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Consensus and Variable Region PCR Analysis of *Helicobacter pylori* 3′ Region of *cagA* Gene in Isolates from Individuals with or without Peptic Ulcer

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The clinical outcome of *Helicobacter pylori* infection may be associated with the *cagA* bacterial genotype. To investigate the *cagA* status of *H. pylori*-infected patients and the relationship between *cagA* and peptic ulcer disease, gastric biopsy specimens from 103 Caucasian patients in Brazil were analyzed by PCR. Since allelic variation in *cagA* exists and distinct *H. pylori* subgenotypes may circulate in different regions, PCR using primers for a variable 3′ region of the *cagA* gene according to a Japanese methodology and for a consensus *cagA* 3′ region used in Western methods was used for *cagA* detection. *cagA* was present in 53 (71%) of 75 *H. pylori*-positive cases when analyzed by the consensus region method and was associated with duodenal ulcer disease ($P = 0.02$), but not with gastric ulcer ($P = 0.26$), when compared to patients with duodenitis or gastritis. The variable region PCR method was able to detect 43 (57%) *cagA*-positive cases within the same group of *H. pylori*-positive patients and showed three subtypes of *cagA* (A, B/D, and C) that were not associated with clinical outcome. However, in 8 (18%) of the cases, more than one subtype was present, and an association between patients with multiple subtypes and disease outcome was observed when compared to patients with isolated subtypes ($P = 0.048$). *cagA* was a marker of *H. pylori* strains for duodenal ulcer disease in our population, and in spite of the differences in the 3′ region of the *cagA* gene, the Japanese methodology was able to detect the *cagA* status in most cases. The presence of multiple subgenotypes of *cagA* was associated with gastric ulcer.

Helicobacter pylori infection affects more than half of the world population, but symptomatic disease appears in only a minority of infected subjects (4, 16, 24, 32). The clinical spectrum of this infection ranges from asymptomatic gastritis to peptic ulcer and gastric cancer (19, 29). It is unclear why only a minority of the subjects infected by *H. pylori* develop peptic ulcer and gastric cancer while most people harboring this bacterium remain asymptomatic or have only chronic gastritis.

The causes of the different outcomes of *H. pylori* infection may include host and environmental factors and differences in prevalence or expression of bacterial virulence factors (2, 17, 45). Several genes have been identified that may play a role in the pathogenicity of the bacterium (8, 28, 40). The *cagA, vacA*, and *iceA* genes have been used as molecular markers of *H. pylori* virulence (44). The *cagA* gene (cytotoxin-associated gene) is considered a marker for the presence of one pathogenicity island of about 40 kbp (5). The structure of the gene reveals a 5′ highly conserved region and a variable 3′ region, in which the presence of a variable number of repeat sequences results in a protein with a molecular mass of 120 to 140 kDa (7, 40, 48). CagA-producing *H. pylori* strains induce inflammatory cytokines, especially interleukin-8 (9, 10). The presence of *cagA* is associated with duodenal ulcer, gastric mucosal atrophy, and gastric cancer (12, 38, 44), and the majority of *H. pylori* strains can be classified into one of two groups (*cag* positive or *cag* negative), based on the possession of the *cagA* gene and the associated genes in the *cag* pathogenicity island (37). The gene *vacA* encodes the vacuolating cytotoxin that is involved in epithelial cell injury (8). The production of the cytotoxin is associated with pathogenicity and depends on the bacterium genotype (14). The gene is present in all *H. pylori* strains and comprises two variable parts. The s region (encoding the signal peptide) exists as an s1 or s2 allele, and s1 can be further subdivided into subtype s1a, s1b, or s1c. The m region (middle) occurs as an m1 or m2 allele (43). *vacA* s1m1 strains produce a large amount of toxin, s1m2 strains produce moderate amounts, and s2m2 strains produce very little or no toxin (1). The presence of cytotoxic activity has been suggested as a marker for strains with enhanced virulence acting either directly via cytotoxicity or indirectly via an increased inflammatory and immune response (1). Since the vast majority of *vacA* s1 strains are also *cagA* positive, the two markers are closely related (1, 35, 42). The *iceA* gene (induced by contact with epithelium) has two main allelic variants, designated *iceA1* and *iceA2*. The expression of *iceA1* is up-regulated on contact between *H. pylori* and human epithelial cells and may be associated with peptic ulcer disease (28). As suggested by van Doorn et al. (44), strains typed as *vacA* s1 *cagA*$^+$ *iceA1* can be considered the most pathogenic and are found predominantly in patients with ulcer disease. In contrast, strains typed as *vacA* s2m2 *iceA2* (*cagA* negative) appear to be the least pathogenic and do not occur in peptic ulcer patients (44).

