JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2000, p. 2853–2857 Vol. 38, No. 8
0095-1137/00/$04.00+0

vacA Genotypes in *Helicobacter pylori* Strains Isolated from Children with and without Duodenal Ulcer in Brazil

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Received 30 December 1999/Returned for modification 23 February 2000/Accepted 15 May 2000

Data concerning the association between *vacA* genotypes and disease in children in both developed and developing countries are scarce, especially because of the small number of children with a duodenal ulcer studied. The *vacA* genotypes of *Helicobacter pylori* strains obtained from 65 children (24 with a duodenal ulcer and 41 without a duodenal ulcer; 33 girls; mean age, 10.2 years; age range, 1 to 17 years) were investigated as described by J. C. Atherton et al. (J. Clin. Microbiol. 37:2979–2982, 1999). Ten (15.4%) children were infected with more than one *H. pylori* strain. None of these patients were included in our analysis of the relationship between gastric disorders and specific *vacA* genotypes. The s1 allele was detected in all *H. pylori* strains isolated from patients with a duodenal ulcer and from 21 (58.3%) patients without a duodenal ulcer ($P = 0.003$). Strains with the s2 allele were found only in patients without ulcer ($n = 15$; 41.7%). Most s1 strains had the s1b allele (97.5%), a result similar to that reported for adults from the Iberian peninsula, which could reflect the Brazilian population origin. One untypeable s1 strain was isolated. The m1 allele was also more frequently found in strains obtained from duodenal ulcer patients ($P = 0.028$). The m2 allele was found in strains obtained from 20 (36.4%) children, 3 (15.8%) with an ulcer and 17 (47.2%) without an ulcer. Only one m hybrid strain (m1 and m2 hybrid) was detected. It was demonstrated for the first time that the frequencies of colonization with strains with the s1 allele (14.3% in children up to 8 years of age and 85.7% in older patients; $P = 0.012$) and of strains with the m1 allele (11.1% in patients up to the age 8 years and 88.9% in older children; $P = 0.013$) increase with age.

Helicobacter pylori infection occurs all over the world, and more than 50% of adults are infected with this pathogen (21). The prevalence of the infection in the pediatric population varies greatly depending on the socioeconomic status of the country concerned. In developed countries only a minority of children are colonized with *H. pylori* (11), whereas in developing countries, in general, there is a higher prevalence of colonization (5, 8), so that the majority of young adults are chronically infected (25, 28). Once established, the pathogen may reside in the gastric mucosa for years, possibly for the whole life of the host (31). In some subjects, the long-term colonization can lead to the development of severe gastroduodenal diseases such as duodenal ulcer and gastric adenocarcinoma (6, 11, 24).

Even though most *H. pylori* infections are clinically silent, the organism is associated with substantial morbidity and mortality (6, 8, 10, 11, 21, 24). The reason for such a clinically diverse outcome of infection remains uncertain but may include host and environmental factors (20) as well as differences in the prevalence or expression of bacterial virulence factors (3, 7). During the past decade, the products of several *H. pylori* genes have been used as markers for different clinical outcomes (6, 7). A cytotoxin that may damage epithelial cells by inducing the formation of vacuoles, frequently associated with duodenal ulcer or gastric adenocarcinoma etiopathogenesis (3), is encoded by *vacA*. Even though all *H. pylori* strains possess the gene, only about 40% of strains in the United States express HeLa cell vacuolating activity in vitro (33). Analysis of *vacA* from different strains has shown that the gene differs in its signal sequence, which could be s1a, s1b, s1c, or s2, and in its midregion sequence, which could be, at least, m1 or m2 (2, 35).

Although *H. pylori* is cosmopolitan, little is known about the geographic distribution of specific *H. pylori* strains, especially in developing countries (34). Also, data concerning the association between *vacA* genotypes and disease in children in both developed and developing countries are scarce, and few or no patients with a duodenal ulcer were included in the previous studies (1, 9, 16). Thus, the relationship between ulcerogenesis and specific *vacA* alleles could not be assessed. Furthermore, we are not aware of any study with a pediatric population that includes the subtyping of the s1 allele of the gene.

Classification of *vacA* mosaicism may be important for determination of whether distinct *H. pylori vacA* genotypes can be used to predict the clinical outcomes of *H. pylori* infection. It may also be important to provide an understanding of the distribution of different strains of the bacterium in the world. This study was undertaken to investigate *vacA* mosaicism in *H. pylori* strains obtained from children with and without a duodenal ulcer in Brazil.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the University Hospital, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Informed consent to participate in the study was obtained from the children and from their parents or guardians.

