CagA phosphorylation EPIYA-C motifs and the *vac*A i genotype in *Helicobacter pylori* strains of asymptomatic children from a high-risk gastric cancer area in northeastern Brazil

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Helicobacter pylori *infection is one of the most common infections worldwide and is associated with gastric diseases. Virulence factors such as VacA and CagA have been shown to increase the risk of these diseases. Studies have suggested a causal role of CagA EPIYA-C in gastric carcinogenesis and this factor has been shown to be geographically diverse. We investigated the number of CagA EPIYA motifs and the* vac*A i genotypes in* H. pylori *strains from asymptomatic children. We included samples from 40 infected children (18 females and 22 males), extracted DNA directly from the gastric mucus/juice (obtained using the string procedure) and analysed the DNA using polymerase chain reaction and DNA sequencing. The* vac*A i1 genotype was present in 30 (75%) samples, the i2 allele was present in nine (22.5%) samples and both alleles were present in one (2.5%) sample. The* cag*A-positive samples showed distinct patterns in the 3' variable region of* cag*A and 18 of the 30 (60%) strains contained 1 EPIYA-C motif, whereas 12 (40%) strains contained two EPIYA-C motifs. We confirmed that the studied population was colonised early by the most virulent* H. pylori *strains, as demonstrated by the high frequency of the* vac*A i1 allele and the high number of EPIYA-C motifs. Therefore, asymptomatic children from an urban community in Fortaleza in northeastern Brazil are frequently colonised with the most virulent* H. pylori *strains*.

Key words: *Helicobacter pylori* - *H. pylori* CagA-EPIYA - *H. pylori/vac*A

Helicobacter pylori infection is associated with the development of chronic gastritis, peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue (Marshall 1994). The microorganism is one of the most genetically diverse bacterial species; its diversity has been enhanced by extensive interstrain gene transfer and recombination (Danon et al. 1998). Several studies have indicated geographic differences among the strains, particularly in respect to virulence factors such as the vacuolating cytotoxin (*vac*A) gene (Atherton et al. 1997, Van Doorn et al. 1998). VacA, which is one of the most important *H. pylori* virulence factors, causes a multiplicity of alterations in the gastric cells, including the formation of cytoplasmic vacuoles, the fragmentation of mitochondria and the induction of autophagy and cell death. Although *vac*A is present in all strains, there is considerable variation in vacuolating activities among the strains because of sequence heterogeneity at the signal (s) and middle (m) regions within the gene (Atherton et al. 1995, Umit et al. 2008). A third polymorphism was recently identified in the p33 *vac*A region, which is known as the intermediate (i) region; the i1 and i2 alleles were more frequently associated with the s1m1 and s2m2 variants, respectively (Rhead et al. 2007). It has been proposed that the i1 allele is the most important determinant of cytotoxicity and the best marker of disease outcome among the *vac*A polymorphic alleles (Argent et al. 2008, Jones et al. 2011, González-Rivera et al. 2012, Ameer et al. 2014).

The cytotoxin-associated gene (*cag*A) is another major *H. pylori* virulence factor that is strongly associated with the more severe gastrointestinal diseases in western countries (Blaser et al. 1995, Censini et al. 1996, Queiroz et al. 1998). *cag*A belongs to the *cag* pathogenicity island that codes a type IV secretion system (T4SS) associated with increased secretion of interleukin-8, a strong pro-inflammatory chemokine that participates in the genesis of *H. pylori*-induced gastritis. The T4SS is also responsible for the entrance of the CagA protein into the gastric epithelial cells where CagA is phosphorylated on a tyrosine residue within the phosphorylation motifs in the carboxy-terminal variable region of the protein. These motifs are defined as EPIYA (Glu-Pro-Ile-Tyr-Ala) A, B, C and D according to their different flanking amino acids. The cagA protein generally possesses EPIYA A and B segments that are followed by none, one, two or

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three C segments in strains circulating in the western countries or a D segment in the East Asian countries. EPIYA C and D are the main sites for the phosphorylation of CagA. Phosphorylated CagA forms a physical complex with SHP-2 phosphatase and triggers abnormal cellular signals leading to the deregulation of cell growth, cell to cell contact and cell migration, the elongation of epithelial cells and an increase in epithelial cell turnover, all of which enhance the risk of damaged cells acquiring precancerous genetic changes. Carrying the type D EPIYA or multiple C repeats is associated with increased SHP-2 phosphatase activity induced by CagA (Higashi et al. 2002, Naito et al. 2006) and increased risks of precancerous lesions and gastric cancer (Hatakeyama 2006, Batista et al. 2011).

