Prevalence of vacA, cagA and babA2 genes in Cuban Helicobacter pylori isolates

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AIM: To investigate the prevalence of vacuolating cytotoxin (vacA), cytotoxin associated gene A (cagA) and babA2 genes in Cuban Helicobacter pylori isolates from functional dyspeptic patients.

METHODS: DNA was extracted from H pylori-positive cultures taken from 130 dyspeptic patients. Genotyping was performed by PCR, using specific primers for vacA (s1, s2, m1, m2), cagA and babA2 genes. Endoscopic observations and histological examinations were used to determine patient pathologies.

RESULTS: vacA alleles s1, s2, m1 and m2 were detected in 96 (73.8%), 34 (26.2%), 75 (57.7%) and 52 isolates (40%), respectively, while the cagA gene was detected in 95 isolates (73.2%). One hundred and seven isolates (82.3%) were babA2-positive. A significant correlation was observed between vacAs1m1 and cagA and between vacAs1m1 and babA2 genotypes (P < 0.001 and P < 0.05, respectively) and between babA2 genotype and cagA status (P < 0.05); but, no correlation was observed between vacAs1 and babA2 genotypes. Eighty five (65.4%) and 73 (56.2%) strains were type 1 (vacAs1-cagA-positive) and ”triple-positive” (vacAs1-cagA-babA2-positive), respectively, and their presence was significantly associated with duodenal ulcer (P < 0.01 and P < 0.001, respectively).

CONCLUSION: The distribution of the main virulence factors in the Cuban strains in this study resembled that of the Western-type strains, and the more virulent H pylori isolates were significantly associated with duodenal ulcer, ulcer disease being the worst pathology observed in the group studied.

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Key words: Cuban dyspeptic patients; Helicobacter pylori; vacA, cagA and babA2

INTRODUCTION

Helicobacter pylori (H pylori), a spiral-shaped microaerophilic bacterium infects more than 50% of the world’s population, the rate of infection being higher in developing countries[1]. H pylori is a major etiological agent in several gastroduodenal diseases, such as functional dyspepsia, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue lymphoma. The clinical outcome following infection with this pathogen has been related to environmental conditions, host
immunological factors and microorganism virulence.

Vacuolating cytotoxin (VacA), cytotoxin associated gene A (cagA), and blood adhesion binding antigen (babA) are the most commonly studied virulence markers of \( H \) \( pylori \). However, there are other bacterial proteins with pathogenic potential, such as sialic acid-binding adhesin (SabA), outer inflammatory protein (sipA), and duodenal ulcer promoting gene (dupA); but, the influence of these proteins on \( H \) \( pylori \) pathogenesis is still under study.

The VacA protein induces vacuolation and apoptotic processes in epithelial cells, as well as immunosuppressive actions in immunological cells. The vacA gene comprises two main regions: the signal zone (s1 or s2) and the middle region (m1 or m2). The vacA s1m1 allelic combination exhibits the highest activity, while s2m2 and the rare s2m1 combinations are non-toxic.

Recently, a new polymorphic region in the vacA gene called the intermediate region (i) has been discovered and its i1 active allele seems to be a better predictor of gastric cancer than the s1 or m1 allele.

Hydrophilic protein CagA contains the so-called EPIYA motifs, which interact with several eukaryotic proteins, promoting changes in the signal transduction pathway, cytoskeletal plasticity and IL-8 secretion in epithelial cells. CagA-positive \( H \) \( pylori \) isolates are associated with a higher rate of gastric inflammation and damage, when compared with CagA-negative strains. The cagA gene is located at the end of the cag pathogenicity island, a system that introduces CagA and a peptidoglycan into epithelial cells. Several epidemiological studies have shown the correlation between cagA-positive strains and a higher risk of developing peptic ulceration, gastric atrophy and gastric cancer.

