Factors Associated with *Helicobacter pylori* Infection by a cagA-Positive Strain in Children

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Although infection with a cagA-positive *Helicobacter pylori* strain is considered a risk factor for the development of duodenal peptic ulcer in adults, this association has not been demonstrated in children. The presence of cagA was investigated by polymerase chain reaction in *H. pylori* strains isolated from 27 children with duodenal ulcer and 53 without duodenal ulcer. All patients (100%) with duodenal ulcer and 33 (62.3%) without ulcer were colonized by a cagA-positive strain (*P* = .00007). A cagA-positive status was also associated with a more marked macroscopic gastritis, with a greater inflammatory infiltrate of both mononuclear and polymorphonuclear cells in the antral and oxyntic gastric mucosa and degenerative and regenerative changes of the gastric mucosa. Increased cagA positivity was also associated with increased age, but no association between cagA-positive status and sex was observed.

There is now evidence that *Helicobacter pylori* is an essential factor in the pathogenesis of duodenal peptic ulcer [1]. However, it remains unclear why a minority of *H. pylori*-positive patients develop the disease. It has been suggested that *H. pylori* strains that contain the cag pathogenicity island (cag-PAI) are more virulent and are associated with the complications of the infection, such as peptic ulcer [2, 3]. The cag-PAI contains genes that encode proteins with similarities to components of secretion systems and seems to induce an increased inflammation in the gastric mucosa through release of interleukin-8 (IL-8) by epithelial cells [4]. The cagA gene, located in the cag-PAI, encodes a highly immunogenic outer-membrane protein of molecular mass 120–140 kDa [5]. Although its function remains unknown, it may be considered a marker of enhanced IL-8 factor release and consequently a marker of greater inflammation in the gastric mucosa.

In adults, several studies have demonstrated the association between the presence of cagA and peptic ulceration [2, 3, 5–7]. However, geographical differences have been demonstrated. Studies from Korea and China have shown that the majority of *H. pylori* strains are cagA-positive, regardless of clinical presentation [8, 9].

Few studies evaluating cagA status in children are available [10–14]. In most of them, few or no patients with duodenal ulcer were included, and so the relationship between ulcerogenesis and cagA could not be assessed. In 2 studies, however, 13 and 12 children with duodenal ulcer, respectively, were evaluated, but the results were conflicting [10–13]. In the first study, cagA status was investigated by a serum anti-CagA IgG assay, and association between the presence of ulcer and cagA-positive status was observed [10]. In the second study, cagA was investigated by polymerase chain reaction (PCR), and because the authors did not observe association between the presence of the gene and duodenal ulcer, they concluded that cagA was not a marker of duodenal ulcer in children [13]. In the latter study, some children were from countries in eastern Europe and Asia, where the general population has a high prevalence of cagA-positive strains.

Therefore, we evaluated prospectively *H. pylori*-positive children with and without duodenal ulcer to investigate the relationship between ulcerogenesis and infection with a cagA-positive *H. pylori* strain in children. We also compared the endoscopic and histologic alterations of the gastric mucosa and the age and sex of the children colonized by a cagA-positive or -negative *H. pylori* strain.

Patients and Methods

We studied 80 *H. pylori*-positive children (43 girls and 37 boys; mean age, 10.4 years; range, 3–16) who underwent endoscopy for...
investigation of upper abdominal pain and who did not have a history of underlying disorder. Almost all of them were of low socioeconomic level. Twenty-seven children had duodenal peptic ulcer and 53 did not.

The endoscopic alterations of the gastric and duodenal mucosae were recorded for each child, and gastritis was scored as absent (0), mild (1), moderate (2), or marked (3).

**Microbiological study.** Tissue samples for culture were maintained in sodium thiglycollate broth (Difco, Detroit) at 4°C for a maximum of 1 h, ground separately in a tissue homogenizer (Kontes, Vineland, NJ), and plated onto Petri dishes containing freshly prepared Belo Horizonte medium [15]. The plates were incubated at 37°C in a micro-aerophilic atmosphere obtained by use of a gas generation system (Anaerocult C; Merck, Darmstadt, Germany). The plates were evaluated after 3, 6, 9, and 12 days of incubation. *H. pylori* was identified by macroscopic and microscopic morphology, by a rapidly positive urease test, by positive oxidase and catalase reactions [16], and by the presence of ureA. A pool of strains of the microorganism(s) of each patient was maintained at −80°C in Brucella broth supplemented with 30% glycerol.