However, until now, studies on *H. pylori* CagA EPIYA sites have focused on adults. Studies evaluating children are scarce and only symptomatic children who underwent an endoscopy have been included in previous studies (Panayotopoulou et al. 2007, Yamaoka et al. 2010). Furthermore, with respect to the *vac*A i region, we are aware of only one study evaluating 11 symptomatic Bulgarian children (Yordanov et al. 2012). Previously, we have shown a high prevalence of infection by *H. pylori* strains carrying the *cag*A gene and the *vac*A s1 allele in asymptomatic children from northeastern Brazil, a high-risk area for gastric cancer, by evaluating gastric juice/mucus DNA obtained by a string test (Gonçalves et al. 2013). In this study, we aimed to characterise the number of CagA EPIYA motifs and the *vac*A i genotypes in *H. pylori* DNA from these children.

SUBJECTS, MATERIALS AND METHODS

This study was approved by the Ethical Committee of the Federal University of Ceará, Brazil. All the children and their parents signed an informed consent.

We included 40 *H. pylori*-positive children (18 females and 22 males) from eight-18 years old with a mean age of 13.2 (standard deviation = 3.2) years. These children had previously participated in *H. pylori* epidemiological studies in Parque Universitário, an urban community in Fortaleza, Brazil, and had their *H. pylori* status determined by a ^{13}C urea breath test (UBT) according to the protocol previously validated for the Brazilian population (Cardinali et al. 2003). Seven *H. pylori*-negative children were also evaluated (3 females and 4 males) who were eight-18 years old with a mean age of 14.8 years.

The DNA was extracted directly from the gastric mucus/juice [obtained by the string procedure (Gonçalves et al. 2013)] using the QIAmp kit (QIAGEN, Germany) according to manufacturer's recommendations with minor modifications. The DNA concentration was determined by spectrophotometry using NanoDrop 2000 (Thermo Scientific, USA) and the samples were stored at -20ºC until use. Distilled water was used as a reaction control.

The thermocycler GeneAmp PCR System 9700 (Applied Biosystems, USA) was used for all the reactions. The amplified products were electrophoresed in 2% agarose gel, stained with ethidium bromide and analysed in an ultraviolet (UV) light transilluminator. An *H. pylori* strain from our collection (BH1010-95), that was known to be *vac*A s1i1m1 and *cag*A-positive, in addition to an s2i2m2 *vac*A genotype and *cag*A-negative standard Tx30a *H. pylori* strain, were used as controls. Distilled water was also used as a negative control.

The presence of the *H. pylori*-specific *ure*A gene was evaluated using nested polymerase chain reaction (PCR) to confirm the presence of the bacterium DNA (Gonçalves et al. 2013).

The *vac*A i region was amplified using the genotyping method recently proposed by Figueiredo's group (Ferreira et al. 2012) with minor modifications that generated smaller amplicons (145 bp for i1 and 151 bp for i2). This method was expected to easily amplify the DNA from samples with low concentrations of specific DNA and containing inhibitors such as DNAse produced by the oral bacteria; this method was used on the gastric mucus/juice samples that were obtained using the string test. Briefly, the PCR mixtures were prepared in a final volume of 25 μL, containing 1X PCR Buffer (Applied Biosystems), 2,5 mM $MgCl_2$, 2 mM concentrations of deoxynucleotide triphosphates, 1,25 U of Amplitaq Gold (Applied Biosystems) and 5 mM concentrations of the forward and reverse primers. PCR was performed with a 5 min pre-denaturation at 95ºC, followed by 35 cycles of 30 s at 95ºC, 30 s at 50ºC and 30 s at 72ºC. A final extension was performed for 5 min at 72ºC. The PCR products were electrophoresed in 2.5% agar gels and examined under UV light according to standard procedures. The PCR amplification of the *vac*A s and m regions was performed using the primers described by Atherton et al. (1995).

