Prevalence of *cag*A and *vac*A Genes in Isolates from Patients with *Helicobacter pylori*-associated Gastroduodenal Diseases in Recife, Pernambuco, Brazil

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Geographical differences in the prevalence of Helicobacter pylori *genes and their association with disease severity have been identified. This study analyzes the prevalences of the* cag*A gene and alleles of the* vac*A gene in* H. pylori-*associated gastroduodenal diseases in isolates from Recife, PE, Brazil. Gastric biopsy of 61* H. pylori-*positive patients were submitted to DNA extraction and gene amplification by polymerase chain reaction. Among the 61 patients, 21 suffered from duodenal ulcer (DU) and 40 from gastritis (GT). The prevalence of* H. pylori *strains harbouring the* cag*A gene was higher in the DU group (90.5%) than in the GT group (60%) (p = 0.02). The* vac*A gene was amplified in 56 out of 61 biopsies, of which 43 (76.8%) contained bacteria carrying the s1 allele and 13 (23.2%) the s2. However, the prevalence of the* vac*A s1 genotying was the same in either DU or GT group. The majority of the s1-typed strains, 39 (90.7%) out of 43, were subtype s1b. In resume there was a strong association between the* H. pylori cag*A+ gene and DU. However, there were no differences between the DU and GT groups in relation to the* vac*A s1 and s2 alleles distribution, albeit the subtype s1b was predominat.*

Key words: *cag*A - *vac*A- genotype - *Helicobacter pylori* - Recife - Brazil

Helicobacter pylori is a polyflagellated spiral gram-negative bacterium and the main etiologic agent of duodenal ulcer (DU) (Coghlan et al. 1987, Marshall et al. 1988) and chronic gastritis. It is also associated with the development of MALT lymphoma (Wotherspoon et al. 1993, Zucca et al. 1998) and carcinoma of the stomach (Uemura et al. 2001, Passaro et al. 2002).

It colonizes the stomach of about 50% of the world's population, having a prevalence of over 70% in underdeveloped countries (Perez-Perez et al. 1990, Rocha et al. 1992, Souto et al. 1998). The imbalance between the bacterium aggressive factors and the host defence mechanisms leads to the disease in small part of the population.

Two virulence factors are found only in strains regarded as having greater pathogenicity and the ability to lead to the development of disease, namely the protein associated with cytotoxin, *cag*A, and the vacuolizing cytotoxin, *vac*A.

The *cag*A gene represents a marker for the "island of pathogenicity", a 40-Kb genomic region that codifies virulence factors, including one which induces the synthesis of IL-8 cytokine (*cag*E, *cag*G), a potent inducer of the inflammatory response (Tummuru et al. 1995, Censini et al. 1996, Hsu et al. 2002). The *cag*A gene-expressing *H. pylori* strains have been more prevalent in those suffering from DU than patients with gastritis (GT) or nonulcerous dyspepsia (NUD) (Cover et al. 1990, Takata et al. 1998, Van Doorn et al. 1998). However, it has been observed some geographic variations on the type of *H. pylori* strains isolated in Houston (US), for instance, where *cag*A positive are the predominant strain in the population (Graham et al. 1996, Miehlke et al. 1996, Matsukura et al. 1997).

Another virulence factor, the product of the *vac*A gene, is able to induce vacuolization in eucariotic cells (Leunk et al. 1988). Polymorphism at the signal sequence (s1a, s1b, s1c or s2) and/or the middle region (m1 or m2) of the *vac*A gene generates allelic variations. The s1 type allele is said to be associated with cytotoxic activity and development of peptic DU (Atherton et al. 1997, Evans et al. 1998). A high prevalence of the s1a subtype was observed in different regions in the world (Atherton et al. 1995, Ito et al. 1997). A recent publication showed a greater prevalence of subtype s1b in Central and South American countries (Van Doorn et al. 1999, Ashour et al. 2002).