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The proportion of *H. pylori* isolates which are *cagA* positive varies from one geographic region to another (13). Studies from Japan (21, 37), Korea (26), and China (25) have shown that more than 90% of *H. pylori* strains are *cagA* positive, while in the United States (27), Canada (30), and Europe (18, 35, 43) these percentages are lower. Therefore, *cagA* cannot be used as a marker for the presence of peptic ulcer disease in those regions where the prevalence of *cagA*-positive *H. pylori* strains is uniformly high. Since allelic variation in *cagA* exists and distinct *H. pylori* subgenotypes may circulate in different regions (25), differences in *cagA* subgenotype may provide a marker for differences in virulence among *cagA*-positive *H. pylori* strains (48). Some studies have demonstrated that PCR using primers designed from Western *H. pylori* isolates were ineffective in the detection of Eastern Asia *H. pylori* strains, and vice versa (25, 41, 47). The aim of this study was to verify the *cagA* genotype of *H. pylori* isolates, using primers designed according to the 3′ variable region of Japanese isolates (48) and primers designed according to a consensus region (3), and to determine the prevalence of the *cagA* genotype and the relationship between *cagA* subgenotypes and peptic ulcer disease in Caucasian patients from southern Brazil.

MATERIALS AND METHODS

Patients and endoscopy method. A total of 103 Caucasian patients (48 male and 55 female; mean age = 51.9 ± 15.4 years; range, 15 to 81 years) with dyspeptic symptoms underwent esophagogastroduodenoscopy in a private clinic in Porto Alegre, Rio Grande do Sul, Brazil. Endoscopies were performed by one of the investigators (J. C. P.-L.), and the diagnosis was based on visual examination of the stomach and the duodenum. Twenty-six patients presented with duodenal ulcer, 31 with gastric ulcer, 21 with duodenitis, and 21 with erosive gastritis, and 4 were considered to have a normal endoscopic examination. The endoscopic diagnosis was grouped into three main categories: (i) nonulcer patients, for those patients presenting only gastritis or duodenitis ($n = 42$); (ii) gastric ulcer patients ($n = 31$); and (iii) duodenal ulcer patients ($n = 26$). The four patients who were considered to have a normal endoscopy were excluded from analysis. During endoscopic examination, four biopsy specimens were obtained from the antrum. The specimens were examined for the presence of *H. pylori* by histopathology (two specimens), urease test, and PCR. Exclusion criteria were the use of nonsteroidal anti-inflammatory drugs (NSAIDs) or recent (within the previous month) intake of antibiotics. The patients were asked about documented history of peptic ulcer disease and previous anti-*Helicobacter* therapy. Informed consent was obtained from all participants. The Ethics Committee of our institutions granted ethical approval.

After each endoscopic examination, endoscopes were cleansed with combined manual and mechanic cleaning procedures for washing and disinfection. A new autoclaved biopsy forceps was used for each patient. PCR for detection of *H. pylori* was randomly performed in the last cleaning-water wash as a contamination control. All wash samples were negative for *H. pylori* by PCR, even when the analysis was performed after *H. pylori*-positive patients (data not shown).

Histological analysis. The biopsy specimens for each patient were fixed in Formol solution, dehydrated, and embedded in paraffin wax. Sections were cut and stained with hematoxylin and eosin to grade the severity of gastritis and with Giemsa stain to detect *H. pylori*. The classification and grading of gastritis were made in accordance with the Sydney system (33).

Urease test. The biopsy specimens were introduced with a sterile needle into solid urea agar and incubated at room temperature. Results were recorded up to 24 h after inoculation. If urease enzyme of *H. pylori* is present in the specimen, the resulting degradation of urea causes the pH to rise and the color of the gel turns from yellow to pink.

Preparation of DNA for PCR amplification. The biopsy specimens for DNA analysis were placed in 0.85% NaCl, and the DNA was isolated directly from the biopsy specimens using the QIAamp tissue kit (Qiagen Inc., Santa Clarita, Calif.) according to the manufacturer's instructions. The DNA was eluted in 200 μl of elution buffer, and 10 μl was used for the amplification reaction.