Study group. Sixty-five nonconsecutive *H. pylori*-positive children (mean age, 10.2 years; age range, 1 to 17 years) from the state of Minas Gerais in Brazil who

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TABLE 1. Oligonucleotide primers used for *ureA* detection and *vacA* genotyping

Region detected	Primer designation	Primer sequence	Size of PCR product (bp)	Reference
ureA	HPU1	5′-GCCAATGGTAAATTAGTT-3′	411	10
	HPU2	5′-CTCCTTAATTGTTTTTAC-3′		
s1 and s2	VA1-F	5′-ATGGAAATACAACAAACACAC-3′	259	2
	VA1-R	5′-CTGCTTGAATGCGCCAAAC-3′	286	
s1a	SS1-F[a]	5′-GTCAGCATCACACCGCAAC-3′	190	2
s1b	SS3-F[a]	5′-AGCGCCATACCGCAAGAG-3′	187	2
s1c	S1C-F[a]	5′-CTYGCTTTAGTRGGGYTA-3′	213	37
m1	VA3-F	5′-GGTCAAAATGCGGTCATGG-3′	290	2
	VA3-R	5′-CCATTGGTACCTGTAGAAAC-3′		
m2	VA4-F	5′-GGAGCCCCAGGAAACATTG-3′	352	2
	VA4-R	5′-CATAACTAGCGCCTTGCAC-3′		
m hybrid	VA7-F[b]	5′-GTAATGGTGGTTTCAACACC-3′	705	4
	VA7-R[b]	5′-TAATGAGATCTTGAGCGCT-3′		

[a] Used with primer VA1-R.
[b] Used with primers VA4F and VA4R.

underwent upper gastrointestinal endoscopy for investigation of gastric complaints were included in this study: a group of 24 patients (8 girls and 16 boys; mean age, 11.7 years; age range, 1 to 17 years) with an endoscopically documented duodenal ulcer and 41 patients (25 girls and 16 boys; mean age, 9.3 years; age range, 1 to 15 years) without a gastric or duodenal ulcer at endoscopy. No subject had received antimicrobial drugs, H2-receptor antagonists, acid pump inhibitors, nonsteroidal anti-inflammatory drugs, or any medication for at least 30 days before the endoscopy.

Biopsy fragments were obtained from the antrum and corpus of the stomach of each patient. They were maintained in sodium thioglycolate broth (Difco, Detroit, Mich.) at 4°C for up to 2 h.

***H. pylori* isolation.** Diagnosis of *H. pylori* infection was performed by culture. Two fragments from each region of the stomach were ground separately in a tissue homogenizer and were plated onto freshly prepared Belo Horizonte medium (29). The plates were incubated at 37°C in a microaerophilic atmosphere (Anaerocult C; Merck, Darmstadt, Germany) and were examined after 3 and 7 days of incubation. *H. pylori* was identified by macroscopic and microscopic morphologies, preformed urease test, and positive oxidase and catalase reactions (30), as well as by the presence of *ureA*, as described in a subsequent section. A pool of colonies obtained from each patient was transferred to a microcentrifuge tube that contained 500 μl of sterile distilled water. After centrifugation at 6,000 × *g* for 5 min, the supernatant was discarded and the pellet was stored at −80°C for DNA extraction.

DNA extraction. DNA isolation was performed as described previously by Fox et al. (14). In brief, a buffer solution that contained 8.0% sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, and 0.1% Triton X-100 was added to the bacterial pellets. Lysozyme (from chicken egg white; Calbiochem, La Jolla, Calif.) was added to a final concentration of 3 mg/ml, and the solution was incubated for 12 min at 37°C. Sodium dodecyl sulfate and RNase A (Sigma Chemical Co., St. Louis, Mo.) were added to final concentrations of 1.0% and 0.5 mg/ml, respectively, and the mixture was incubated for 1 h at 37°C. Pronase (Sigma) and proteinase K (Life Technologies, Gaithersburg, Md.) were added to final concentrations of 0.8 and 0.5 mg/ml, respectively, and samples were incubated overnight at 37°C. A 1:10 volume of 5% (wt/vol) cetyltrimethylammonium bromide (Sigma)–0.7 M NaCl solution was added, and the mixture was gently rocked and incubated at 56°C for 10 min. DNA was extracted in an equal volume of a phenol (Life Technologies)-chloroform mixture (1:1) and was precipitated overnight at −20°C in the presence of 0.3 M sodium acetate (Sigma) and 3:1 volumes of absolute ethanol. The DNA was then pelleted by centrifugation at 12,000 × *g* for 30 min and was allowed to dry in air. The pellets were suspended in sterile distilled water, and the DNA was quantified by measuring the optical density at 260 nm and used for investigation of the presence of the *ureA* and *vacA* s and m alleles.