In spite of the efforts in the past decade to prove an association of the infection by different genotype *H. pylori* strains and the development of a specific disease, the findings were not conclusive (Weel et al. 1996, Takata et al. 1998).

In this study the molecular epidemiology of the *H. pylori*-associated disease has been analyzed in patients from the city of Recife, Northeastern Brazil.

MATERIALS AND METHODS

Sixty-one *H. pylori*-infected patients with dyspetic complaints were selected and submitted to upper gastrointestinal endoscopy at the gastroenterology outpatient clinic in the Pernambuco Federal University Hospital. On confirmation of the histology, they were divided into two groups: those with DU and those suffering from GT. The criteria of exclusion was: patients with peptic ulcer presenting haemorrhage at the time of the diagnosis, those suffering from a severe concurrent condition, portal hypertension and those who had taken nonsteroidal anti-inflammatory drugs in the previous four months or undergone any therapy for *H. pylori* eradication.

The study protocol received the approval of the "Ethics in Research Committee" of the Health Science Centre at the Federal University of Pernambuco. The research protocol started after the patients were explained about the project and sign the informed consent form.

Biopsy and histology of the gastric mucosa - Three fragments of the gastric mucosa were removed from both corpus and antrum. One fragment of each was placed in a sterile Eppendorf tube containing a 0.9% NaCl solution and frozen at -70°C for further polymerase chain reaction (PCR) analysis. The four remaining fragments were placed in a flask containing 10% formalin and sent for histopathology. The microscopic identification of the *H. pylori* was performed by Giemsa staining.

Extraction of DNA - The genomic DNA was extracted from the biopsy sample using the Rapidprep Genomic DNA isolation kit for cell and tissue (Pharmacia, US), in accordance with the manufacturer's instructions and stored at -20°C.

PCR - The genomic DNA was used as a DNA template for the amplification of the *cag*A gene fragment by means of the PCR technique with pairs of primers previously described in the literature and shown in Table I. The primers F1 and F2 give a PCR product of 349 bp and were designated P1 (Navaglia et al. 1998). The other pair of primers (HPC1 and HPC2), which were designated P2, amplifies a region of 194 bp (Ito et al. 1997). The PCR was prepared with 1 µl of template DNA, 100 µl of dNTP, 20 pmol of each primer and 1U of *Taq*-DNA polymerase (Pharmacia) in 25 µl of PCR solution containing 10 mM tris-HCl (pH 8.8); 50 mM KCl; 3 mM MgCl$_2$ and 0.1 mg/ml of bovine serum albumin. The reaction conditions for the *cag*A gene amplification with both pairs were as follows: 30 cycles with denaturation at 94°C for 1 min; annealing temperature at 55°C for 1 min and synthesis at 72°C for 1 min.

For the *vac*A gene typing we performed the PCR with primers specific for its signal sequence. The PCR-product generated by VA1-F and VA1-R primers were 259 bp or 286 bp in size for respectively type s1 or s2 *vac*A$^+$ strains. The use of the antisense VA1-R primer with the SS1-F or SS2-F primers render respectively a PCR fragment of 190 bp for s1a and 187 bp for s1b subtyped *vac*A$^+$ strains. The PCR conditions were as follows for the both set of primers used: 30 cycles of denaturation at 94°C for 1 min; annealing temperature at 53°C for 1 min; and synthesis at 72°C for 1 min (Atherton et al. 1995).

The PCR reaction negative control consisted of the reaction performed without bacterial DNA or human DNA extracted from epidermal biopsies added. Positive control was carried by replacing the *H. pylori* specific primers to that which amplifies the human constitutive *gpd*H gene. PCR products were visualized by eletrophoresis on 1.5% (*cag*A) or 2% (*vac*A) agarose gels stained with ethidium bromide under UV light and photographed with a polaroid film.

Negative controls were as described above. PCR products were visualized by eletrophoresis on 2% agarose gels stained with ethidium bromide under UV light and photographed with a polaroid film.