***H. pylori* detection by PCR.** PCR primer sets specific for the *H. pylori* 16S rRNA (15) and *ureA* (6) genes were used. The primer pair HPU18 and HPU54 was designed by Clayton et al. (6) and optimized by Furuta et al. (15). The primers HPU18N (5′-CCCATTTGACTCAATGCGATG-3′) and HPU54N (5′-TGGGATTAGCGAGTATGTCGG-3′) amplify a 132-bp product from the 16S rRNA gene. To confirm *H. pylori* identification, another PCR test, with primers annealing to the *H. pylori* urease structural gene subunit A (*ureA*) (6), was performed. The primers employed, UREA1 (5′-GCCAATGGTAAATTAGTT-3′) and UREA2 (5′-CTCCTTAATTGTTTTTAC-3′) amplify a 394-bp product from the *ureA* gene. PCRs were performed in a volume of 50 μl containing 20 nmol of Tris-HCl (pH 8.4) per liter, 50 mmol of KCl per liter, 2.5 (for 16S rRNA) and 1.5 (for *ureA*) mmol of $MgCl_2$ per liter, 200 μmol of deoxynucleoside triphosphate per liter, 2.5 U of *Taq* DNA polymerase (GIBCO BRL, Grand Island, N.Y.), and 25 pmol of both forward and reverse primers. PCR was performed in a PTC-1196 DNA thermocycler (MJ Research Inc., Watertown, Mass.) under the following conditions: 5 min of preincubation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C (for 16S rRNA) or 45°C (for *ureA*), and 1 min at 72°C. Final extension was performed for 7 min at 72°C. The 16S rRNA and *ureA* amplimers (20-μl aliquots) were examined by electrophoresis on 3% and 2% agarose gels according to standard procedures (36). The results were considered positive when the product that was equivalent to the fragment described was found. Negative and positive *H. pylori* controls, as well as water as a negative contamination PCR control, were included in each assay. PCR amplification was performed in duplicate for each DNA sample.

***cagA* detection by PCR.** The primers 5′-ACCCTAGTCGGTAATGGGTTA-3′ (CAG1) and 5′-GTAATTGTCTAGTTTCGC-3′ (CAG2) (48) were used to amplify the 3′ region of the *cagA* gene. These primers were designed to amplify the variable 3′ region of the *cagA* gene in a Japanese population. Depending on the type and number of repeats present in the sequence of the gene, the products were classified into fragment A (sizes ranging from 600 to 650 bp), fragment C (around 800-bp products), and fragments B/D (around 750 bp). Another primer set for a constant region near the 3′ end of *cagA* described by Bukanov and Berg (3) was also used to detect the *cagA* gene. These forward and reverse primers, CagA/ConF (5′-GTGCCTGCTAGTTTGTCAGCG-3′) and CagA/Con-R (5′-TTGGAAACCACCTTTTGTATTAGC-3′), amplify a 402-bp fragment. PCRs were performed in a volume of 50 μl containing 20 nmol of Tris-HCl (pH 8.4) per liter, 50 mmol of KCl per liter, 2.5 (for CagA/Con) and 1.5 (for CAG1/2) mmol of $MgCl_2$ per liter, 200 μmol of deoxynucleoside triphosphate per liter, 2.5 U of *Taq* DNA polymerase (GIBCO BRL), and 25 pmol of both forward and reverse primers. PCR was performed in a PTC-1196 DNA thermocycler (MJ Research Inc.) under the following conditions for the CAG1/2 amplification: 35 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. For the CagA/Con amplification, each reaction mixture was amplified for 35 cycles as follows: 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. A preincubation of 3 min at 94°C and a final extension for 7 min at 72°C were performed in both *cagA* detection procedures. PCR amplification was performed in duplicate for each DNA sample, and positive and negative controls were included in each assay. The products of amplification were examined by electrophoresis on 2% agarose gels according to standard procedures (36).

Statistical analysis. Data were analyzed by using SPSS software, version 6.1.3 (SPSS Inc., Chicago, Ill.). Pearson's chi-square and Fisher exact tests were used to assess relationships between categorical variables. Analysis of variance with post hoc tests (Tukey's) was used for statistical comparisons of continuous variables among the diagnostic groups. Relative risk and 95% confidence intervals (CI) were calculated by using EPI-INFO's version 6.04 Statcalc. Significance was defined as P values of <0.05.

RESULTS

Patients. *H. pylori* was analyzed in biopsy specimens of 103 Caucasian Brazilian patients who underwent endoscopy. Fifty-seven (55%) patients had peptic ulcer disease: 26 (25%) with duodenal ulcer and 31 (30%) with gastric ulcer. Twenty-one (20%) of the patients had gastritis only and 21 (20%) had duodenitis; these patients were classified as nonulcer cases ($n = 42$; 41%). Four patients (4%) were normal and since they were *H. pylori* negative, they were excluded from the statistical analysis. Patients with gastric ulcer were significantly older than the nonulcer cases ($P = 0.02$) (Table 1).

