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Importance in Diagnosis of Gastritis of Detection by PCR of the *cagA* Gene in *Helicobacter pylori* Strains Isolated from Children

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The *cagA* gene has been detected by PCR and DNA hybridization in 45 *Helicobacter pylori* strains isolated from children. For each child, clinical symptoms, endoscopic aspect of the gastric mucosa, and histological gastritis were evaluated. Gene-positive strains were associated with hemorrhagic gastritis in 66.6% of the children, while gene-negative strains were associated with hemorrhagic gastritis in 11.2% of the children ($P = 0.0001$). In addition, 88.8% of gene-positive strains were associated with severe histological gastritis (scores of 3 and 4), and gene-negative strains were collected from the gastric mucosa with the same type of infiltration of neutrophils and lymphocytes in the lamina propria in 55.5% of the children. These differences were statistically significant ($P = 0.017$). Gene-positive strains were also isolated more frequently from children with vomiting ($P = 0.04$), while the absence of clinical signs was not significantly different in *cagA* gene-positive or -negative patients. All of these observations confirmed the role of this *cagA* gene as a marker of gastric inflammation in children. The detection of this gene might be helpful to determine the degree of inflammation of the gastric mucosa in the absence of abdominal symptoms. We might better understand the natural history of *H. pylori* infection if we studied the evolution of gastritis in children with regard to the *cagA* gene status of isolated strains.

Helicobacter pylori is now recognized as an important pathogen of humans. This bacterium causes chronic gastritis, which is a risk factor for the development of peptic ulcer disease and adenocarcinoma of the stomach (1). However, although nearly all infected persons develop gastritis (6), it remains unclear why the majority of *H. pylori*-infected persons remain asymptomatic (9), whereas in a minority of persons, *H. pylori* infection leads to tissue inflammation with development of gastric, duodenal ulcers, and gastric adenocarcinoma. Many studies (2, 3) demonstrate heterogeneity among strains of *H. pylori* with regard to the production of an antigenic protein migrating at approximately 120 to 128 kDa on reducing sodium dodecyl sulfate-polyacrylamide gels. This high-molecular-mass *H. pylori* antigen is associated with cytotoxin production and is named CagA (cytotoxin-associated gene A). Recently, the gene encoding this protein was cloned and sequenced (16), and the expression of this protein was exactly correlated with the presence of the gene (16, 20). The association of CagA antigen with an increased antral mucosal polymorphonuclear leukocyte infiltration and with the presence of peptic ulceration is known in adults (2). In children, clinical and histological manifestations of *H. pylori* infection are often moderate; controversies concerning the relationships between the presence of this bacterium, histological features, and clinical symptoms remain.

The aim of this study was the detection of the *cagA* gene in *H. pylori* strains isolated from children. A DNA-specific fragment of 393 bp was detected by PCR and DNA hybridization for screening of the *cagA* gene.

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MATERIALS AND METHODS

Forty-five children (mean age, 12.4 years; range, 1 to 18 years) referred for upper gastrointestinal tract endoscopy were included in this study. All were known to have a positive serodiagnosis for *H. pylori* by an enzyme-linked immunosorbent assay (Cobas Core anti-*H. pylori* enzyme immunoassay; Roche, Basel, Switzerland). For each child, clinical signs were noted before endoscopy. Twenty of the children were symptomatic, with abdominal pains, vomiting, weight loss, or hematemesis. The 25 other children had none of these clinical symptoms. Informed consent was obtained from each patient or his or her parents before the patient entered the study. This study was approved by the Lille University Hospital Ethical Committee. The macroscopic aspect of the gastric mucosa was noted.

Two antral biopsies (one for microbiology and one for histology) were performed. The biopsy specimens for histology were fixed in Bouin's fluid, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Gastric histopathology was classified as follows: grade 1, normal mucosa or isolated nodular lymphoid hyperplasia; grade 2, lymphoid chronic superficial gastritis; grade 3, focal chronic active gastritis; grade 4, diffuse chronic active gastritis. The grading used was that described by Whitehead et al. (18) as modified by Warren and Marshall (17). Each parameter was evaluated without previous knowledge of the *cagA* gene status.