Statistical analysis - The Epi-info program, Version 6.04B was used for the statistical analysis. The continuous quantitative variables were represented by mean, standard deviation, minimum and maximum values, using the Student t test for the analysis of significance. The qualitative and discrete variables were represented by absolute frequency (n) and relative frequency (%) and their significance analyzed using the chi-square test or Fisher's exact test. The level of rejection for the hypothesis of nullity was always less than 0.05%.

TABLE I

List of primers used for amplification of the fragments of the *vac*A and *cag*A

Primer (Amplified region)	Size	Sequence
VA1-F, (s1 and s2) [a]	259 and 286 bp	5'ATGGAAATACAACAAACACAC3'
SS1-F, (s1a) [a]	190 bp	5'GTCAGCATCACACCGCAAC3'
SS3-F, (s1b) [a]	187 bp	5'AGCGCCATACCGCAAGAG3'
VA1-R [a]		5'CTGCTTGAATGCGCCAAAC3'
F1, (*cag*A- P1) [b] B1	349 bp	5'GATAACAGGCAAGCTTTTGAGG3' 5'CTGCAAAAGATTGTTTGGCAGA3'
HPC1, (*cag*A-P2) [c] HPC2	194 bp	5'TCAAATACACCAACGCCTCC3' 5'AGCTTCTTGTGGGGACAATC3'

*a*: Atherton et al. 1995; *b*: Navaglia et al. 1998; *c*: Ito et al. 1997

RESULTS

Characteristics of the population - Among 61 patients selected, 21 had DU and 40 GT. Their ages ranged from 16 to 69 years, the mean age being 40.5 years. It was not observed a significative difference on the mean ages in both groups studied. The majority of patients (77.2%) were females.

Regard to the patient socioeconomic status, their monthly income ranged from US$120 to US$180. The education level of the studied population was in 50% of the cases incomplete at primary elementary school, of which half were illiterate. The majority lived in a good sanitation condition with 93.7% having running water and 97.4% a sewage system. The drinking water was filtered (71.3%), tap water (27.5%) or from a well (1.3%).

cagA$^+$ *status* - All the biopsy samples were submitted to DNA extraction and PCR analysis (Figure). Among the 43 *H. pylori cagA*$^+$ strains (70.5% of total), 31 matched for both set of primers, five strains were only positive for the P1 pair (three with ulcers and two with GT) and seven were only positive for the P2 pair (three with and four without ulcer). Nineteen out of 21 (90.5%) patients with duodenal ulcer were infected by *H. pylori cagA*$^+$ strains with a significant difference (p = 0.01), when compared with only 24 out of 40 with GT (60%) (Table II).

vacA$^+$ *status* - The *vacA* gene was amplified in 56 (91.8%) out of the 61 *H. pylori* isolates. Five out of 40 patients with GT group were not amplified. As the initial selection for sampling was the detection of *H. pylori* at histology, it is unlikely that the *H. pylori* DNA was not extracted. Furthermore, there were not PCR inhibitors in the sample preparation, as shown by the amplification of the human constitutive *gpd*H gene. Therefore, it was assumed that there were no obstacle to *vacA* gene amplification if the gene is present.
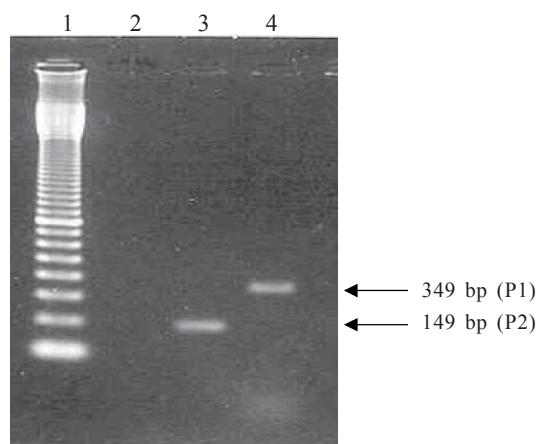
Identification of the *cag*A by polymerase chain reaction (PCR). Lanes - 1: molecular weight marker; 2: negative control (without DNA); 3: *Helicobacter pylori*, strain A4 *cag*A$^+$, PCR with primer P2; 4: *H. pylori*, strain A21 *cag*A$^+$, PCR with primer P1

By means of the signal sequence amplification using different pairs of primers it was possible to classify the strains as type s1 or s2 and subsequently as subtype s1a or s1b.