Prevalence of *H. pylori* infection. PCR with primers for the 16S rRNA gene was performed on DNA extracted directly from biopsy specimens of all 103 patients. A total of 75 (75%)

TABLE 1. Characteristics of the study groups and prevalence of *H. pylori* infection evaluated by different methods in patients with peptic ulcer and in nonulcer patients

Characteristic	Clinical status[a] (*n*)				*P* value
	Nonulcer		Peptic ulcer		
	G (21)	D (21)	GU (31)	DU (26)	
Mean age, yr (±SD)	48.6 (±16.9)	46.1 (±12.3)	59.2 (±13.6)	50.8 (±15.3)	0.02
Sex ratio (M/F)[b]	7/14	14/7	13/18	14/12	0.06
No. (%) positive for *H. pylori* infection as determined by:					
Histology	9 (43)	14 (67)	27 (87)	15 (63)	
Urease test	8 (38)	13 (62)	21 (68)	16 (67)	
ureA PCR	9 (43)	10 (48)	20 (65)	19 (73)	
16S rRNA PCR	10 (48)	13 (62)	28 (90)	24 (92)	

[a] Abbreviations: G, gastritis; D, duodenitis; GU, gastric ulcer; DU, duodenal ulcer.
[b] M, male; F, female.

samples were positive, including 24 (92%) of the 26 patients with duodenal ulcer, 28 (90%) of the 31 patients with gastric ulcer, and 23 (55%) of the 42 patients in the nonulcer group. PCR using primers for the *ureA* gene showed that all of the 28 samples negative for the 16S rRNA primers were also negative for *ureA*, but of the 75 16S rRNA-positive samples only 58 were *ureA* positive. The analysis of the 17 16S rRNA-positive and *ureA*-negative samples showed that 11 were also *cagA* positive. On histological examination, 66 patients were positive for *H. pylori*, 34 were negative, and in 3 patients the examination was not possible. Using the urease test, 58 patients were positive, 41 were negative, and the test was not performed in 4 patients. *H. pylori* status was defined according to the agreement of 16S rRNA positivity and two or more of the diagnostic tests used. However, three patients were positive only by 16S rRNA PCR, and they were considered *H. pylori* positive because of the *cagA* positivity, as determined by two different assays. With the PCR using 16S rRNA as the "gold standard," the sensitivity of the *ureA* PCR was 77.3% (95% CI, 65.0 to 85.9), the urease test sensitivity was 78.1% (95% CI, 68.6 to 87.6), and the histology sensitivity was 83.3% (95% CI, 74.7 to 91.9). The specificity of the *ureA* PCR was 100% (95% CI, 85.0 to 100.0), the urease test specificity was 96.2% (95% CI, 88.9 to 100.0), and the histology sensitivity was 78.6% (95% CI, 63.9 to 93.6).

The prevalence of *H. pylori* in patients with duodenal ulcer (24 of 26; 92%) and gastric ulcer (28 of 31; 90%) was found to be significantly higher than in the nonulcer group (23 of 42; 55%; χ^2 for trend = 14.0; $P < 0.001$). The relative risk for duodenal ulcer in patients positive for *H. pylori* was 5.36 (95% CI, 1.39 to 20.63; $P = 0.003$) and the relative risk for gastric ulcer in *H. pylori*-positive patients was 4.03 (95% CI, 1.37 to 11.86; $P = 0.003$), when compared to *H. pylori*-negative patients.

Detection of *cagA*. The *cagA* gene was detected in 43 (57%) samples among 75 *H. pylori* samples positive for 16S rRNA by PCR when the 3′ variable sequence primers were used, while 53 (71%) samples were *cagA* positive using the consensus sequence primers. According to the presence of repeat sequences in the 3′ variable region, as determined by the analysis of the molecular size of the PCR products (17), the following amplification products were observed: 28 (65%) patients were subtype A (products ranging from 600 to 650 bp), 5 (12%) patients were subtypes B or D (around 750 bp), and 2 (5%) patients showed subtype C (around 850 bp). Since the subtypes B and D have the same PCR product length and can be distinguished only by sequencing, they were grouped together. Eight (18%) patients presented two or three subgenotypes: three (7%) patients had A, B/D, and C genotypes; three (7%)

