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doi:10.3748/wjg.15.204

World J Gastroenterol 2009 January 14; 15(2): 204-210
World Journal of Gastroenterology ISSN 1007-9327

BRIEF ARTICLES

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

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Supported by The National Centre for Scientific Research of Cuba, No. 220207
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Received: October 17, 2008 Revised: December 2, 2008
Accepted: December 9, 2008
Published online: January 14, 2009

Abstract

AIM: To investigate the prevalence of vacuolating cytotoxin (*vacA)*, cytotoxin associated gene A (*cagA)* and blood adhesion binding antigen (*babA2)* genotypes of *Helicobacter pylori* (*H pylori*) isolates from Cuban 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.

Key words: Cuban dyspeptic patients; *Helicobacter pylori*; *vacA*; *cagA* and *babA*

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Torres LE, Melián K, Moreno A, Alonso J, Sabatier CA, Hernández M, Bermúdez L, Rodríguez BL. Prevalence of *vacA*, *cagA* and *babA2* genes in Cuban *Helicobacter pylori* isolates. *World J Gastroenterol* 2009; 15(2): 204-210 Available from: URL: http://www.wjgnet.com/1007-9327/15/204.asp DOI: http://dx.doi.org/10.3748/wjg.15.204

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[2].

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 (*oipA*), and duodenal ulcer promoting gene (*dupA*); but, the influence of these proteins on *H pylori* pathogenesis is still under study[3].

The VacA protein induces vacuolation and apoptotic processes in epithelial cells, as well as immunosuppressive actions in immunological cells[4]. The *vacA* gene comprises two main regions: the signal zone (s1 or s2) and the middle region (m1 or m2)[5]. The *vacA s1m1* allelic combination exhibits the highest activity, while *s2m2* and the rare *s2m1* combinations are non-toxic[5]. 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[6].

Hydrophilic protein CagA contains the so-called EPIYA motifs[7], which interact with several eukaryotic proteins, promoting changes in the signal transduction pathway, cytoskeletal plasticity and IL-8 secretion in epithelial cells[8]. CagA-positive *H pylori* isolates are associated with a higher rate of gastric inflammation and damage, when compared with CagA-negative strains[8,9]. The *cagA* gene is located at the end of the cag pathogenecity island, a system that introduces CagA and a peptidoglycan into epithelial cells[10]. Several epidemiological studies have shown the correlation between *cagA*-positive strains and a higher risk of developing peptic ulceration, gastric atrophy and gastric cancer[8,9].

The blood group binding antigen mediates adherence of *H pylori* to human gastric epithelium[11]. This antigen is encoded by the polymorphic gene called *babA2*, while allele *babA1* is non-functional[11]. Some studies have suggested that BabA plays a crucial role in the development of severe functional dyspepsia, peptic ulcer and gastric adenocarcinoma[12,13]. 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[14].

The clinical outcome of this bacterial infection seems to be influenced by the distribution of the above-mentioned pathogenic factors in *H pylori* strains[15]; 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℃ for 5 to 8 d. All *H pylori* isolates were positive for oxidase, catalase and urease. The reference strain J99[16] 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[17]. Purified DNAs were stored at -20℃ until use. In all cases, PCR amplification was carried out in a 25 μL reaction mixture containing 2.5 μL 10X 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℃ for 1 min, followed by 40 cycles at 94℃ for 1 min, 60℃ for 1 min and 72℃ for 1 min, and a final extension at 72℃ 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 χ^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

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

Primer	Sequence (5'-3')	AT ℃	Size (bp)	Ref.
glmMF	CCCTCACGCCATCAGTCCCAAAAA	60	417	[18]
glmMR	AAGAAGTCAAAAACGCCCCAAAAC			
cagF1	GATAACAGGCAAGCTTTTGA	60	349	[7]
cagB1	CTGCAAAAGATTGTTTGGCAGA			
vacAsF	ATGGAAATACAACAAACACAC	52	*s1*-259/*s2*-286	[20]
vacAsR	CTGCTTGAATGCGCCAAAC			
vacAmF	CAATCTGTCCAATCAAGCGAG	56	*m1*-567/*m2*-642	[20]
vacAmR	GCGTCAAAATAATTCCAAGG			
bab7-F	CCAAACGAAACAAAAAGCGT	60	271	[21]
bab7-R	GCTTGTGTAAAAGCCGTCGT			
babA2F[1]	AATCCAAAAAGGAGAAAAAGTATGAAA	60	832	[13]
babA2R	TGTTAGTGATTTCGGTGTAGGACA			
babA2R607[2]	GTTTTCTTTGAGCGCGGGTAAGC	60	607	[14]

[1]Forward primer used with primer babA2R or babAR607 to amplify *babA2* gene; [2]Five nucleotides (GTTTT) were added to the original primer designed by Zambon *et al*[14] to increase specificity.