The *cag*A gene was amplified using two previously described sets of primer pairs (Kelly et al. 1994, Peek et al. 1995). The *H. pylori* strains were considered to be *cag*A-positive when at least one of the two reactions was positive.

For the PCR amplification of the 3' variable region of the *cag*A gene, 20-100 ng of DNA was added to 1% Taq DNA polymerase buffer solution (50 mM KCl and 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 100 μM of each deoxynucleotide, 1.0 U Platinum Taq DNA polymerase (Invitrogen, Brazil) and 10 pmol of each primer, for a total solution volume of 20 μL. The primers used were previously described (Yamaoka et al. 1998). The reaction conditions were as follows: 95ºC for 5 min, followed by 35 cycles of 95ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min, ending with 72ºC for 7 min. The amplified products were electrophoresed in 1.5% agarose gel that was stained with ethidium bromide and the products were analysed in an UV light transilluminator. The reaction yielded products of 500-850 bp according to the number of EPIYA-C.

A subset of samples (n = 10) was randomly selected for sequencing to confirm the PCR results (Batista et al. 2011). The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's recommendations. The purified products were sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit in an ABI 3130 Genetic Analyser (Applied Biosystems). The sequences obtained were aligned using the CAP3 Sequence Assembly Program (available from: pbil.univ-lyon1.fr/cap3.php). After alignment, the nucleotide sequences were transformed into amino acid sequences using the BLASTX program

TABLE I

Distribution of the *Helicobacter pylori vac*A signal (s) and middle (m)[a] alleles and *cag*A status according to the *vac*A intermediate (i) genotypes

	n (%)				*cag*A status	
	s1	s2	m1	m2	Positive	Negative
*vac*A i1	30 (90.9)	0	14 (100)	5 (35.7)	27 (90)	2 (22.2)
*vac*A i2	3 (9.1)	6 (100)	0 (0)	9 (64.3)	3 (10)	7 (77.8)
p	< 0.001		< 0.001		< 0.001	

a: amplified in 29 samples; n = 40 (1 sample with a mixed genotype was not included in the analyses).

(available from: blast.ncbi.nlm.nih.gov/Blast.cgi) and compared to the sequences deposited in the GenBank database (ncbi.nlm.nih.gov/Genbank/).

The data were analysed with the two-tailed chi-square or Fisher's exact tests using the Statistical Package for the Social Sciences v.17 (SPSS Inc, USA). The level of significance was set at a p value ≤ 0.05.

RESULTS

The presence of *H. pylori*-specific *ure*A was successfully confirmed by the nested PCR in all 40 strains and was negative in the seven strains of the *H. pylori*-negative children.

*vac*A i was amplified in all *ure*A-positive samples and the i1 genotype was present in 30 (75%) samples, the i2 allele was present in nine (22.5%) samples and both alleles were present in one (2.5%) sample. The mixed infection was not included in the analyses. All *vac*A i1 alleles were also *vac*A s1, six of the nine (66.67%) *vac*A i2 strains were s2 and three strains were *vac*A s1i2 ($p < 0.001$) (Table I). The m region of *vac*A was amplified in 29 samples: 14 (48.2%) samples were m1, 14 (48.2%) samples were m2 and one (3.6%) sample m1m2 (the last sample was not included in the analyses). All m1 genotypes were i1, nine of the m2 genotypes were i2 and five of the m2 genotypes were i1 ($p < 0.001$) (Table I). Considering the cases in which the m allele was amplified, 14 (50%) samples were s1i1m1, five (17.86%) samples were s1i1m2, three (10.71%) samples were s1i2m2 and six (21.43%) samples were s2i2m2 (Table I). The *vac*Ai1 allele was associated with the *cag*A-positive status ($p < 0.001$, odds ratio: 21.00, 95% confidence interval: 2.70-212.01) (Table I).