**PCR analysis.** All *H. pylori* strains isolated were evaluated for the presence of cagA and ureA. Two cagA-positive strains (ATCC 49503 and NCTC 11637) and one cagA-negative strain (Tx30A) were used as positive and negative controls, respectively, for cagA gene detection. The same *H. pylori* strains and 2 Proteus mirabilis and *Escherichia coli* human isolates were used as positive and negative controls in the reaction for ureA detection. A reagent-negative control reaction, in which DNA samples were replaced with distilled water, was done with each batch of amplification to exclude contamination.

The *H. pylori* strains were thawed, plated onto brain-heart infusion agar plates supplemented with 10% sheep blood, and incubated at 37°C under micro-aerophilic conditions for 3 days. An abundant bacterial growth from a Petri dish measuring 60 mm in diameter was transferred with a sterile swab to a tube containing 1 mL of PBS at pH 7.4.

DNA from the bacteria was prepared as follows: bacterial samples were suspended in a tube containing 1 mL of PBS, centrifuged twice in PBS at 13,000 g for 10 min, and suspended in a buffer containing 8% sucrose, 50 mM Tris HCl at pH 8.0, 50 mM EDTA, and 0.1% Triton X-100. Lysozyme from chicken egg white (Boehringer Mannheim, Indianapolis) was added to a final concentration of 3 mg/mL, and the solution was incubated for 12 min at 37°C. SDS and RNase A from bovine pancreas (Boehringer, Mannheim, Germany) were added to final concentrations of 1% and 50 μL/mL, respectively, and the mixture was incubated for 1 h at 37°C. Pronase and proteinase K (Boehringer, Mannheim, Germany) 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 cetyl-trimethylammonium bromide (CTAB, Sigma, St. Louis)–NaCl solution (5% [w/v] CTAB, 0.7 M NaCl) was added, and the solution was gently mixed and incubated at 55°C for 10 min. The DNA was extracted in an equal volume of phenol chloroform (1:1) and was precipitated overnight at −20°C in the presence of 0.3 M sodium acetate and 3.1 volumes of absolute ethanol. The DNA precipitates were then pelleted by centrifugation at 16,000g for 30 min and allowed to air-dry [17]. The pellets were suspended in sterile distilled water, and DNA was quantified by measuring optical density at 260 nm.

**Detection of cagA.** Genomic DNA from the bacterial strains was PCR-amplified by use of 2 sets of synthetic oligonucleotide primers as described elsewhere by Kelly et al. [18] and Peek et al. [19]. The amplified PCR products were resolved in 1% agarose gels containing Tris/borate/EDTA by using 100 bp (Gibco BRL, Gaithersburg, MD) as a molecular weight marker. The agarose gels were stained with ethidium bromide and viewed under short-wavelength UV light. The strains were considered to be cagA positive when at least one of the reactions was positive.

**Detection of ureA.** Genomic DNA from bacterial samples was amplified by use of a set of synthetic oligonucleotide primers, as described by Clayton et al. [20].

**Histologic study.** Endoscopic biopsy samples of the antral and oxyntic mucosae were fixed in 10% formalin and embedded in paraffin wax by routine methods: 5-micron-thick histologic sections stained with hematoxylin and eosin and with carbolfuchsin were obtained for histologic analysis and for detection of *H. pylori*. The mucosae were analyzed in terms of degree of inflammatory reaction and activity, which were scored as follows according to the Revised Sydney System [21]: none (0), mild (1), moderate (2), or marked (3). Regenerative and degenerative changes were also recorded (present or absent). Changes were considered regenerative when the proliferative compartment (neck of the glands) was expanded, with cells presenting large basophilic nuclei and conspicuous nucleoli, with or without small tufts of epithelial cells in the foveolae or foveolar hyperplasia with a globoid or a corkscrew-like appearance. Degenerative changes were characterized by the presence of mucin depletion, cytoplasmic vacuolation, and/or focal or diffuse cellular desquamation in the foveolar and surface epithelia.

