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iceA Genotypes of *Helicobacter pylori* Strains Isolated from Brazilian Children and Adults

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Data concerning the geographic distribution of *iceA* alleles are scarce, and information on the association of the gene with the disease is rare and still controversial. Furthermore, no such study has been developed in Brazil, where duodenal ulcer and gastric adenocarcinoma are very common. We investigated, by PCR, the frequency of *iceA* alleles and *cagA* status in *Helicobacter pylori* strains isolated from 142 patients (62 children and 80 adults; 66 female; mean age, 30.0 years; age range, 3 to 78 years) with gastritis, duodenal ulcer, or gastric adenocarcinoma. *iceA* was identified in bacterium samples obtained from all patients. Eleven (7.7%) of them were infected with multiple strains. Among the patients with nonmixed infection, *iceA2* allele was detected in 118 (90.1%). *iceA2* allele was associated with ulcer ($P = 0.02$) and with carcinoma ($P = 0.001$). *iceA2* amplicons of 229, 334, or 549 bp were detected, but none of them was associated with the patient's disorder. *iceA2* strains were more frequent in patients older than 7 years ($P = 0.001$). The gene was also more frequent in strains obtained from males ($P = 0.02$). *cagA* was more common in strains obtained from carcinoma ($P = 0.0008$) and ulcer patients ($P < 0.006$). *cagA*-positive strains were more frequent in children older than 7 years ($P < 0.003$). No association between *cagA* status and sex was found ($P = 0.28$). In conclusion, we think *iceA* should not be used as a reliable marker for predicting the clinical outcome of *H. pylori* infection.

Helicobacter pylori is a microaerophilic organism that is carried by more than half of all persons worldwide. It is now accepted as the most important cause of gastritis in human beings, as an essential factor in the etiopathogenesis of peptic ulcer disease (an important cause of morbidity), and as a risk factor for gastric cancer, a very common malignancy all over the world (42). The organism is one of the most genetically diverse bacteria, as demonstrated by DNA fingerprinting and other genotyping and DNA sequencing techniques (1, 36). There are also evidences of important geographic differences among *H. pylori* strains (15, 22, 39, 42). For example, from 50 to ca. 70% of the *H. pylori* strains obtained from patients from United States and Europe are *cagA* positive, a genotype that, at least in these places, appears to be related to the development of peptic ulcer disease and gastric adenocarcinoma (19). Otherwise, almost all strains isolated from East Asian patients are *cagA* positive, despite the disease status (14). Diverse geographic distribution of s alleles of *vacA*, which encodes a vacuolating cytotoxin, has also been observed. It has been reported that s1a variant is more common in American and Northern European strains, that s1b is more frequent in Latin American, Portuguese, and Spanish strains, and that s1c is almost exclusively found in patients from Eastern Asia (39). With regard to *iceA*, a gene induced by contact with gastric epithelial cells, little is known about its two distinct genotypes relationship to disease and, also, about their geographic distribution. It was reported that the *iceA1* allele is related to the development of peptic ulcer disease in the United States (28) and The Netherlands (39), a result that has not been confirmed in other countries, such as Japan (15, 42), Korea (42), Colombia (42), and India (22). Recently, Peek et al. (27) detected higher acute inflammatory scores in the gastric mucosa of patients colonized with *iceA1* strains, a result that could explain the association between this genotype and ulcer disease previously reported by Peek et al. (28) and van Doorn et al. (39).

Similar studies are still scanty in South American countries, where both peptic ulcer disease and gastric adenocarcinoma are very common, and we are not aware of any investigation, in Brazil, of *iceA* genotype distribution among *H. pylori* strains isolated from patients presenting with the different clinical situations associated with the bacterium. Furthermore, no such study has yet been performed on strains obtained from children. For these reasons, we undertook this study in order to investigate, in Brazilian children and adults, the distribution of *iceA* in *H. pylori* strains isolated from gastritis, duodenal ulcer, and gastric carcinoma patients. Studies on the diversity of *H. pylori* genes may be important not only for predicting the clinical outcomes of the infection but also to understand better the worldwide distribution of the microorganism and its evolutionary origins. Also, we aimed to investigate the frequency of infection by more than one *H. pylori* genotype, an aspect still

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little explored, especially in developing countries, where the high prevalence of the diseases associated with the bacterium could contribute to a high frequency of infection with multiple strains.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Federal University of São Paulo (UNIFESP), Brazil. Consent to participate in the study was obtained from all patients; in the case of children, consent was also obtained from their parents or guardians.