The blood group binding antigen mediates adherence of \( H \) \( pylori \) to human gastric epithelium. This antigen is encoded by the polymorphic gene called babA2, while allele babA1 is non-functional. Some studies have suggested that BabA plays a crucial role in the development of severe functional dyspepsia, peptic ulcer and gastric adenocarcinoma. Furthermore, the combined presence of vacAs1 and cagA genotypes (type 1 strains) or even the “triple-positive” strains (vacAs1, cagA and babA2), has shown a higher correlation with the appearance of peptic ulcer, intestinal metaplasia and gastric cancer.

The clinical outcome of this bacterial infection seems to be influenced by the distribution of the above-mentioned pathogenic factors in \( H \) \( pylori \) strains, but, complete genotyping of Cuban \( H \) \( pylori \) strains has never been carried out. Therefore, the aim of this study was to determine the frequency of the main virulence factor genes in Cuban \( H \) \( pylori \) isolates and establish their associations with the clinical outcome.

**MATERIALS AND METHODS**

**Patients**

\( H \) \( pylori \) isolates were obtained from 130 consecutive \( H \) \( pylori \)-positive patients (77 male and 53 female) with a mean age of 49.1 years (range, 18 to 88) who underwent routine endoscopy due to dyspeptic complaints at CIMEQ Hospital, Havana, Cuba. Endoscopic observation and histological confirmations were used to determine patient pathologies. This study was approved by the ethics committee at CIMEQ Hospital. All patients provided informed consent to participate in the study.

**Microorganism culture**

Antrum gastric biopsy specimens obtained from all patients were homogenized, inoculated into Columbia agar base plates with 7% human blood and SR0147E selective supplement (Oxoid, England, UK), and grown under microaerophilic conditions at 37°C for 5 to 8 d. All \( H \) \( pylori \) isolates were positive for oxidase, catalase and urease. The reference strain J99 was kindly provided by Professor Francis Megraud from Pellegrin Hospital, Bordeaux, France.

**DNA extraction and cagA, vacA and babA2 genotyping**

Genomic DNA was extracted by CTAB methodology with phenol/chloroform and isopropanol precipitation as previously described. Purified DNAs were stored at -20°C until use. In all cases, PCR amplification was carried out in a 25 μL reaction mixture containing 2.5 μL 10× PCR buffer (Roche, Germany), 0.2 mmol/L of each deoxynucleotide triphosphate, 0.6 mM sense and antisense primers, 4 mmol/L magnesium chloride, 1,25 U Taq DNA polymerase (CIGB, Cuba) and 100 ng genomic DNA. The PCR had an initial step at 94°C for 1 min, followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min, using a Master Cycler apparatus (Eppendorf, Germany).

The primers used and their details are shown in Table 1. Primers to the glmM gene of \( H \) \( pylori \) were used to control DNA integrity and specificity. PCR products were analyzed on 1.5% agarose gel electrophoresis with ethidium bromide. Images were taken through the Gene Genius system (Syngene, England, UK).

**Statistical analysis**

Differences among groups were tested using the \( \chi^2 \) test. \( P \) values < 0.05 were considered to be significant. The statistic software, version 8 for Windows, was used for statistical analysis.

**RESULTS**

**Detection of \( H \) \( pylori \) genotypes**

\( H \) \( pylori \) was successfully cultured from 130 Cuban dyspeptic patients. DNA integrity and specificity was confirmed by glmM PCR, which rendered the expected 417 bp band from all isolates (data not shown). PCR product sizes of vacA s and m alleles were used to differentiate them in agarose gels (Figure 1, panel A). The most virulent vacAs1 allele was predominantly present in Cuban \( H \) \( pylori \) isolates (Table 2), and...
was visualized as a band of 259 bp on agarose gel electrophoresis (Figure 1, panel A), whereas 26.2% of isolates had the vacA s2 genotype (Table 2). The middle region of the vacA gene was detected in only 127 of the 130 isolates, m1 and m2 genotypes were more equally distributed than s genotypes (Table 2). On the other hand, s1m1 and s2m2 genotypes were the most common allelic combinations of the vacA gene among Cuban isolates, and only one strain harbored the s2m1 genotype (Table 2).