***ureA* detection.** The genomic DNA from the bacterial samples was amplified with synthetic oligonucleotide primers (Table 1) and by the methodology described by Clayton et al. (10). An *Escherichia coli* strain (human isolate) was used as a negative control, *H. pylori* strain ATCC 49503 was used as a positive control, and distilled water was used as internal-reaction negative control.

***vacA* detection.** PCR amplification of the *vacA* signal sequences and midregions was performed by using the oligonucleotide primers (Table 1) described by Atherton et al. (2). The strains were initially characterized as type s1 or s2 and type m1 or m2. All *H. pylori* strains with the s1 allele were further characterized into s1a or s1b variants (2).

For PCR of each sample the PCR mixture contained 25 pmol of each primer, 50 to 100 ng of genomic DNA, each deoxynucleoside triphosphate (Life Technologies) at a concentration of 100 μM, and 1 U of *Taq* DNA polymerase. The reactions were performed in an automated MJ Research thermal cycler (MJ Research, Watertown, Mass.) with 35 cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min (2).

To check our results and to confirm whether seven strains which gave positive reactions for both m1 and m2 had mixed or hybrid m1 and m2 sequences in the middle region of the gene, another strategy recently described by Atherton et al. (4) was used. The primers used in this reaction are shown in Table 1. The PCR protocol was similar to that described in the previous paragraph, except that the reaction mixtures were heated first to 95°C for 90 s and then underwent 35 cycles of 30 s at 95°C, 60 s at 56°C, and 90 s at 72°C. After cycling, the products were extended for 5 min at 72°C.

H. pylori strains ATCC 49503 (s1a-m1), 435-95 (isolated at the Laboratory of Research in Bacteriology and identified as s1b-m2), and Tx30A (s2-m2) were used as controls for *vacA* allele detection. Distilled water was used as an internal-reaction negative control.

PCR product detection. All amplified PCR products were resolved in agarose gels, stained with ethidium bromide, and detected under a short-wavelength UV light source. Standards of 50 or 100 bp (Life Technologies) were used as molecular size markers.

Statistical methods. Statistical analysis was performed by the two-tailed χ^2 test with Yates' correction or by Fisher's exact test. The significance was set at a P value of <0.05.

RESULTS

More than one *H. pylori* strain was detected in 10 (15.4%) of the 65 patients studied, 5 of whom had a duodenal ulcer (Table 2). None of these patients were included in the analysis of the relationship between gastric disorders and specific *vacA* genotypes.

Among the 55 patients infected with *H. pylori* strains with nonmixed *vacA* allelic types, the s1 signal sequence was found in strains isolated from all 19 patients with a duodenal ulcer and from 21 (58.3%) children without an ulcer. The s2 allele was found only in strains isolated from patients without an ulcer ($n = 15$; 41.7%). There was a strong correlation between the presence of the s1 allele and the presence of a duodenal ulcer ($P = 0.003$; odds ratio [OR] = noncalculable).

When s1 strains were subtyped into s1a and s1b variants, only s1b strains were mostly found; one (2.5%) strain, however,

TABLE 2. *vacA* genotypes of *H. pylori* strains isolated from patients infected with multiple strains

Subject no.	Endoscopic diagnosis	*vacA* allele	
		s region	m region
1	Duodenal ulcer	s1b	m1 and m2
2	Duodenal ulcer	s1b	m1 and m2
3	Duodenal ulcer	s1b and s2	m2
4	Duodenal ulcer	s1b and s2	m1 and m2
5	Duodenal ulcer	s1b and s2	m1 and m2
6	No ulcer	s1b and s2	m2
7	No ulcer	s1b and s2	m1 and m2
8	No ulcer	s1b and s2	m1 and m2
9	No ulcer	s1b and s2	m1 and m2
10	No ulcer	s1a and s1b	m2

isolated from a patient without an ulcer, did not react with primers designed to detect s1a and s1b types.

In regard to the middle region, the m1 allele was found in 34 (61.8%) patients: 16 (84.2%) with an ulcer and 18 (50.0%) without an ulcer. The m2 *vacA* allelic type was identified in 20 (36.4%) strains: 3 (15.8%) isolated from ulcer patients and 17 (47.2%) from children without an ulcer. A significant association between the presence of the m1 allele and duodenal ulceration was observed ($P = 0.028$; OR = 5.33, 95% confidence interval [CI] 1.15 to 27.95). One strain was identified as an m1-m2 (m1,2) hybrid, as described below.