Within a maximum of 2 h after collection, the biopsy specimens for histology were weighed before homogenization in 1 ml of saline solution, cultured on Skirrow selective medium, and incubated for 3 to 7 days at 37°C under microaerophilic conditions. The isolates were identified by Gram stain, colonial morphology, and positive oxidase, catalase, and urease reactions. The number of CFU per milliliter and then the number per milligram were noted. The isolates were grown on Müeller-Hinton agar supplemented with 7% fetal calf serum for 3 days at 35°C under microaerophilic conditions for PCR tests. This study included one strain known to have the *cagA* gene (*H. pylori* ATCC 49503) and another *cagA* gene-negative strain (strain Tx30a).

Two oligonucleotide primers, 5′-CCATGAATTTTTGATCCGTTCGG-3′ and 5′-GATAACAGGCAAGCTTTTGAGAGGGA-3′, chosen from the sequenced *cagA* gene by Tummuru (16), amplified a 393-bp product from this gene. To confirm *H. pylori* identification and to use an amplification control, another PCR test, with primers of the *H. pylori* urease structural gene, was performed (8). Therefore, primers 5′-GCCAATGGTAAATTAGTT-3′ and 5′-CTCCTTAAT TGTTTTTAC-3′ amplified a 411-bp product from the *ureA* gene (nucleotides 304 to 714). Suspensions of each *H. pylori* strain were prepared in sterile distilled water (200 μl) from agar plates with a standard loop. The samples were boiled for 10 min and then centrifuged at 14,000 × *g* for 5 min. An aliquot of 10 μl of

TABLE 1. Correlation between *cagA* gene detected by PCR in *H. pylori* isolates and clinical signs of each child[a]

Clinical sign		No. of isolates positive for *cagA* gene (n = 18)	No. of isolates negative for *cagA* gene (n = 27)	P
Type	Presence			
Abdominal pain	Yes	8	4	0.04
	No	10	23	
Vomiting	Yes	6	2	0.04
	No	12	25	
Weight loss	Yes	1	2	NS[b]
	No	17	25	
Hematemesis	Yes	3	1	NS
	No	15	26	
None	Yes	10	15	NS
	No	8	12	

[a] Twenty children had clinical symptoms (abdominal pain, vomiting, weight loss and/or hematemesis); 25 children had no clinical symptoms.
[b] NS, not significant.

supernatant was used as the target DNA in amplification assays. The PCR mixture contained 2.5 U of *Taq* polymerase (Boehringer Mannheim Laboratories), 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide, and 0.5 μM each oligonucleotide primer. PCR was performed with a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, Conn.) as follows: denaturation at 94°C for 1 min, annealing at 45°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Tubes containing distilled water instead of DNA samples (negative control) and DNA samples of control strains with and without the *cagA* gene were used with each batch of amplification mixture. A total of 35 cycles were executed. The amplified products were analyzed by agarose gel electrophoresis and Southern hybridization. A 10-μl sample of PCR-amplified products was electrophoresed on a 3% agarose gel. The gel, which was stained with ethidium bromide (1 μg/ml), was examined under UV light for the presence of the 393-bp fragment of amplified DNA. The specificity of the amplified DNA was confirmed by Southern blot hybridization with a *cagA* DNA probe. This was obtained from the PCR products after amplification of the DNA of the *cagA* gene-positive reference strain. The amplified PCR product was purified by electrophoresis. The appropriate fragment was cut out of the gel, placed into a 0.22-μm Spin-X tube (Costar, Cambridge, Mass.), centrifuged at 10,000 × *g* for 30 min, and labelled with horseradish peroxidase by the ECL labelling system (Amersham International plc Laboratories). Southern transfers with a VacuGene XL apparatus (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and DNA hybridizations were carried out by standard methods (13) on each DNA sample after amplification by *cagA* gene primers.

Statistical analysis was done by use of chi-square analysis for the data shown in the tables and by the Wilcoxon sum test for the data illustrated in Fig. 1. A P of <0.05 was considered significant.

RESULTS

All *H. pylori* DNA samples were positive for the *ureA* gene by PCR. The presence of the gene coding for CagA was found in 18 (40%) strains by PCR.

Correlations between *cagA* gene-positive strains and clinical symptoms are summarized in Table 1. Abdominal pain and vomiting were observed more frequently in children infected with strains having the *cagA* gene than in those infected with gene-negative strains. However, among the 25 *H. pylori* strains collected from children with no abdominal pain, vomiting, weight loss, or hematemesis, 10 had the gene and 15 did not. Thus, the absence of clinical signs was not significantly different in CagA-positive or -negative patients.