**Statistical analysis.** Differences between groups were evaluated by use of the 2-tailed x² test with Yate’s correction or by use of the Fisher exact test. Mean ages were compared by use of Student’s t test, and scores of macroscopic and microscopic gastritis were compared by use of the Mann-Whitney U test. The level of significance was set at P < .05.

**Results.**

The patients’ characteristics are listed in table 1. The mean age (± SD) of children with duodenal ulcer (11.6 ± 1.8 years) was significantly higher (P = .009) than that of children without duodenal ulcer (9.7 ± 2.8 years). An increased risk of developing duodenal ulcer was observed in boys (odds ratio [OR], 3.3; 95% confidence interval [CI], 1.1–9.8; P = .026).

**Specificity of the PCR assays.** The expected 411-bp product was seen after amplification of genomic DNA from *H. pylori*.
strains ATCC 49503, NCTC 11637, and Tx30A with primers for detection of ureA. No band was observed when DNA from E. coli or P. mirabilis control strains was employed.

With respect to cagA detection, DNA from H. pylori standard strains ATCC 49503 and NCTC 11637 yielded 349-bp and 400-bp products on agarose gel electrophoresis after amplification with primers designed by Peek et al. [19] and Kelly and 400-bp products on agarose gel electrophoresis after amplification with primers designed by Peek et al. [19] and Kelly et al. [18], respectively. DNA from H. pylori strain Tx30A, E. coli, and P. mirabilis did not yield any amplification product (data not shown).

cagA status and peptic ulcer. Twenty-seven (100%) of 27 patients with duodenal ulcer and 53 (62.3%) of 53 patients without duodenal ulcer were colonized by a cagA-positive strain. A significant difference between patients with and without duodenal ulcer was observed (P = .00007; OR cannot be calculated, because none of the patients with duodenal ulcer were colonized by a cagA-negative strain).

cagA status and demographic characteristics. The mean age (± SD) of children without duodenal ulcer who were colonized by a cagA-positive strain (10.7 ± 2.1 years) was significantly higher (P = .002) than that of children colonized by a cagA-negative strain (8.1 ± 3.2 years). When we stratified children by age (3–6 years [1 cagA-positive and 7 cagA-negative], 7–10 years [13 cagA-positive and 7 cagA-negative], and 11–14 years [19 cagA-positive and 6 cagA-negative]), we found that increased cagA positivity was associated with increased age (P = .005) (figure 1).

When all patients were analyzed together, boys were found to be more frequently colonized by a cagA-positive strain than girls (86.8% vs. 64.3% [OR, 3.7; 95% CI, 1.1–13.4; P = .04). However, when only the subgroups of children without duodenal ulcer were analyzed, cagA status was not linked to sex (P = .2).

cagA status and macroscopic aspects of the gastric mucosa. Antral nodularity was not associated with cagA status either in the group of patients without duodenal ulcer (P = .3) or in all patients analyzed together (P = .1). In contrast, the presence of endoscopic gastritis was associated with a positive cagA status both when all children were evaluated (OR, 9; 95% CI, 2.5–34.6; P = .0002) and when only those without duodenal ulcer were considered (OR, 6; 95% CI, 1.5–25.5; P = .008). More intense macroscopic gastritis was also associated with a cagA-positive status (P = .00005 when all children were analyzed together and P = .001 when only those without duodenal ulcer were studied; table 2). In regard to the localization of the macroscopic gastritis, patients without duodenal ulcer who were colonized by a cagA-positive strain presented more frequently with alterations in both antrum and corpus (OR, 8.5; 95% CI, 1.5–36.9; P = .01) than did those colonized by a cagA-negative strain. This association was more significant when patients with and without duodenal ulcer were analyzed together (OR, 11; 95% CI, 2.2–42.3; P = .001; table 2).

cagA status and microscopic aspects of the gastric mucosa. The intensity and activity of gastritis in both the antral and oxyntic mucosae of patients without duodenal ulcer were greater in those colonized by a cagA-positive strain than in those colonized by a cagA-negative strain (table 3). Conversely, no difference was observed between cagA-positive patients with and without duodenal ulcer (intensity of the antral [P = .1] and oxyntic [P = .9] gastritis and activity of the antral [P = .7] and oxyntic [P = .6] gastritis; table 3).