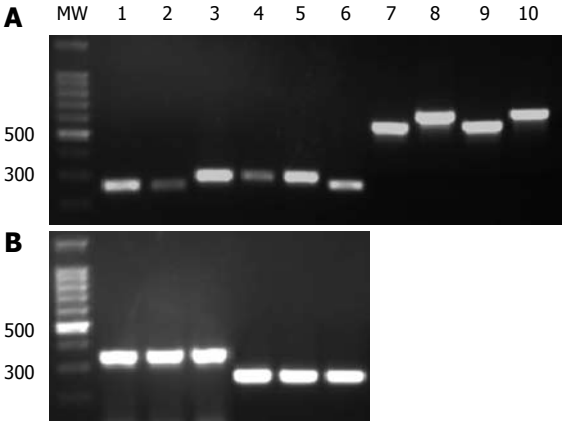

Figure 1 Genotyping of main virulence factor genes in Cuban *H pylori* isolates. The images shown are from a representative gel electrophoresis of two independent PCR amplification products of *vacA* (*s1*, *s2*, *m1*, *m2*), *cagA* and *babA2* genes from Cuban isolates and J99 control strain. A: Lanes 1 and 7, reference strain J99 (*vacAs1* and *m1* alleles, respectively); Lanes 2 and 6, *vacAs1* strains; Lanes 3-5, *vacAs2* strains; Lanes 8 and 10 *vacAm2* strains; Lane 9, *vacAm1* strain. B: Lanes 1 and 4, J99 strain (*cagA* and *babA2* gene, respectively); Lanes 2 and 3, *cagA*-positive strains; Lanes 5 and 6 *babA2*-positive strains. MW: 100 bp DNA Ladder (Promega, USA).

was visualized as a band of 259 bp on agarose gel electrophoresis (Figure 1, panel A), whereas 26.2% of isolates had the *vacAs2* 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 2 Correlation between *vacA* alleles and *cagA* and *babA2* genotypes in 130 Cuban *H pylori* isolates

vacA	*s1m1*	*s1m2*	*s2m2*	*s2m1*	*s1m-*	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 (35.4)	1 (0.8)	3 (2.3)	130

Table 3 Correlation between virulence factor genotypes and disease outcome

Genotypes	Pathologies		
	FD *n* = 51 (%)	GU *n* = 33 (%)	DU *n* = 46 (%)
vacAs1m1	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 (87)
babA2+	36 (70.6)	28 (84.8)	43 (93.5)[b]
Type 1	29 (56.9)	16 (48.5)	40 (87)[d]
Triple-positive	24 (47.1)	12 (36.4)	37 (80.4)[f]

FD: Functional dyspepsia; GU: Gastric Ulcer; DU: Duodenal Ulcer; *P* values were calculated with the χ^2 test; [b, d, f]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* genotypes ($P = 0.047$), 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	*cagA* prevalence (%)	*babA2* prevalence (%)
	s1	*s2*	*m1*	*m2*		*cagA* +	*babA2* +
Europe	48-89	11-51	37	63	[14,23,26,27]	66-73[14,23,26,27]	34-72[13,14,34]
America	57-68	16-48	37-44	29-63	[19,20,24]	57-75[7,19,24]	46-69[24,32]
East Asia	100	0	41-94	5-55	[12,25,28]	90-100[12,25,28,35]	80-100[12,21,35]

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 *vacAs1m1* 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[15]. 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 *vacAs2* allele[20], which has a reduced capacity to secrete VacA toxin[22]. According to our results, the most virulent *vacAs1* 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)[23,24]. In the present study, the prevalence of *vacAm1* and *vacAm2* 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[19,20]. 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)[25,28]. In addition, a highly significant correlation was observed between *cagA* status and *vacAs1* and *vacAs1m1* 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 Mattar *et al*[24], 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[31,32]. 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[3,11]. Here, Cuban *H pylori* isolates exhibited a high frequency (82.3%) of the *babA2* allele when the primers of Sheu *et al*[21] 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[33], which should lead to an underestimation of *babA2*-positive strains. Our results add new data to previous observations[24,34] 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[13,14,32] 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[12,35], suggesting that underestimation due to allelic variation in the *babA* gene could have a variable impact in different geographic areas, as was previously suggested[34]. 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]. However, other studies have claimed no association between this genotype and more severe pathologies[24].

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[14]. 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[24]. Our data indicate that type 1 and triple-positive strains increase the risk of developing DU in Cuban dyspeptic patients, a 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[24,25].

It is interesting to note that despite the high rate of *H pylori* infection in Cuban dyspeptic patients[36-38], and the relatively high pathogenic potential of Cuban isolates found previously[37,38] and in the present study, a low incidence of gastric adenocarcinoma has been found in Cuban patients with dyspepsia[36-38]. 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.

ACKNOWLEDGMENTS

We acknowledge Yampier Roblejo, Marcia Samada, Juan González, Rafael Fando, Margot Martínez and Orlando Reyes for their genuine contributions to endorse the data and conclusions of the manuscript. We are also grateful to Francis Megraud for the reference strain J99 and to Ana Laura Lopez for her technical assistance.

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, pathologies 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 gastroduodenal diseases.

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