The PCR-amplified products from all 30/40 (75%) *cag*A-positive samples showed distinct patterns in the 3' variable region of *cag*A. The PCR results were confirmed by sequencing 10 randomly selected PCR products. No EPIYA-D motifs were found in the *H. pylori* strains studied. The presence of CagA EPIYA-A and B motifs were detected in all *cag*A-positive isolates followed by one or two EPIYA-C motifs arranged in the ABC or ABCC combination, respectively. Eighteen of the 30 (60%) strains had one EPIYA-C motif and 12 (40%) strains had two EPIYA-C motifs. The distributions of the EPIYA-C motifs and the *vac*A i genotypes are shown in Table II. Neither *vac*A nor the EPIYA motifs were amplified in the samples of the seven ^{13}C UBT-negative children.

TABLE II

Distribution of the CagA EPIYA-C genotypes according to the *Helicobacter pylori vac*A intermediate (i) alleles

	CagA EPIYA-C Motifs (n)		
	ABC	ABCC	Total
*vac*A i1	15	12	27
*vac*A i2	3	0	3

n = 40 (1 sample with a mixed genotype was not included in the analyses).

DISCUSSION

In the present study, we evaluated the prevalence of *H. pylori* virulence markers, the high number of CagA EPIYA-C motifs and the *vac*A i1 genotype in gastric juice/mucus DNA obtained by the string test (Gonçalves et al. 2013) in asymptomatic children from northeastern Brazil. To the best of our knowledge, this is the first study evaluating these *H. pylori* virulence markers, which are considered the best predictors of *H. pylori*-associated disease in asymptomatic children. Although the infection is mainly acquired in childhood and there are substantial knowledge gaps with respect to different aspects of the infection, including the factors linked to *H. pylori* infection outcomes, it has been difficult to evaluate the bacterium virulence markers circulating in the general population because the studies on this subject are biased by the fact that the child samples are often obtained from children selected for endoscopy who may harbour the most virulent strains. Previously, we showed that in this gastric cancer high-risk Brazilian region, *H. pylori* infection is acquired early in childhood (Rodriguez et al. 2004) and asymptomatic children are colonised more frequently by *H. pylori* strains carrying the toxigenic *vac*A s1 allele (Gonçalves et al. 2013); this has also been observed in asymptomatic children from another gastric cancer high-risk country, Colombia (Sicinschi et al. 2012).

Of note, we confirmed that the studied population was colonised early by the most virulent *H. pylori* strains, which was demonstrated by the high frequency of the *vac*A i1 allele; this allele was strongly associated with the *vac*A s1, *vac*A m1 and *cag*A-positive virulent genotypes. These results suggest that the *cag*A and *vac*A genotypes may not be considered as independent variables in *H. pylori* pathogenesis, which is consistent with the study by Argent et al. (2008) that showed the potential of a functional association between *vac*A and *cag*A. The authors also showed that polymorphisms within each virulence gene may affect the functional interactions between their products.

The observation of the high prevalence of *H. pylori* infection with strains harbouring high numbers of EPIYA-C motifs reinforces the fact that the population is strongly exposed to the most virulent *H. pylori* strains. In China, symptomatic children also frequently carry *H. pylori* strains with the more virulent EPIYA-D that circulates in East Asian countries (Juan et al. 2009), contrary to that demonstrated in the United States of America, which is a gastric cancer low-risk country (Yamaoka et al. 2010).

It must be emphasised that the EPIYAs of the strains of the children we studied have the typical western sequences. A study evaluating two Amerindian populations in a gastric cancer low-risk region in the Peruvian Amazon demonstrated differences in the *cag*A sequence of the EPIYA B and the region between EPIYA B and C. These strains were considered less virulent than those carrying the Western or East Asian prototype EPIYA (Suzuki et al. 2011).

One limitation of this study is the sample size. Further studies on the subject including a larger number of children are warranted. In addition, it would be worthwhile to evaluate whether symptomatic children from the same region are also colonised with highly virulent *H. pylori* strains.

In conclusion, despite the small number of children evaluated, the results of this study demonstrated a high prevalence of *H. pylori* infections with the most virulent strains present in asymptomatic children in northeastern Brazil. Our findings highlight the importance of the early diagnosis of *H. pylori* to identify populations at a greater risk of developing severe gastrointestinal diseases.

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