When the methodology recently proposed by Atherton et al. (4) was used to amplify m variants of *vacA*, our previous results were confirmed for all except one strain. The latter strain was previously identified as m1 and was obtained from a patient without an ulcer but was found to be an m1,2 hybrid. All samples which had previously given final PCR products for both m1 and m2 alleles were confirmed to be mixed.

Among the 55 nonmixed *H. pylori* isolates, *vacA* homologs that contained three of the possible combinations of the signal sequence and middle region types (s1-m1, s1-m2, and s2-m2) were identified. Strains with the s1-m1 genotype were found in 34 (61.8%) children: 16 (84.2%) of 19 with ulcers and 18 (50.0%) of 36 without ulcers ($P = 0.028$; OR = 5.33, 95% CI = 1.18 to 32.52). When the frequency of occurrence of the s1-m2 genotype was compared to those of the other genotypes, no difference was observed among duodenal ulcer patients and children without an ulcer ($P = 0.33$, OR = 3.19; 95% CI = 0.32 to 40.76). All strains with the s2-m2 combination (15 of 55; 27.3%) were obtained from patients without an ulcer. The s2-m1 combination was not detected.

When the relationship between *vacA* alleles and age was analyzed for the 36 children without an ulcer but infected with nonmixed *H. pylori* strains, strains of both the s1 (14.3% for patients up to 8 years of age and 85.7% for older children) and the m1 (11.1% for patients up to the age of 8 years and 88.9% for older children) allelic types were more frequently detected in children older than 8 years ($P = 0.012$; OR = 9.0, and 95% CI = 1.48 to 64.71 and $P = 0.013$, OR = 10.0, and 95% CI = 1.48 to 107.47, respectively) (Table 3).

DISCUSSION

Particular *vacA* genotypes have been considered markers of pathogenesis for individual *H. pylori* strains, at least in the United States and in European countries, since in vitro production of cytotoxin, in vivo epithelial damage, and development of peptic ulcer disease or gastric adenocarcinoma are all related to *vacA*-specific genotypes, as demonstrated for adults (3, 12, 35). However, studies of *H. pylori* virulence factors have been difficult to interpret because observations reported for one geographic region or ethnic group have not always been confirmed in different places (15, 22, 26, 27, 36, 37). In Brazil, where the prevalence of *H. pylori* infection is high, such studies are still scanty, and this paper provides the first description of *vacA* genotyping of *H. pylori* strains from a pediatric population in Brazil.

Our findings demonstrate a strong association between the presence of the s1 allele and the presence of a duodenal ulcer in children and are in agreement with those reported for adults (2, 3, 12, 34). As the most likely explanation for the high prevalence of strains with the s1 genotype in adults with peptic ulcer, it has been proposed that the elevated toxicities of s1 strains might contribute to the development of ulcerations (2, 3). Previous descriptions of the different vacuolating activities of strains with different *vacA* genotypes (2) demonstrate that, in vitro, s1 strains secrete larger amounts of cytotoxin than s2 strains, which are known to be less virulent (2, 3, 13). In fact, we found s2 *H. pylori* strains only in the gastric mucosa of children without an ulcer. Our results also demonstrate an association between the presence of the m1 allele and the presence of a duodenal ulcer in children. A similar result was also reported for adults from Japan, where the prevalence of m1 strains is high (36). According to Atherton et al. (3), m1 *H. pylori* strains are more frequently associated with gastric epithelial injury, and the midregion type of *vacA* is the most important genotypic correlate of a high level of cytotoxin activity in vitro, which could explain the association that we observed.

Conversely, an association between duodenal ulcer in children and infection with toxigenic *H. pylori* strains, identified by the detection of cytotoxin activity in vitro or by *vacA* genotyping, was not observed by others (1, 9, 19). These results may be related both to the small number of children with duodenal ulcers evaluated and to the population studied. Among investigations that used PCR to detect the presence of *vacA* in children (1, 9, 16), none characterized s1 strains into their different variants, s1a, s1b, and s1c. The prevalence of subtype s1b (97.5%) among *H. pylori* strains isolated from the children who we studied is in contrast to that described among strains obtained from adults from some Eastern and Western developed countries, where subtype s1c and s1a strains, respectively, are more frequently found (34). Type s1b strains are also more common in countries with extensive cultural and socioeconomic relationships with Brazil in past centuries, such as Portugal and Spain (34), from which *H. pylori* may have spread to the Brazilian population. For these reasons, it should be considered that differences in the frequency of particular *H. pylori* genotypes could play a role in the incidence of *H. pylori*-associated diseases.