The correlation between the number of *H. pylori* (in CFU per milligram) and the *cagA* gene status of strains is summarized in Fig. 1. There was no difference between the two groups with regard to the presence or absence of the *cagA* gene.

The macroscopic aspect of the gastric mucosa from infected patients and the *cagA* gene status of strains are summarized in Table 2. There was no difference in the two groups for antral nodularity. Conversely, gene-positive strains were associated with hemorrhagic gastritis in 66.6% of patients, while gene-negative strains were associated with hemorrhagic gastritis in 11.2% of patients (P = 0.0001). The presence of ulcer disease was not oberved by the endoscopist during our study.

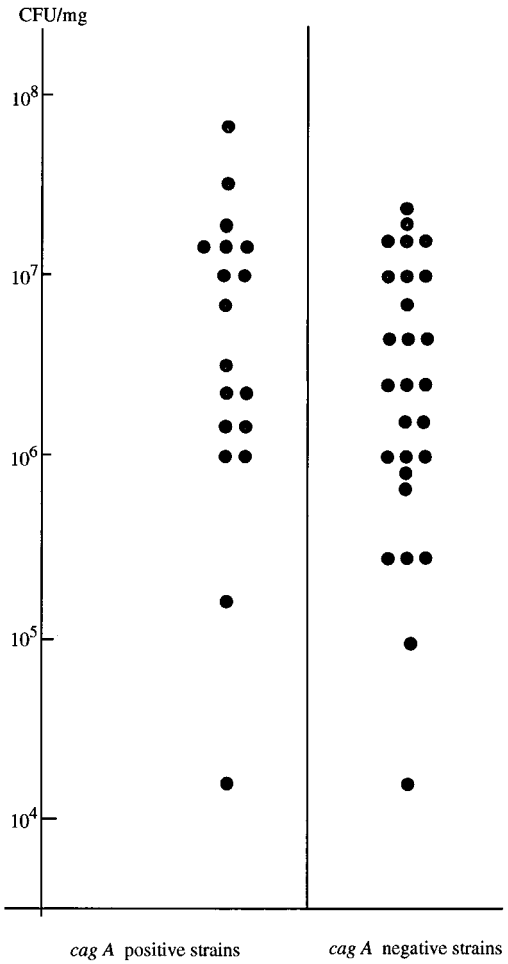

FIG. 1. Correlation between *cagA* gene status and number of CFU of *H. pylori* per milligram isolated for each patient.

Classification of gastritis was determined by the number of polymorphonuclear leukocytes and lymphocytes, the presence of leukocytes and lymphocytes as components of the lamina propria either individually isolated or in groups, and whether these cells invaded neck glands, leading to epithelial surface degeneration. In this scheme, severe gastritis was scored as a grade 4, whereas grade 1 or 2 gastritis was of doubtful significance. Of the 18 strains having the *cagA* gene, 16 (88.8%) were isolated from patients with severe gastritis (grade 4, 6 patients; grade 3, 10 patients), while only 2 were isolated from patients with gastritis with a score of 1 or 2. Of the 27 *cagA* gene-negative strains, 15 (55.5%) were isolated from patients with gastritis of grade 4 (1 patient) or grade 3 (14 patients; P = 0.017), while 12 were from patients with gastritis with a score of 1 or 2. These differences observed between infections caused by strains with or without the *cagA* gene and the degree of inflammation of gastric mucosa were statistically significant.

DISCUSSION

A high-molecular-mass (120- to 128-kDa) *H. pylori* antigen has been described as being closely related to cytotoxin pro-

TABLE 2. Correlation between *cagA* gene detected by PCR in *H. pylori* isolates with endoscopic features and severity of gastritis defined by a score of 1 to 4

Endoscopic feature		No. of patients	No. of isolates positive for *cagA* gene (n = 18)	No. of isolates negative for *cagA* gene (n = 27)	P
Type	Presence				
Antral nodularity	Yes	25	10	15	NS[a]
	No	20	8	12	
Hemorrhagic gastric mucosa	Yes	15	12	3	0.0001
	No	30	6	24	
Gastritis					
Score 1	Yes		1	6	
Score 2	Yes		1	6	
Score 3	Yes		10	14	0.017[b]
Score 4	Yes		6	1	

