagnose the presence of this bacterium and its genotype accurately, in the patient. There is increasing evidence that the presence of *H. pylori* genes and their different genotypic combinations in the strain colonizing a patient affects the development of gastric disease.

The predominant combination of *vacA* alleles in both countries was s1/m1 and the prevalence was similar in both countries. The mosaic combination of s and m region allelic types correlates with the production of the cytotoxin and is thereby associated with the virulence of the strain; the fact that the majority of the strains found in Cuban and Venezuelan patients are s1/m1, the most virulent type, is of importance to the regional health systems of both countries. These results are in accordance with several other studies (De Gusmão et al. 2000,

Figueiredo et al. 2001, Chen et al. 2005, Martins et al. 2005, Garcia et al. 2006).

We found a significant difference in amplification efficiency between the two primers used, with the 335-bp primers being more effective at amplifying this region of *cagA* in *H. pylori* isolates from Cuban patients. There have been indications of sequence variability in *cagA* in isolates of different geographic origins (Pan et al. 1997, van Doorn et al. 1998) and this may lead to an underestimation of the true prevalence of *cagA*-positive *H. pylori* strains.

Further studies carried out in our lab, where we have amplified other regions of *cagA*, suggest that the percentage of *cagA*-positive strains, as measured using only one set of primers, is usually underestimated.

The results of this study provide important information in the epidemiological study of the frequency of *cagA* in Caribbean populations. When we simultaneously evaluated the presence of *cagA* and *vacA* alleles, we found that the predominant genotype in both coun-

tries was *cagA*⁺/*slm1*. This finding is in accordance with other studies performed in Latin American populations. Faundez et al. (2002) found that *cagA*⁺/*slm1* strains were the most common in Chilean patients and Morales-Espinosa et al. (1999) also found that *cagA*⁺/*slm1* strains had a higher prevalence in Mexican patients. Additionally, Martins et al. (2005) found an association between the *cagA*⁺ and *vacAm1sl1* genotypes in Northern Brazil and Citty et al. (2002) found that the *cagA*⁺/*slm1* phenotype was the most frequent in Colombian patients. Given the fact that the genotype *cagA*⁺/*slm1* is highly associated with a more severe gastroduodenal disease, these data are of great importance for the regional health systems of Cuba and Venezuela.

PCR amplification of the major virulence factor genes of *H. pylori* proved to be an efficient method for genotyping the strains isolated from both countries. Even though we did not find any significant differences between the genotype distributions in both countries, we found that a significantly higher number of strains isolated from Cuban patients were amplified with the 335-bp primers. Sequencing of the entire *cagA* gene will be performed to complete the study and to elucidate the genetic differences that account for this preferential primer amplification. We also found that the predominant genotype in both countries was *cagA*⁺/*vacA* *slm1*. Because this is the genotype most commonly associated with more severe gastric disease, we strongly suggest that *H. pylori* infection should be considered as a public health problem in our countries and that preventive measures be taken. In addition, this study demonstrates that diversity mainly in *cagA* sequences needs to be evaluated carefully for investigation of the isolates circulating among human populations in epidemiological studies of *H. pylori* genotypes.

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