Amplification of the cagA gene was visualized as a band of 349 bp (Figure 1, panel B) and was present in 73.2% of the strains (Table 2). When primers babA7F/babA7R (Table 1) were used to amplify the babA2 gene, over 80% of the Cuban strains carried this gene (Table 2). In contrast, a low prevalence of babA2 genotype was observed among the Cuban isolates when using primers babA2F/babA2R and babA2F/babA2R607 (Table 1).

Table 1 Primer used for PCR genotyping of Cuban *H pylori* strains

| Primer   | Sequence (5'-3') | AT | Size (bp) | Ref. |
|----------|------------------|----|-----------|------|
| glmMF    | CCCCCAGCATAGCCCACCCAA | 60 | 417       | [18] |
| glmMR    | AAGAAGTGACACGCTCCACAC | 60 | 349       | [7]  |
| cagF1    | GATGACAGGGACAGCTTCTG | 52 | 1-259/2-286 | [20] |
| cagB1    | CTCCCTGTTAATCGGCAACC | 56 | m1-567/m2-642 | [20] |
| vacAsF   | ATGCAATAACCAAAACACAC | 74 | 56 (56.9) |       |
| vacAsR   | CTCCCTGTTAATCGGCAACC | 74 | 52 (20)   |       |
| vacAmF   | CAATCTGTTAATCGGCAACC | 24 | 28 (54.9) |       |
| vacAmR   | GCCATCTGTTAATCGGCAACC | 56 | 52 (20)   |       |
| bab7-F   | CCAACAGGACAAACAGGCGT | 60 | 60 (36)   |       |
| bab7-R   | GCCATCTGTTAATCGGCAACC | 24 | 28 (54.9) |       |
| babA2F   | AATCCCAAAGGAGAAGGAGATAGAA | 60 | 832 (13) |       |
| babA2R   | TGTATGTGATTGTTTGACAG | 40 | 40 (87)   |       |

Figure 1 Genotyping of main virulence factor genes in Cuban *H pylori* isolates. The images show the representative gel electrophoresis of two independent PCR amplification products of vacA s1, s2, m1, m2, cagA and babA2 genes from Cuban isolates and J99 control strain. A: Lanes 1 and 7, reference strain J99 (vacAs1 and m1 alleles, respectively); Lanes 2 and 6, vacAs1 strains; Lanes 3-5, vacAs2 strains; Lanes 6 and 8 vacAm2 strains; Lane 9, vacAmR strain. B: Lanes 1 and 4, J99 strain (cagA- and babA2-positive strains, Lane 2 and 3, cagA-positive strains; Lanes 5 and 6 babA2-positive strains. MW: 100 bp DNA Ladder (Promega, USA).

Table 2 Correlation between vacA alleles and cagA and babA2 genotypes in 130 Cuban *H pylori* isolates

| vacA | s1m1 | s1m2 | s2m2 | s2m1 | s1m- | s2m1 | Total (%) |
|------|------|------|------|------|------|------|-----------|
| cagA+ | 70   | 14   | 8    | 1    | 2    | 95 (73.2) |
| cagA- | 4    | 5    | 25   | 0    | 1    | 35 (26.8) |
| babA2+ | 67   | 14   | 24   | 1    | 1    | 107 (82.3) |
| babA2- | 7    | 5    | 9    | 0    | 2    | 23 (17.7) |

Total (%) 74 (56.9) 19 (14.6) 33 (25.4) 1 (0.8) 3 (2.3) 130

Table 3 Correlation between virulence factor genotypes and disease outcome

| Genotypes | FD | GU | DU |
|-----------|----|----|----|
| vacAs1m1  |    |    |    |
| s1m1      | 28 (54.9) | 16 (48.5) | 30 (65.2) |
| s1m2      | 5 (9.7) | 9 (27.3) | 5 (10.9) |
| s2m2      | 16 (31.4) | 6 (18.2) | 11 (23.9) |
| s1m-      | 1 (2) | 2 (6) |    |
| s2m1      | 1 (2) |    |    |
| cagA+     | 36 (70.6) | 19 (57.6) | 40 (67) |
| babA2+    | 36 (70.6) | 28 (84.8) | 43 (93.5) |
| Type 1    | 29 (56.9) | 16 (48.5) | 40 (67) |
| Triple-positive | 24 (47.1) | 12 (36.4) | 37 (80.4) |

FD: Functional dyspepsia; GU: Gastric Ulcer; DU: Duodenal Ulcer; P values were calculated with the χ² test; b Statistically significant differences (P values < 0.01).