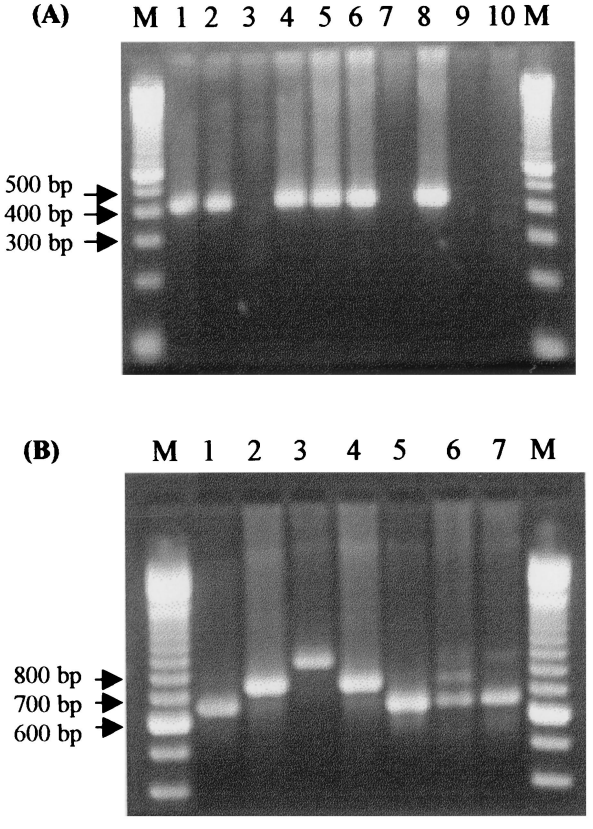

FIG. 1. Analysis of the *cagA* gene by PCR using primers for the consensus region (A) and for the variable 3′ region (B). (A) PCR products (400 bp) from positive *cagA* isolates (lanes 1, 2, 4, 5, and 6) and negative isolates (absence of PCR products) (lanes 3 and 7). Lane 8, positive control; and lanes 9 and 10, negative controls. (B) PCR products ranging from 650 to 850 bp from *cagA*-positive isolates. Fragments of 650 bp (type A) (lanes 1 and 5), fragments of 750 bp (types B/D) (lanes 2 and 4), and fragments of 850 bp (type C) (lane 3) were obtained. Lane 6, from a patient with types A, B/D, and C; lane 7, from a patient with types A and C; lane M, molecular size marker ladder, 100 bp.

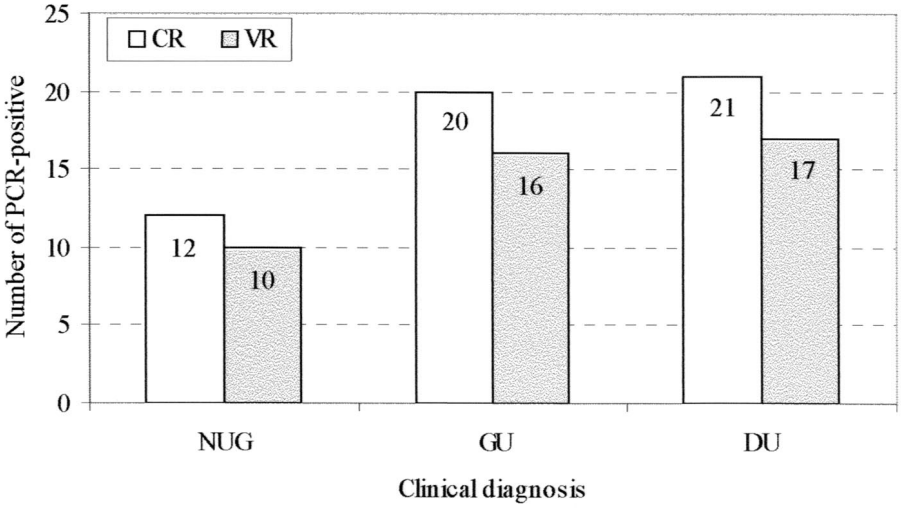

FIG. 2. PCR for *cagA* gene detection using primers for the consensus region (CR) or variable region (VR). NUG, nonulcer group; GU, gastric ulcer; DU, duodenal ulcer.

patients had A and B/D; one (2%) patient had C and B/D; and another (2%) patient had A and C genotypes. PCR products of the type A subgenotype were always more intense on gel electrophoresis than B/D or C in cases with mixed strain infections. The amplification products of the PCR methods using the consensus region primers (400 bp) or the variable region primers (ranging from 600 to 850 bp) are shown in Fig. 1. The consensus region primers could detect 10 patients that were *cagA* negative by the technique using the variable region primers. Four of the patients belonged to the duodenal ulcer group, four belonged to the gastric ulcer group, and two belonged to the nonulcer group (Fig. 2).