Patients and gastric specimen collection. A total of 142 patients, including 80 adults (32 female and 48 male; mean age, 52.4 years; median, 51.0 years; age range, 18 to 78 years) and 62 children (34 girls and 28 boys; mean age, 10.2 years; median, 10.5 years; age range, 3 to 17 years), most of them from the lower socioeconomic stratus, from the state of Minas Gerais in Brazil, were included in this study. All of them presented with gastric complaints and had culture proven *H. pylori* infection.

In the group of adult patients, 44 were endoscoped at the Dr. Celso Affonso de Oliveira Endoscopy Service or at the Gastrointestinal Endoscopy Service of the University Hospital–UFMG, when biopsy fragments from the gastric antrum and corpus were taken for microbiology and for histology. Among them, 24 (10 female and 14 male; mean age, 40.1 years; median, 39.0 years; age range, 18 to 61 years) had duodenal ulcer endoscopically proven, and 20 (10 female and 10 male; mean age, 55.1 years; median, 57.0 years; age range, 20 to 78 years) did not have gastric or duodenal lesions at endoscopy. After each procedure, endoscopes were cleansed with detergent, disinfected by immersion in 2.0% glutaraldehyde for 20 min, and then rinsed well in water.

The remaining 36 adult patients (12 female and 24 male; mean age, 59.1 years; median, 58.0 years; age range 32 to 77 years) were selected, if the diagnosis was histologically confirmed, among those who were scheduled for gastric surgery to remove distal gastric adenocarcinoma at the Surgical Clinic of University Hospital–UFMG, Luxemburgo Hospital, Santa Casa Hospital or Mário Penna Hospital of Oncology. Specimens of the stomach of these patients were also taken from the antrum and body regions, at least 2 cm away from the tumors, for microbiology. Fragments from the tumor were also removed for histology. All materials employed for sampling the stomach were cleansed in a way similar to that described for endoscopes, except that they were autoclaved when possible.

The 62 pediatric patients were submitted to upper endoscopy at the Pediatric Gastrointestinal Endoscopy Service of the University Hospital–UFMG. Among them, 23 (10 girls and 13 boys; mean age, 11.1 years; median, 12.0 years; age range, 3 to 17 years) had duodenal ulcer endoscopically proven and 39 (24 girls and 15 boys; mean age, 9.4 years; median, 10.0 years; age range, 3 to 15 years) did not have gastric or duodenal alterations at endoscopy. Endoscopy and biopsy collection were performed as described previously for adults.

All gastric fragments obtained for microbiology were immediately placed in a tube containing sodium thioglycolate broth (Difco, Detroit, Mich.), transported in an ice bath, and processed within 2 h. Fragments from the antral and body mucosa of the stomach and from the tumor were fixed in formalin for histological examination.

Histology. Fragments fixed in formalin were dehydrated in an alcohol-xylene series and embedded in paraffin wax. Adjacent sections of 5 μm were obtained, placed on slides, stained with hematoxylin and eosin, and examined under a light microscope to confirm the presence of gastritis and gastric adenocarcinoma. All *H. pylori*-positive patients had some degree of gastritis ranging from mild to severe. The diagnosis of gastric adenocarcinoma was confirmed in all suspected cases.

***H. pylori* isolation.** Diagnosis of *H. pylori* infection was performed by culture as previously described (29, 30). Two kinds of *H. pylori* samples were obtained: a pool of colonies or isolated clones.

Pools of colonies were obtained from 90 patients, 49 children (18 with duodenal ulcer and 31 without ulcer) and 41 adults (21 with gastric carcinoma, 11 with duodenal ulcer, and 9 without ulcer or gastric carcinoma). *H. pylori* colonies grown onto Belo Horizonte medium were identified as described previously (31, 32) and were removed from the plates with a cotton swab and 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 pellets were maintained at −80°C for DNA extraction. Other portion of the growth was maintained at −80°C as previously described (29).