**Combinations of vacA, cagA and babA2 genotypes**

On examining the association of the main virulence genes in each strain, a statistically significant correlation was observed between s1m1 genotype and cagA status (P = 0.00001), between s1m1 and babA2 genotype (P = 0.047), and between cagA and babA2 genotypes (P = 0.049). A significant association was also observed between vacAm1 allele and cagA status or babA2 genotype (P = 0.00001 and P = 0.035, respectively), while most s2m2 strains carried a cagA-negative genotype (Table 2). However, no correlation was observed between vacAs1 and babA2 genotypes (P = 0.12). Additionally, 85 isolates were classified as type 1 strains and 73 were triple-positive strains (Table 3).
### Table 4 Worldwide distribution of main H. pylori virulence factors

| World Area  | vacA alleles prevalence (%) | References |
|-------------|-----------------------------|------------|
| Europe      | s1  | s2  | m1  | m2  | capA prevalence (%) | babA2 prevalence (%) |
| America     | 48-89 | 11-51 | 37  | 63  | 66-75% | 34-78% |
| East Asia   | 57-68 | 16-48 | 37-44 | 29-63 | 57-79% | 46-69% |

### Relationship between genotypes and gastric diseases

Of the 130 *H. pylori* infected patients studied, 39.2% were diagnosed with functional dyspepsia, 35.4% had a duodenal ulcer (DU) and 25.4% had a gastric ulcer (GU). Table 3 shows that the *vacA* s1m1 genotype was detected at a higher frequency in isolates from patients with DU, and in strains obtained from patients with functional dyspepsia; but, the presence of this genotype did not correlate with the presence of duodenal or gastric ulcer (P = 0.21 and P = 0.4, respectively). On the other hand, the *vacA* s1m1 genotype had a higher frequency in DU patients; but, no association was observed between s1m1 or any other *vacA* genotype, and the presence of severe pathologies in this study (Table 3). GU patients exhibited the highest frequency of s2m2 strains, followed by patients with functional dyspepsia (Table 3). No correlation was found between the *cagA* genotype and duodenal or gastric ulcer (P = 0.051 and P = 0.22, respectively); but, an association between *cagA*-positive strains and DU may be assumed as a clear tendency (Table 3). Meanwhile, the *babA2* genotype was significantly associated with DU (P = 0.004), but not with GU (P = 0.13). Type 1 and triple-positive strains (Table 3) were also associated with DU (P = 0.001 and P = 0.0007, respectively) but not with GU (P = 0.45 and P = 0.33, respectively).

### Discussion

Several studies have shown that the incidence and/or severity of gastroduodenal pathologies related to *H. pylori* may vary between geographic areas. This phenomenon is partly due to a different distribution of pathogenic markers in circulating strains.[13] Several pathogenic factors of *H. pylori* have been described and their association with the clinical outcome studied.[19-21]. Distribution of the main virulence factors around the world is summarized in Table 4, showing the high variation between geographic areas. This is the first report to examine the three main *H. pylori* virulence associated genes, *vacA*, *cagA* and *babA2* in Cuban isolates.