Among the 56 *vacA* gene-harbouring *H. pylori* isolates, 43 (76.8%) carried s1 and 13 (23.2%) s2 alelles. In the DU group, 17 (81%) isolates were s1 type and four (19%) s2. Despite of the high percentage it was not statistically different from that found in the GT group, in which 26 (74.3%) isolates were s1 and 9 (25.7%) s2 (p = 0.24).

Among the 43 isolates carrying the s1 allele of the *vacA* gene, 39 (90.7%) were s1b subtype and four (9.3%) s1a, having a similar distribution in both DU and GT groups (p > 0.2) (Table III).

TABLE II

Prevalence ratio of the *cag*A gene among the patients infected with *Helicobacter pylori* with endoscopic findings of duodenal ulcer (DU) or gastritis (GT)

	DU	GT	Total	PR (CI$_{95\%}$)	p
Strains *cag*A$^+$	19 (90.5%)	24 (60%)	43 (70.5%)	3.98 (1.03-15.33)	0.01 ^{a}
Strains *cag*A$^-$	2 (9.5%)	16 (40%)	18 (29.5%)		
Total	21	40	61		

a: prevalence ratio (PR) of *H. pylori*$^+$ *cag*A gene among patients with DU or GT (PR = 3,98; CI$_{95\%}$ 1,03-15,33; p = 0,01)

TABLE III

Prevalence of the s1, s2, s1a and s1b alleles of the *vac*A in the strains isolated from patients with duodenal ulcer (DU) and gastritis (GT)

	s1	s2	Total	s1a ^{a}	s1b ^{b}
DU	17 (81%) ^{a}	4 (19%)	21	1 (5.9%)	16 (94.1%)
GT ^{c}	26 (74.3%)	9 (25.7%)	35	3 (11.5%)	23 (88.5%)
Total	43 (76.8%)	13 (23.2%)	56	4 (9.3%)	39 (90.7%)

a: prevalence ratio (PR) of *Helicobacter pylori*$^+$ allele s1 of the *vac*A among patients with DU or GT (PR = 1.09; CI$_{95\%}$ 0.82-1.45; p = 0.41); *b*: PR of *H. pylori*$^+$ allele s1b of the *vac*A gene among patients with DU or GT (PR = 1.06; CI$_{95\%}$ 0.89-1.28; p = 0.47); *c*: five samples from GT group were not amplified

Association of the vacA and the cagA gene - Among the 43 isolates of the *vac*A s1 allele-carrying *H. pylori*[+], 33 (76.6%) were *cag*A[+] while nine out of the 13 (69.2%) s2 allele-carrying *H. pylori*[+] were also *cag*A[+] (p = 0.58). Considering the *vac*A subtyping, there were 39-*vac*A[+] s1b-carrying strains, in which 31 were also *cag*A[+]. The small number of the *vac*A[+] sla allele-carrying strains made it impossible to compare the two populations prevalence ratio (PR) for the *cag*A gene presence among carriers of the *vac*A gene (PR = 1.11; $CI_{95\%}$ 0.74-1.65; p = NS)

DISCUSSION

In the present study a higher prevalence of the *cag*A gene was observed in patients with DU (90.5%) when compared with the GT group (60%). Several studies have been shown a prevalence of the *cag*A gene in *H. pylori* isolates from patients with DU ranging from 80 to 100% (Cover et al. 1990, Weel et al. 1996, Van Doorn et al. 1998, Uemura et al. 2001). Geographical differences, however, have been found. It has been reported in west countries a presence of *cag*A gene in 95% of the *H. pylori*-infected patients with DU, which did not differ significantly from the GT group with a prevalence of 80% (Miehlke et al. 1996). The same was published for east countries, where the *cag*A gene-expressing *H. pylori* was found in 89.3% of the 28 isolates from patients with DU and in 92.3% of the 24 with GT (Matsukura et al. 1997). Studies carried out in Houston showed also similar results (Graham et al. 1996).