To obtain isolated clones, 5 to 25 colonies from each region of the stomach, presumptively identified as *H. pylori* by macroscopic morphology, were individually transferred to sheep blood agar plates which were incubated as described by Queiroz et al. (29). After 3 to 7 days of incubation, *H. pylori* was identified as described previously (31, 32). Part of the growth from each blood agar plate was removed and processed as described above for DNA isolation. The other part of the growth was maintained at −80°C (29). Isolated clones were obtained from 52 patients, 13 children (5 with duodenal ulcer and 8 without ulcer), and 39 adults (15 with gastric carcinoma, 13 with duodenal ulcer, and 11 without ulcer or gastric carcinoma). A total of 682 clones was obtained: 336 from the antrum (mean number, 9.3, from 1 to 25) and 346 from the corpus (mean number, 9.1, from 1 to 31).

The identification of *H. pylori* samples, both the pool of colonies and the isolated clones, was later confirmed by the presence of *ureA*, a gene that is present in all *H. pylori* strains (9), as described below.

DNA extraction. All bacterial pellets were employed for DNA isolation by using a method previously described by Fox et al. (10). DNA was quantified by measuring the optical density at 260 nm and used for investigation of the presence of *ureA, iceA* alleles, and *cagA*.

***ureA* detection.** Genomic DNA from all samples was used for detection of *ureA*. Only samples which were *ureA* positive were employed in this study. Synthetic oligonucleotide primers and methodology reported by Clayton et al. (9) were used for the amplification of a 411-bp fragment of *ureA*, in an M. J. Research thermal cycler (M. J. Research, Watertown, Mass.). An *Escherichia coli* (human isolate) was used as negative control, *H. pylori* ATCC 49503 was used as positive control, and distilled water was used as internal-reaction negative control.

***iceA* identification.** PCR amplification was performed by using the synthetic oligonucleotide primers and the methodology described by van Doorn et al. (39), though slightly modified. According to these authors, positive *iceA1* reaction generates a fragment of 247 bp and the *iceA2* allele can be detected as 124-, 229-, or 334-bp fragments, depending on the number of 105-bp repeated insertions.

In summary, allele-specific PCR assays were used to detect *iceA1* and *iceA2* (39). For each sample the PCR mixture (20 μl, final volume) contained approximately 50 ng of genomic DNA, 25 pmol of each primer, each deoxyribonucleoside triphosphate (Life Technologies) at a concentration of 200 μM, and 1 U of *Taq* DNA polymerase. The reaction mixtures were cycled in an automated M. J. Research thermal cycler under the following conditions: 9 min of preincubation at 94°C followed by a touchdown program which consisted of 30 s at 95°C; 45 s at the annealing temperature, which started at 65°C and decreased 1°C each two cycles up to 50°C, at which temperature 10 cycles were carried out; and 45 s at 72°C. The final cycle was an extension step at 72°C for 5 min. *H. pylori* ATCC 49503 was used as a positive control for *iceA1, H. pylori* ATCC 43504 was used as positive control for the *iceA2* allele (334-bp amplicon), an *Escherichia coli* (human isolate) was used as a negative control for both reactions, and distilled water was used as the internal-reaction negative control.

***cagA* detection.** PCR amplification of *cagA* was performed with synthetic oligonucleotide primers and by using the methodology reported by Peek et al. (26). *H. pylori* Tx30A was used as a negative control, *H. pylori* ATCC 49503 was employed as a positive control, and distilled water was used as an internal-reaction negative control. Positive reactions yielded a 349-bp final product.

PCR product detection. *ureA, iceA*, and *cagA* amplimers were resolved in 5% polyacrylamide gels stained with silver nitrate as described by Bassam et al. (4) and modified by Rocha (33). Standards of 50 and 100 bp (Life Technologies) were employed as molecular size markers.

Statistical analysis. Data were analyzed statistically by the two-tailed χ^2 test with Yates' correction or by Fisher's exact test. The significance level was set at P value of <0.05.