[a] NS, not significant
[b] Calculated by comparing values for scores of 1 and 2 with values for scores of 3 and 4.

duction. It did not mediate toxin activity directly, but it was called cytotoxin-associated gene A or CagA antigen. This protein is immunogenic (4, 19). In adults, the presence of antibodies to the CagA protein in either serum or mucosal secretions was more common among those with peptic ulcer disease than among those with chronic gastritis alone. In addition, mucosal neutrophil infiltration and epithelial surface degeneration were significantly greater in patients with a local gastric immune response to CagA than in those without such a response (4). These studies suggested that only bacteria expressing CagA were associated with duodenal ulcers and, therefore, that this protein was linked to disease. Recent studies showing that *H. pylori*-positive patients with gastric cancer had significantly greater serologic recognition of the 120- to 128-kDa protein in an enzyme immunoassay relative to that of infected subjects with non-ulcer dyspepsia confirmed this hypothesis (5).

In children, clinical and histological manifestations of *H. pylori* infection are often moderate. *H. pylori* infection can be associated with an absence of clinical signs or with antral nodularity, moderate gastritis, and sometimes a hemorrhagic or normal gastric mucosa (12). The correlation between the presence of *H. pylori* in the gastric mucosa, clinical signs, and gastritis is difficult to define, and the prevalence of the *cagA* gene in isolates is not studied in this patient population. Since the presence of the antigen was correlated with the presence of the gene (20), we detected the *cagA* gene by PCR in 45 strains isolated from children. Of these, 18 (40%) were infected by a *cagA* gene-positive strain. This rate is lower than that described in adults (4, 5, 19). In our population of children infected with a strain having the gene, abdominal pain and vomiting were often observed, and the presence of a hemorrhagic mucosa and severe gastritis were often observed: 66.6% of the gene-positive strains were isolated from a hemorrhagic mucosa, and 88.8% of gene-positive strains were associated with severe histological gastritis (scores of 3 and 4). Conversely, in the population of children infected by gene-negative *H. pylori* strains, a hemorrhagic gastric mucosa and infiltration of neutrophils and lymphocytes, corresponding to gastritis with scores of 3 and 4, in the lamina propria of this mucosa were observed in 11.2% and in 55.5% of children, respectively. No gastric ulcer disease was observed in our population, and no correlation between the *cagA* gene status and the importance of gastric mucosa colonization was found. Therefore, if a correlation between gastric ulcer disease and gastric adenocarcinoma and *cagA*-positive *H. pylori* strains was described in adults, our results demonstrate that such strains were also associated with severe gastritis in children.

These observations confirm the role of the *cagA* gene as a marker of gastric inflammation in children (2, 20). The best-known virulence factors are urease, which is supposed to play an important role in the neutralization of gastric acid secretion (8), flagella, which are essential for swimming through the mucus layer (10, 15), superoxide dismutase (14), and several molecules that are involved in specific adhesion to the superficial epithelial cells of the stomach (11). However, these factors are produced by all of the strains and, therefore, could not explain the different clinical manifestations of *H. pylori* infections. In our work, we demonstrated a heterogeneity in the genotypic characteristics of the strains. This heterogeneity was in relation to the degree of gastric inflammation these strains induced. If we consider the strong association of this protein with severe gastroduodenal pathologies in adults, this detection might be helpful to determine which children should be treated or monitored.

However, although abdominal pain and vomiting were observed often in our population of children, for those infected with a *cagA* gene-positive strain, the absence of clinical symptoms was of little value in the *H. pylori* gastritis diagnosis. We could find strains having this gene in the stomachs of children without clinical signs. On the other hand, some *cagA* gene-negative strains were isolated from children with abdominal pain. These observations confirm the importance of the detection of the *cagA* gene in *H. pylori* strains isolated from children as a marker of gastric inflammation in children since the absence of clinical signs is not significantly different in positive and negative patients.

A chronic *H. pylori* infection may persist for years (7). In the present study, we demonstrated the presence of strains without the *cagA* gene in the gastric mucosa of children. Little information is available regarding the persistence of such strains in the stomach. Further investigations are needed to determine whether *cagA* gene-negative strains could persist for a long time and their role in the pathogenesis of gastritis and duodenal ulcer diseases. We might better understand the natural history of *H. pylori* infection if we knew the *cagA* gene status of *H. pylori* strains.

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