TABLE 3. Distribution of signal sequence and midregion alleles by age group for strains from 55 children infected with *H. pylori* strains with nonmixed *vacA* allelic types

Age (yr)	No. of strains from children:								
	With ulcer ($n = 19$)				Without ulcer ($n = 36$)				
	s1	s2	m1	m2	s1	s2	m1	m2	m1,2[a]
1–8	1	0	1	0	3	9	2	10	0
9–17	18	0	15	3	18	6	16	7	1
Total	19	0	16	3	21	15	18	17	1

Among the 65 patients who we studied, a duodenal ulcer was more frequently observed in children older than 8 years of age ($P = 0.027$; OR = 6.35; 95% CI = 1.23 to 61.71). s1-m1 was the predominant genotype found in ulcer patients older than 8 years of age (15 of 18; 83.3%) when the 55 children with nonmixed *H. pylori* infection were considered (Table 3). When the group of children without duodenal ulcer was analyzed, we demonstrated for the first time that the frequencies of occurrence of strains with the s1 allele and of midregion type m1 increase with age, with such strains being more frequently found in children older than 8 years of age. In regard to the s1 allele, Alarcon et al. (1) also observed an increase in the prevalence of the s1 allele, with strains with the s1 allele found in about 30% of infected children (ages, 3 to 20 years) and 70.0% of infected adults (ages, 21 to 60 years).

Klein et al. (18) have shown that the initial infection with *H. pylori* could be less established in the gastric mucosa of young children, who acquire, lose, and reacquire the infection. We hypothesize that infants may acquire a multiplicity of different *H. pylori* strains and that the prevalence of strains of a particular genotype, such as s1-m1, could be a consequence of bacterial factors that facilitate colonization with some kinds of strains. Further studies on the competition or interaction among *H. pylori* isolates are still needed to improve our understanding of this bacterium-gastric mucosa relationship.

Many studies have demonstrated that adult patients may carry more than one *H. pylori* strain (17, 32, 34). Infections with multiple strains have been reported in adults from the Iberian peninsula, Mexico, and Central and South America, infections with multiple strains may be associated with the high prevalence of *H. pylori* infection in these populations (23, 34). In Brazil, where the prevalence of *H. pylori* infection reaches rates of about 80% in symptomatic and asymptomatic children older than 12 years of age (8, 25), infection with more than one *H. pylori* strain was found in 15.4% of patients, a frequency that is lower than that reported for adults from Portugal (44.5%), Mexico (65.0%), and other countries in South America (23.8%). Certainly, the risk of infection with multiple strains is not related to age only but is related to several other host characteristics, as well as bacterial and environmental factors, such as living conditions and habits of hygiene (25).

Even though some studies have demonstrated that the s2-m1 combination, although rare, can be found (12, 23), we did not find strains of this genotype among the strains that we studied.

Although mutation could explain the finding of combinations of signal sequence and midregion alleles in *vacA*, frequent recombination between strains could also generate high levels of diversity in *H. pylori*. It could explain, for example, the finding of an m1,2 hybrid and also of a strain with an untypeable s1 allele (negative reactions for s1a and s1b). A new s1 subtype, named s1c and considered a specific recombinant of s1a and s1b, was recently reported by van Doorn et al. (35). We tested one strain that was positive for both s1a and s1b variants and the s1 untypeable strain with primers specific for s1c, but we obtained negative results. Thus, these strains could be different ones or the isolate positive for both s1a and s1b could have a mixture of alleles. Further studies are being performed to make these findings clearer.

We conclude that, different from the results reported for adults in the United States and other Western developed countries, *vacA* s1b variants are the most prevalent types of *H. pylori* strains isolated from Brazilian children. The strong association between *vacA* s1 strains and peptic ulcer is complemented by the equally important finding that the *vacA* s2 allele occurred only in strains isolated from patients without an ulcer. These observations confirm the potential importance of *vacA* genotyping for assessment of whether *H. pylori* strains with specific alleles are associated with particular diseases. Thus, *vacA* detection might be helpful for determination of which children are at the highest risk for severe clinical outcomes, such as duodenal ulcer or gastric carcinoma, and, eventually, to define strategies for the treatment or prevention of *H. pylori* infection.

ACKNOWLEDGMENTS

This work was supported by grants from FAPEMIG and CNPq-Brazil. V.R.G. is taking her master's degree at the Department of Microbiology, Institute of Biology, Federal University of Minas Gerais, and A.A.R.A. is taking his doctoral degree at the Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo; they were supported by CAPES-Brazil scholarships.

We acknowledge the generosity of E. Kalapothakis for providing the *Taq* DNA polymerase used in this study.

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