### vacA alleles

The *vacA* s1 and s2 leader sequences are different in a small insert, totaling 27 bp, carried by the *vacA* s2 allele,[20] which has a reduced capacity to secrete VacA toxin.[22]. According to our results, the most virulent *vacA* s1 allele was predominant in Cuban *H. pylori* isolates (Table 2), a finding which has also been observed in other studies of Western strains (Table 4).[22-24]. In the present study, the prevalence of *vacA* s1m1 and *vacA* m2 were similar compared to that of the s1 and s2 allele; meanwhile, the s1m1 and s2m2 genotypes were the most common allelic combinations of the *vacA* gene from Cuban isolates (Table 2), a finding reported in several studies from various countries.[10,26]. Furthermore, the middle region of *vacA* was not detected in three isolates, while only one strain harbored the s2m1 genotype. Genotyping of the *vacA* middle region failed in three strains, probably due to heterogeneity in the *vacA* gene, a finding described previously.[12,24]. Additionally, only one strain harbored the s2m1 genotype, the *vacA* allelic combination relating to lower incidence in several studies.[23,24]. On the other hand, the *vacA* s1m1 genotype was noted at a higher frequency in DU patients; but, no significant correlation was observed between *vacA* genotypes and the appearance of peptic ulcer disease, which is in agreement with previous reports.[19,25].

### cagA genotype

*H. pylori* *cagA*-positive strains have been associated with more severe gastroduodenal diseases.[8,14,15]. Here, 73.2% of the *H. pylori* strains were *cagA*-positive, a prevalence similar to that reported in many studies from Western countries (e.g. USA, 60%[7]; Spain, 66%[26]; and England, 68%[27]) but lower than that reported in some East Asian studies, which encountered over 90% of *cagA*-positive isolates (Table 4).[23,28]. In addition, a highly significant correlation was observed between *cagA* status and *vacA* s1m1 and *vacA* s2m1 genotypes (Table 2), which is commonly linked to an increase in *H. pylori* virulence.[13,14,29,30]. An association was also observed between the presence of the *cagA* gene and the *babA2*-positive genotype, due to the fact that most *cagA*-positive isolates carried the *babA2* allele. Our data support the relationship between *cagA* and *babA2* genes found in previous reports, which could be caused by selective pressure[13,14], although other authors, such as Matarr et al.[34], did not find any correlation between these virulence factors in the isolates investigated. On the other hand, previous studies have shown a high association between the *cagA*-positive genotype and the appearance of DU.[11,12]. In this study, however, no correlation was observed between *cagA* status and DU. Moreover, a high frequency of *cagA*-positive strains was observed in DU patients (Table 3), indicating that a statistical association could be reached by increasing the number of patients in future studies.
**babA2 genotype**

Adherence of *H pylori* to epithelial cells is a relevant step in the development of gastroduodenal pathologies. *BabA2* attaches *H pylori* to these cells, allowing delivery of VacA and CagA toxins near the gastric epithelium and therefore enhancing gastric tissue damage[^11]. Here, Cuban *H pylori* isolates exhibited a high frequency (82.3%) of the *babA2* allele when the primers of Sheu *et al.*[^12] were used to amplify the gene. In contrast, a low prevalence of the *babA2* genotype was observed when the primers reported by Gerhard *et al.*[^13] and a variant of Zambon *et al.*[^14] were used, respectively (Table 2). Interestingly, these last two primers are located in a high polymorphic zone of the *babA* gene[^15], which should lead to an underestimation of *babA2*-positive strains. Our results add new data to previous observations[^16,17,18] that support the ineffectiveness of the Gerhard *et al.*[^13] primers to detect the *babA2* gene, and for the first time relate this to deficiencies in the primers used by Zambon *et al.*[^14]. Consequently, the low levels of *babA2* alleles reported in several previous studies[^13,14,15] may be underestimated, due to the use of Gerhard primers[^13]. However, the prevalence of the *babA2* gene was above 70% in Asian countries using the same primers[^16,17,18], suggesting that underestimation due to allelic variation in the *babA* gene could have a variable impact in different geographic areas, as was previously suggested[^18]. This study showed a high association between the presence of the *babA2* allele and DU disease (Table 3), which is in agreement with several reports which associate the presence of this gene with the appearance of severe gastric damage[^13,14,15]. However, other studies have claimed no association between this genotype and more severe pathologies[^16].