A Brazilian study from the southeastern state of Minas Gerais showed a *cag*A gene prevalence of 52.4% in 21 *H. pylori* isolates from patients with GT and of 88% in isolates from patients with DU, which were similar to the results presented here (Ashour et al. 2002).

The *vac*A s1 allele-carrying *H. pylori* was predominantly detected in the isolates. Despite its greater prevalence in DU-patient isolates, there was no significant differences when compared with the GT group. A study in Tennessee showed a strong association of the *vac*A s1 allele-harbouring *H. pylori* and ulcer, having been detected in 21 out of 23 isolates from patients with DU (91%) and in only 16 out of 33 of those with GT (48%), while the *vac*A s2 allele was found in only 11% of 19 *H. pylori*-infected patients with ulcer (Atherton et al. 1995).

The prevalence of the different *vac*A alleles has been reported in *H. pylori* isolates from different regions. A high prevalence of *vac*A s1 allele and its uniform distribution among the various diseases has been observed in Asian countries (Ito et al. 1997).

In the Brazilian study from Minas Gerais, published in 1998, it was found a prevalence of 94% for the s1 *vac*A allele-harbouring *H. pylori* isolates in patients with DU, much higher than the 54% found in patients with GT and thus, an association of s1 allele with DU (Evans et al. 1998). Similar results were showed in 2002 by the same researcher group (Ashour et al. 2002). The results presented here suggest the existence of regional differences on the *H. pylori* isolates in this country.

The s1b allele-harbouring *H. pylori* predominated in our study (90.7%) and were equally found in isolates from both DU and GT patient groups. The s1b subtype *vac*A[+]-*H. pylori* strains (89.7%) was also predominante in isolates

from Portuguese patients (Van Doorn et al. 1998). Another study showed a high prevalence of s1a allele (88.8%) among 170 s1 *vac*A allele-harbouring *H. pylori* isolates from Northern and Western Europe, while the isolates from Central and South America were almost complete of the s1b subtype. There were other countries with more uniform distribution between the s1a and s1b subtypes of *vac*A[+]-*H. pylori* strains (Van Doorn et al. 1999). Recently, it was found the *vac*A s1b allele in 82-*H. pylori* isolates in Minas Gerais, which was similar to our findings (Ashour et al. 2002).

The association of high prevalence of *cag*A gene-harbouring *H. pylori* and disease severity as DU were showed and are in concordance with several published works. Nevertheless, it is also found in isolates from patients with mild diseases as GT suggesting that *cag*A gene might be not a specific marker for severity. Futhermore, the inherent genetic polymorphism of regional strains was showed by the differential *cag*A primer annealing and amplification. It was found in 28% of the *cag*A-carrying *H. pylori* strains a discordance on the amplification results generated by the two sets of primers used. We had also observed that few isolates showing multiple *vac*A genotypes. The same was observed in isolates from Portugal were the prevalence of *H. pylori* infection in adult population is also high (80%). Nevertheless, we were not able to correlate the infection by multiple *vac*A genotype *H. pylori* and disease severity.

After the *H. pylori* genome sequencing, it is now expected that other genes be characterized. It might be possible that an association of expressed genes are necessary for the development of specific pathologies and the differential gene expression would characterize the *H. pylori* virulence. This would explain the difference in strain virulence isolated from different parts of the world. Once a molecular marker for *H. pylori* infection severity been described it may be use to help decision on treating some patients.

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