Relationship between *cagA* genotype and pathogenicity. The *cagA* status of *H. pylori* isolates correlated with duodenal ulcer disease when the consensus sequence primers were used to detect *cagA* strains. The *cagA* gene was detected using the consensus primers in 21 of the 24 (87%) *H. pylori*-positive duodenal ulcer patients, in 20 of the 28 (72%) *H. pylori*-positive gastric ulcer patients, and in 12 of the 23 (52%) isolates from *H. pylori*-positive patients with gastritis only or duodenitis ($P = 0.009$) (Table 2). In contrast, using the variable sequence primers, 17 (71%) duodenal ulcer patients, 16 (57%) gastric ulcer patients, and 10 (43%) nonulcer patients showed the *cagA* gene ($P = 0.16$). The presence of *cagA* did show a significant association with duodenal ulcer disease, as opposed to gastritis only or duodenitis (nonulcer group), when the *cagA* gene was determined by the consensus PCR primer set. *cagA*-positive strains were significantly more prevalent among patients with duodenal ulcer ($P = 0.02$) but not among gastric ulcer patients ($P = 0.26$). In patients without ulcer, the prevalence of *cagA*-positive and *cagA*-negative strains was approximately equal. Table 2 shows the statistical analysis for the consensus primer amplification assay. The 3′ variable region subgenotypes (A, B or D, and C) did not show a significant association with specific gastroduodenal disease ($P = 0.84$). However, when patients having more than one subtype (multiple subtypes) were compared with those having a single subtype, there was a significant association between peptic ulcer and multiple *cagA* subgenotypes ($P = 0.048$). The most frequent type of *cagA* 3′ region was type A (81%), followed by types B/D (28%) and C (16%). In 18% of the cases, more than one subtype was observed, and in 75% of the cases with multiple subtypes, this was related to gastric ulcer. Table 3 summarizes these results.

DISCUSSION

H. pylori infection leads to the development of chronic gastritis and is associated with peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue-type lymphoma (19). However, it has not been determined to what extent differences in infecting strains, characteristics of the host, interactions with other ulcerogenic factors, and environmental factors explain why most infected subjects will never be affected by peptic ulcer disease or gastric cancer (2, 17, 45). Specific *H. pylori* genotypes, e.g., *vacA* s1 *cagA*$^+$ *iceA1*, are related to more severe morbidity, whereas others, e.g., *vacA* s2m2 *iceA2* (*cagA* negative), appear to be less pathogenic. Genotyping of *iceA* and *cagA* offers the most effective combination for the identification of patients with ulcers. However, because *cagA* and *vacA* s-region genotypes are so strongly associated, the combination of *iceA* and *vacA* s type is almost as effective as the combination of *iceA* and *cagA* (44). Although the association between *H. pylori*-infected, *cagA*-positive patients and peptic ulcer disease is still controversial, most stud-

TABLE 2. Association between *cagA* status of *H. pylori* isolates and duodenal ulcer, gastric ulcer, and nonulcer patients

Clinical status	*n*	No. with *H. pylori cagA*[a]	RR[c]	95% CI	*P* value[b]
Nonulcer	23	12 (52)			
Gastric ulcer	28	20 (72)	1.48	0.82–2.68	0.26
Duodenal ulcer	24	21 (87)	2.97	1.05–8.37	0.02

[a] *cagA* was analyzed by PCR using the consensus region primers.
[b] χ^2 for trend = 6.77; $P = 0.009$.
[c] RR, relative risk.

TABLE 3. Association between *cagA* subtypes of *H. pylori* isolates and duodenal ulcer, gastric ulcer, and nonulcer patients

Clinical status[a] (*n*)	No. (%) with *cagA* subtype[b,c]						
	Single subtype			Multiple subtypes			
	A	B/D	C	A + B/D + C	A + B/D	B/D + C	A + C
NU (10)	7 (70)	2 (20)					1 (10)
GU (16)	8 (50)	1 (6)	1 (6)	3 (19)	2 (13)	1 (6)	
DU (17)	13 (76)	2 (12)	1 (6)		1 (6)		

[a] Abbreviations: NU, nonulcer; GU, gastric ulcer; DU, duodenal ulcer.
[b] *cagA* was analyzed by PCR with variable region primers.
[c] χ^2 for isolated versus multiple subtypes, $P = 0.048$.

ies to date show that indeed such an association exists. Therefore, the aim of this study was to investigate the association of *cagA* genotype and subgenotype and clinical outcome, as well as to compare the detection of *cagA* positivity between an Eastern Asia-based assay and a Western sample-based assay in a subset of *H. pylori*-infected Caucasian Brazilian patients.