RESULTS

Among the 142 patients studied, 59 had gastritis only, 47 had duodenal ulcer, and 36 had gastric adenocarcinoma.

The distribution of *cagA* status among children and adults is shown in Table 1. The gene was more frequently found in strains obtained from duodenal ulcer ($P < 0.006$) and gastric carcinoma ($P < 0.0008$) patients.

It was also more frequent in *H. pylori* strains isolated from patients older than 7 years ($P = 0.003$). However, when ulcer patients were considered separately, no statistical difference was observed in the distribution of the gene according to age (younger [0 of 1] and older [39 of 46] than 7 years) ($P = 0.2$). Also, no difference was found in *cagA* status between males

TABLE 1. *cagA* status of *H. pylori* strains isolated from children and adults with gastritis only, duodenal ulcer, and gastric carcinoma

cagA group	No. of strains (%) isolated from:				
	Children with:		Adults with:		
	Ulcer	Gastritis	Ulcer	Gastritis	Carcinoma
Positive	18 (78.3)	22 (56.4)	21 (87.5)	11 (55.0)	34 (94.4)
Negative	5 (21.7)	17 (43.6)	3 (12.5)	9 (45.0)	2 (5.6)
Total	23	39	24	20	36

and females ($P = 0.28$), both in children ($P = 0.07$) and in adults ($P = 0.51$).

No mixed infection with *cagA*-positive and *cagA*-negative strains was detected among the 52 patients from whom we obtained *H. pylori* isolated clones.

iceA was identified in bacterium samples obtained from all the patients we studied (Table 2). Among them, 131 (92.3%) patients were colonized with bacterium harboring a single *iceA* genotype (nonmixed infection). The other 11 (7.7%) patients, who were infected with *H. pylori* strains showing more than one *iceA* genotype (mixed infection) (Table 2), were not included in the analysis of the relationship between gastric disorders and specific *iceA* genotypes.

Among patients with a nonmixed infection, *iceA1* was detected in *H. pylori* samples isolated from 13 (9.9%): 11 of 55 (20.0%) with gastritis, 1 of 43 (2.3%) with duodenal ulcer, and 1 of 33 (3.0%) with gastric adenocarcinoma (Table 2).

The *iceA2* allele was detected in samples obtained from 118 (90.1%) patients with nonmixed infection: 44 of 55 (80.0%) with gastritis, 42 of 43 (97.7%) with duodenal ulcer, and 32 of 33 (97.0%) with gastric adenocarcinoma (Table 2). A significant association between *iceA2* and duodenal ulcer was observed when children and adults were considered together ($P < 0.02$) and when the children's group was analyzed separately ($P = 0.04$). An association between *iceA2* allele and gastric carcinoma was found ($P = 0.03$) when all patients with gastritis were considered in the analysis. The distribution of *iceA* genotypes was similar in children and adults, whether carcinoma patients were included ($P = 0.30$) or not ($P = 0.75$) (Table 2).

When all patients with nonmixed infection were considered together, *iceA2* was more frequently observed in patients older than 7 years ($P = 0.001$). The allele was also more frequently found in *H. pylori* strains obtained from patients older than 7 years when gastritis only patients were considered ($P = 0.0009$) and when duodenal ulcer patients were analyzed separately ($P = 0.02$). Considering the whole group of patients with nonmixed infection, an *iceA2* genotype was more frequently found in males ($P = 0.02$).

All *iceA2* isolates could be divided in three types according to PCR product size: 229, 334, or 549 bp (Table 2). No association was observed between the size of *iceA2* amplicon and the patient's disorder, both in adults and children.

Eleven patients (7.7%) were colonized with *H. pylori* strains harboring different *iceA* genotypes (mixed infection). Interestingly, one patient was colonized with a strain presenting an *iceA2* amplified product of 229 bp in the antrum and with other strain showing a PCR product of 334 bp in the gastric body. No difference in the distribution of mixed infection was observed when adults (7 of 11 [63.6%]) were compared to children (4 of 11 [36.4%]) ($P = 0.76$) (Table 2). Also, a statistical difference in the distribution of single and mixed infection was not observed when patients with gastritis were compared to those with duodenal ulcer ($P = 1.00$) or with gastric carcinoma ($P = 0.71$). A mixed infection was more frequently seen in males ($P = 0.03$).