**Combination of virulence genotypes**

Of the 130 Cuban *H pylori* isolates, 65.4% and 56.2% were type 1 and triple-positive strains, respectively. Infection with these strains has been associated with a higher degree of inflammation and gastroduodenal lesions[^19]. Similar percentages of both types of strains were found in this study and in a previous report[^13], while Brazilian dyspeptic patients seem to have a lower rate (32.6%) of triple-positive strains[^20]. Our data indicate that type 1 and triple-positive strains increase the risk of developing DU in Cuban dyspeptic patients, finding consistent with other studies, in which these types of strains were mainly found in subjects with peptic ulcer disease[^13], and in patients with intestinal metaplasia and gastric atrophy[^14].

We hypothesize that the absence of a correlation between the virulence genes analyzed and the development of GU might be influenced by the small number of patients with this pathology in our study, although several other studies have not found any correlation between the presence of *H pylori* main virulence factor genes (alone or in combinations) and peptic ulcer disease[^21,22].

It is interesting to note that despite the high rate of *H pylori* infection in Cuban dyspeptic patients[^23-30], and the relatively high pathogenic potential of Cuban isolates found previously[^31-34], and in the present study, a low incidence of gastric adenocarcinoma has been found in Cuban patients with dyspepsia[^35-38]. This reflects a general tendency in the Cuban population towards low levels of gastric cancer; in fact, a gastric cancer death rate of 7.1/100,000 was observed in Cuba in 2007 (http://www.sld.cu/servicios/estadisticas/). Future studies are required to elucidate the above-mentioned investigative problem, including a full characterization of Cuban *H pylori* isolates.

In conclusion, this study has shown a relatively high prevalence of the main virulence factor genes in Cuban *H pylori* isolates, which is similar to that found in the Western-type strains. In addition, a significant association was found between the virulence genes in Cuban strains. Consequently, the presence of more virulent type 1 and triple-positive strains was relatively high in Cuban dyspeptic patients, and increased their risk of developing duodenal ulcer. On the other hand, more severe gastroduodenal pathologies, such as intestinal metaplasia, gastric atrophy and gastric cancer were not found in this study, or in other similar studies and which might merit further research.

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**COMMENTS**

**Background**

Cuba has a high incidence of *H pylori* infection. The presence and association of the main virulence factors VacA, CagA and BabA2 in *H pylori* strains influences the clinical outcome following infection with this pathogen. So far, no whole genotyping of Cuban *H pylori* strains has been carried out. This study addresses the frequency and association of the main virulence factor genes in *H pylori* isolates, and establishes their relationship to clinical outcome in a Cuban dyspeptic population.

**Research frontiers**

In a dyspeptic population in Cuba, the presence and association of the main virulence factor genes (vacA, cagA and babA2) in the infecting strains was significantly high, and their combined presence is a risk factor for duodenal ulcer (DU), but is not associated with gastric ulcer (GU). More severe pathologies, such as intestinal metaplasia, gastric atrophy and gastric cancer were not present in the group studied.

**Innovations and breakthroughs**

Studies of various populations have indicated an association between the presence of vacA, cagA and babA2 genes in *H pylori* isolates and the appearance of more severe gastroduodenal pathologies. The distribution of these virulence markers in *H pylori* strains varies among populations. The present study showed a relatively high prevalence of the main virulence factor genes in Cuban *H pylori* isolates, similar to that found in the Western-type strains. In addition, the study demonstrated a significant association between the virulence genes in the strains studied, which was related to the risk of developing DU, but not GU in dyspeptic patients. Furthermore, despite the relatively high virulence potential of Cuban *H pylori* isolates, pathogens such as intestinal metaplasia, gastric atrophy and gastric cancer were not present in the dyspeptic population studied.
Applications
In developing countries with a high incidence of *H. pylori* infection and dyspepsia, it is important to screen the isolates for main virulence factors. The information generated here may be used to develop a procedure to detect *H. pylori* pathogenic factors in a given population from biopsy samples. Intervention may then be concentrated on subjects with a higher risk of severe pathologies.

Peer review
This study determined the prevalence of main virulence factor genes vacA, cagA and babA2 in *Cuban H. pylori* isolates and their association with gastrointestinal diseases.

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