In this study, the prevalence of *cagA* strains detected by PCR using primers designed according to the sequence that includes the repeat sequences in the 3′ region from Japanese strains was 57%. Yamaoka et al. (48) examined the possibility that the different structural subtypes of the *cagA* gene (A to D) may be preferentially associated with specific *H. pylori*-related gastric disease, and they found that 86% of type C variants were obtained from patients with gastric cancer. In this study from Japan, the most frequent type of *cagA* 3′ region was type A (93%). In our study, type A was also the most frequent type encountered (81%). However, in contrast to what was found in the Japanese study, we observed a much higher frequency of the subtypes B or D (28% in our study versus 2% in the Japanese study) and C (16% versus 4%). Since the *cagA* subtypes were not sequenced but were determined by the analysis of the molecular weight of the PCR products, subtypes B and D, which have the same molecular weight, were grouped together.

A considerable proportion of our cases (18%) contained multiple genotypes, confirming that patients may have more than one *H. pylori* strain (39, 42). The proportion of specimens that have multiple strains may be underestimated if all of them have identical *cagA* genotypes. As described in other studies, the use of nonsterile endoscopes may be an alternative explanation for the high percentage of multiple strains (34), but it seems unlikely that this occurred in our study because we followed several steps for contamination control. Another explanation for the patients presenting with different *cagA* status could be the existence of subclones within the secluded strain. Enroth et al. (11) found that in subclones of *H. pylori* the entire *cag* pathogenicity island or part of it is deleted, which may affect binding capacity and virulence. Finally, the observation that on PCR amplification there seems to be a predominance of a specific *cagA* subgenotype (A) suggests that template competition might be occurring during the PCR amplification process and/or in mixed infections per se, and, therefore, we might have underestimated the number of mixed infections in our patients.

The subgenotypes did not show a significant correlation with specific gastroduodenal disease in our study ($P = 0.84$). However, there was a trend for the association of subtype C as well as subtype B/D, when analyzed as multiple subtypes, with gastric ulcer. When patients having more than one subtype (multiple subtypes) were compared with those having a single subtype, there was a significant association between peptic ulcer and multiple *cagA* subgenotypes ($P = 0.048$). However, if there was an underestimation of mixed-strain infections within our patient group, this conclusion must be reconsidered. Yamaoka et al. (48) reported that both types B and C are associated with severe atrophic gastritis, the precursor lesion of gastric cancer. In our study, six of the eight cases with multiple *cagA* subgenotypes were encountered in patients with gastric ulcer. In another study by Yamaoka et al. (46), they reported that strains with more than three repeat regions were associated with significantly higher scores for gastric mucosal atrophy and metaplasia than those strains with fewer repeat regions. *H. pylori* strains with three repeat regions were also significantly more susceptible to pH 3 than isolates with fewer repeat regions. The variation in the pattern of repeats in strains isolated from western countries and from East Asian strains may reflect different conditions within the stomach in different geographic regions and eventually the outcome disease (46). Type A may be the most prevalent subgenotype around the world, as shown in our study as well as in the study from Japan, i.e., from two widely separated geographic locations (48). However, we observed a higher frequency of the type C subgenotype (16%) than that reported previously in the analysis by Yamaoka et al. in a Japanese population (4%) (48) or in a Korean population where no type C *H. pylori* strains were found, although the sequences of *cagA* repeat regions from Korean strains were very homologous to those from Japanese strains (46). Furthermore, depending on the geographic area analyzed, there may be a variation in the subtypes that are more frequently related to symptomatic *H. pylori*-associated disease.

It has been suggested that the presence of repeat sequences in the 3′ region of the *cagA* gene may result in proteins with different immunogenic properties (7). The structural *cagA* variants result in larger-sized CagA proteins. In a study by Yamaoka et al. (48), the molecular weight of CagA proteins was significantly greater in strains with larger-size PCR products than in strains with smaller-size PCR fragments, and the enzyme-linked immunosorbent assay titer of serum anti-CagA antibody in patients infected with type C strains was significantly higher than that in patients infected with type A strains (48). These data suggest that *cagA* variants may provide new markers for other factors involved in gastric carcinogenesis, or they may be associated with higher levels of immune response, possibly

influencing the outcome of *H. pylori* infection (48). In the near future, we will need more *cagA*-positive cases so that we can evaluate the prevalence of the different subtypes and determine whether there is a relationship between *cagA* subgenotype and *H. pylori* infection outcome. For cancer cases it will also be necessary to verify the *cagA* subgenotype and its association with neoplasia in our population.