An association between *iceA2* and *cagA* was observed when all patients with a nonmixed infection were considered together ($P = 0.0009$).

DISCUSSION

H. pylori infection is now recognized as an important risk factor for peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (12, 13, 25, 34). Despite being one of the most common bacterial infections all over the world, most infected individuals are asymptomatic throughout their lives; only a minority of them develop such severe diseases. Although host and environmental factors could be important, such a discrepancy may be related to the great genetic diversity of the species. In fact, infection with certain *H. pylori* genotypes, among them *cagA*-positive and *vacA* s1 variants, seems to be associated with progression to more severe diseases, such as peptic ulceration and gastric carcinoma (3, 5, 6, 17, 18, 20, 39), at least in Western countries.

A novel gene, designated *iceA* (from "inducible by contact with epithelium" of the gastric mucosa), has recently been reported by Peek et al. (28). It was demonstrated that one of its alleles, *iceA1*, is significantly associated with peptic ulcer disease in the United States (28) and Holland (39). According to Peek et al. (27), *iceA1* expression is associated with a higher activity of the gastric inflammation that is present in virtually all *H. pylori*-infected individuals, a condition that increases the risk for developing ulcer disease and gastric carcinoma. However, as demonstrated previously for *cagA* and *vacA* variants (14, 24, 40) in East Asian populations, *iceA1* genotype was not

TABLE 2. Distribution of *iceA* genotypes in *H. pylori* strains isolated from children and adults with gastritis, duodenal ulcer, and gastric carcinoma

Genotype	No. of strains isolated from:					Total no. of strains
	Children with:		Adults with:			
	Gastritis	Ulcer	Gastritis	Ulcer	Cancer	
Single-strain infection	38	20	17	23	33	131
iceA1	8		3	1	1	13
iceA2	30	20	14	22	32	118
229 bp	15	9	10	15	16	65
334 bp	14	11	4	7	16	52
549 bp	1					1
Multiple-strain infection	1	3	3	1	3	11
iceA1 plus *iceA2* (229 bp)				1	1	2
iceA1 plus *iceA2* (334 bp)					1	1
iceA1 plus *iceA2* (229 plus 334 bp)			2			2
iceA2 (229 plus 334 bp)	1	3	1		1	6
Total	39	23	20	24	36	142

found to be correlated with the presence of peptic ulceration in Japan (15, 42), Korea, Colombia, the United States (Texas) (42), and India (22).

The present study describes, for the first time, an analysis of *iceA* genotypes in *H. pylori* strains isolated from children and adults from Brazil. The number of reports on this subject is scanty, and the results on the association of the gene with specific gastrointestinal disorders are still inconclusive. Also, this study confirms several findings reported for *cagA* in Western countries, such as the correlation between the presence of the gene and peptic ulceration and gastric adenocarcinoma.

All of our strains could have *iceA* typed by a method similar to that reported by van Doorn et al. (39).

In contrast to the results reported for *H. pylori* strains isolated from Dutch (39), Japanese (15, 42), and Korean (42) patients, *iceA2* was found to be the most frequent genotype detected in our population (90.1% among patients with a nonmixed infection).

In regard to the association of *iceA* alleles with gastroduodenal diseases, our results are distinct from those reported by Peek et al. (28) and van Doorn et al. (39), who found a significant association between the *iceA1* allele and peptic ulceration in American and Dutch patients, respectively. We found that the *iceA2* genotype was most frequently found in *H. pylori* strains isolated from patients with duodenal ulcer and with gastric carcinoma. Similar results have been recently reported by Yamaoka et al. (42) for strains obtained from patients from Colombia and from Texas and by Mukhopadhyay et al. (22) for Indian strains. If the activity of the inflammatory response, which increases the risk for peptic ulcer disease and gastric carcinoma, is higher in the gastric mucosa of patients infected with strains that express *iceA1* (27), and if most peptic ulcer and gastric carcinoma patients in Brazil and in the countries cited above harbors *iceA2 H. pylori* strains, it is probable that host and/or other bacterial factors could be more important than *iceA1* transcription in vivo. Are *iceA* and other gene expressions coregulated? We could hypothesize that *iceA* genotypes are not reliable markers of peptic ulcer disease and gastric carcinoma and that the association observed, in some places with *iceA1* and in others with *iceA2*, could be related to other noncontrolled experimental variables. In fact, it is difficult to admit that *iceA2*, a gene that is considered as a protective factor in some regions and that is associated with more severe diseases in other places, could be considered a molecular marker of more virulent *H. pylori* strains. Further studies, including the investigation of *H. pylori iceA* distribution and expression in different populations, are needed to help us understand better the association between the gene and the disease.