Because the sequence of the *cagA* gene differs markedly from one geographic region to another (Western versus East Asia isolates) (40, 46, 47), we tested our *H. pylori*-positive samples using a primer set designed according to a consensus sequence of the *cagA* gene (3). Ten patients that were *cagA* negative after amplification with primers for the variable sequence were positive using primers for the consensus region, showing that some *H. pylori* strains from Brazil were not detectable by PCR using primers that included the repeat sequences from the Japanese isolates (48). The prevalence of *cagA* positivity by the former method was 71%, showing an association between *cagA* and disease outcome ($P = 0.009$). The presence of *cagA* showed a stronger correlation with duodenal ulcer disease (87%) than with gastritis only or duodenitis (52%; $P = 0.020$). In the gastric ulcer group, the prevalence of *cagA* positivity was 72%, and there was probably not enough statistical power to demonstrate the association of gastric ulcer and *cagA* ($P = 0.26$). The fact that the prevalence of *cagA*-positive and *cagA*-negative strains was approximately equal in patients without ulcer implies that individuals without ulcer can be infected with high-risk *H. pylori* genotypes. It has been speculated that patients infected with the more pathogenic strains but who do not have peptic ulcer disease may be younger than ulcer patients with these strains and they may have not yet developed the disease (44). Indeed, in our study, patients without ulcer were significantly younger than the patients with gastric ulcer ($P = 0.021$).

Several important aspects can influence the occurrence of peptic ulcer in patients with or without *H. pylori* infection (23, 31). Not all patients in whom peptic ulcer disease was diagnosed were infected with the virulent genotype of *H. pylori*. Conversely, the virulent genotype was found in patients without peptic ulcer disease. Since the use of NSAIDs can be directly associated with ulcer disease (22), we excluded from our study any patient with current NSAID use. Even so, we were unable to control for past use of these drugs, with *H. pylori* presenting in these patients as an innocent bystander. This could explain why 29% of the *H. pylori*-positive gastric ulcer patients were *cagA* negative. Conversely, patients using acid-suppressing drugs may present without peptic ulcer at endoscopy. Host factors, such as smoking, acid output, and human leukocyte antigen status, may also influence the clinical outcome of *H. pylori* infection and may interact with bacterial virulence factors (31). Additional bacterial virulence factors may also play an important role, such as the bacterial Le^b-binding phenotype, which has been associated with the presence of *cagA* among clinical isolates of *H. pylori* (20). Regarding our sampling methods, a single biopsy specimen from the antrum was analyzed by PCR, and so sampling errors cannot be excluded. In addition, the primer sets used might be unable to detect specific Brazilian sequences. A characterization of our population may be necessary to investigate differences in the primary structure of the gene and to define subgenotype prevalence.

Considering that gastritis caused by *H. pylori* infection could progress to ulcer disease (38), patients with only gastritis at the time of endoscopy may still develop ulcer disease later in life. The quantity and virulence of *H. pylori* strains in the duodenum may play a critical role in the development of duodenal ulcer. Evidence of a high density of *cagA*-positive strains in the duodenum of patients with severe duodenitis is an important determinant of duodenal ulcer disease (18), and it is speculated that this is a relevant factor in the reason why duodenal ulcers develop in only a minority of *H. pylori*-infected subjects.

The unequal distribution of genotypes may contribute to the different prevalence rates of *H. pylori*-associated disease in different parts of the world (44). A recent study reported that *cagA* genotyping is useful for molecular epidemiological studies, showing that strains can be completely separated (East Asian and non-Asian strains) by differences in the *cagA* 3′ region. Yamaoka et al. (47) reported that PCR using specific Japanese *cagA* primers was not able to detect non-Asian strains and could be used to identify the population of origin of *H. pylori* isolates. In our study, we used a primer set that, although not identical to that of Yamaoka et al., was also originally designed for a Japanese population and which nevertheless allowed the identification of several positive strains with similar subgenotype patterns isolated from non-Oriental Brazilian patients. This finding suggests that it might be necessary to sequence a representative number of *H. pylori* isolates from individual populations in order to affirm that one specific method can be used to identify a particular population.

In summary, our results showed a *cagA* prevalence of 71% and a specific association with duodenal ulcer disease when *cagA* gene status was determined by the consensus PCR primer set, suggesting that *cagA* is an important marker for this disease in our country, as already observed by other investigators (13). To completely elucidate whether specific *cagA* subgenotypes have different prognostic implications in *H. pylori*-associated disease, it will be necessary to further study the sensitivity of PCR methods designed to detect different subgenotypes, and this could be achieved by sequencing a representative number of *cagA*-positive *H. pylori* isolates in our population. The identification of the most virulent *H. pylori* strain seems to be an important prognostic factor and may guide prophylactic anti-*Helicobacter* treatment to prevent peptic ulcer disease later in life or to prevent high-risk patients from developing gastric cancer.

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