Similarly to the *vacA* s1 variants, maybe *iceA* genotyping could be used as a marker of *H. pylori* strain geographic distribution and evolutionary origin. We should also consider that host-determined factors, such as the expression of specific receptors or antagonist factors, are certainly involved in the whole process and, consequently, could be determinant of the global distribution of organism strains and eventually of the clinical outcome of the infection. Probably, factors that explain why s1c variant of *vacA* is almost exclusively found in Asian patients, and why s1a and s1b variants are found in people living in the same region (38) and, sometimes, in the same individual (11) could help us understand why *iceA2* is the most prevalent genotype in some regions and why *iceA1* was not found to be related to more severe *H. pylori*-associated diseases in Brazil and in other countries.

The distribution of *iceA2* 229- and 334-bp amplicons was almost the same in the strains we studied. Only one sample yielded an amplicon of aproximately 540 bp, a nondescribed one, probably containing four 105-bp in-frame duplications.

Both *cagA* positivity and the *iceA2* genotype were more frequently found in patients older than 7 years. Correlation of the frequency of some *vacA* alleles and *cagA* positivity with age was recently reported by Alarcon et al. (2), Gusmão et al. (11), and Queiroz et al. (29). The finding that colonization with *iceA2* strains is age correlated, being more common in older patients, has never been reported before. It could be related to age-dependent expression of host factors such as observed by Çelik et al. (8) for Le^b receptors.

iceA2 was more frequently found in males. This finding was only observed when all patients with a nonmixed infection were considered, but not when gastritis only, duodenal ulcer, and gastric carcinoma patients were analyzed separately. It is probably related to the higher number of males in the gastric carcinoma group (69.7%) and not to, for example, hormonal differences between sexes.

Infections with multiple *H. pylori* strains have been reported by many authors (16, 21, 23, 35, 38). Mixed infection was detected in 7.7% patients and was not related to age and disease. The frequency we found is lower than that reported by van Doorn et al. (39) for Dutch patients (14.9%). Taking into account that the higher the prevalence of the infection the higher the risk of becoming infected with different strains, we should expect a higher prevalence of multiple infection in our patients, since the prevalence of *H. pylori* infection in some Brazilian regions reaches rates of 80% in symptomatic and asymptomatic children older than 12 years of age (7). However, if we consider that *iceA2* is observed in more than 90% of our patients, the risk of becoming infected with both *iceA1* and *iceA2* is low. Furthermore, the prevalence of mixed infection that we observed could be underestimated because of the low number of possible combinations between *iceA1* genotype and *iceA2* amplicons.

We found a significant association between *cagA* positivity and the *iceA2* allele. In contrast, van Doorn et al. (39) did not find any association between these genotypes. Our result probably reflects the high prevalence of both *cagA* and the *iceA2* allele in *H. pylori* strains isolated from our population.

In conclusion, we were not able to confirm the reports of van Doorn et al. (39) and Peek et al. (27, 28), who found the *iceA1* genotype to be associated with peptic ulcer disease. We think that the statistical association we found between the *iceA2* genotype and the different *H. pylori*-associated disorders could reflect the high prevalence of this genotype in *H. pylori* strains in the population we studied. We think that the gene should not be used as a reliable marker, at least in a Brazilian setting, for predicting the clinical outcome of *H. pylori* infection. Interpretation of this kind of association should be cautious and must consider genotypic and phenotypic organism characteristics and related disease associations in different geographic regions and ethnic groups.

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