The presence of microscopic gastric erosion was more frequently observed in patients with duodenal ulcer than in those without duodenal ulcer (P = .02). It was also associated with a cagA-positive status (P = .03).

Regenerative and degenerative changes and vacuolation were more frequently observed in the gastric mucosae of patients colonized by a cagA-positive strain than in the gastric mucosae of cagA-negative patients when all patients were analyzed (regenerative changes, P = .00007; degenerative, P = .00003; vacuolation, P = .00003) and when only patients without duodenal ulcer were analyzed. cagA status was not linked to sex (P = .2).

**Table 2.** Endoscopic aspects of the gastric mucosa of patients with and without duodenal ulcer (DU), according to cagA status.

<table>
<thead>
<tr>
<th>Endoscopic aspect</th>
<th>cagA+ patients (n)</th>
<th>cagA- patients (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With DU</td>
<td>Without DU</td>
</tr>
<tr>
<td>No gastritis</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Antral gastritis (mild)</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Pangastriosis</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>Mild</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Moderate</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Marked</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE. +, positive; -, negative.
Activity was scored according to the Revised Sydney System [21]: none (0), mild
in the outcome of the infection. Among the putative bacterial
ability among
duodenal ulcer. Besides host and environmental factors, vari-
infections in the world, few infected patients will develop peptic
Discussion
erative, ; vacuolation, ).
ulcer were considered (regenerative changes, P = .0001; degen-
erative, P = .0007; vacuolation, P = .003).

Table 3. Microscopic aspects of the gastric mucosa of patients with
and without duodenal ulcer (DU) according to cagA status (median
score).

<table>
<thead>
<tr>
<th>Gastritis</th>
<th>cagA+ patients With DU (n = 27)</th>
<th>cagA+ patients Without DU (n = 33)</th>
<th>cagA− patients With DU (n = 20)</th>
<th>cagA− patients Without DU (n = 20)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antral mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
<td>.02</td>
</tr>
<tr>
<td>Activity</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
<td>.005</td>
</tr>
<tr>
<td>Oxyntic mucosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
<td>.06</td>
</tr>
<tr>
<td>Activity</td>
<td>1.5</td>
<td>1</td>
<td>0</td>
<td></td>
<td>.02</td>
</tr>
</tbody>
</table>

NOTE. +, positive; −, negative. Degree of inflammatory intensity and ac-
tivity was scored according to the Revised Sydney System [21]: none (0), mild
(1), moderate (2), or marked (3).
* Analysis of the group of children without DU by Mann-Whitney U test.

Although *H. pylori* infection is one of the most common
infections in the world, few infected patients will develop peptic
duodenal ulcer. Besides host and environmental factors, vari-
ability among *H. pylori* strains may play a role in the differences
in the outcome of the infection. Among the putative bacterial
virulence factors, cagA, which is part of the cagA-PAI, is now
accepted as a risk factor for the development of duodenal ulcer
in adults [2, 3]. However, the impact of cagA status on the risk
of developing duodenal ulcer in childhood has not heretofore
been demonstrated.

Our results clearly demonstrate that infection with cagA-
positive *H. pylori* strains is associated with duodenal peptic
ulcer in children. We know of no other study that reported such
a large number of children with duodenal ulcer whose *H. pylori*
isolates were characterized to determine whether cagA was
present. Our results are in agreement with those of Oderda et
al. [10], who, in a study of 13 children with duodenal ulcer,
evaluated cagA status by the detection of specific antibodies
against CagA protein in the sera of the patients. Caution is
required in the interpretation of serologic tests, because spont-
aneous clearing of *H. pylori* infection has been demonstrated in
childhood [22], and antibodies to CagA protein persist longer
in patient sera [23]. Thus, false-positive results may increase
because of a slower clearing of the antibody. Furthermore, there
is evidence that in children the initial antibody response to *H.
 pylori* infection is to small-molecular-size antigens [24]. The
response to the CagA antigen was observed as late as day 857
in a child whose antibody response was monitored during pro-
gression from acute to chronic infection [24]. Therefore, nega-
tive results may be overestimated.

In contrast, association between infection with a cagA pos-
itive *H. pylori* strain and duodenal ulcer in children was not
demonstrated by others [12–14]. In all of those studies, however,
almost all children with duodenal ulcer were colonized by a
cagA-positive strain. These differing results may be explained
both by the small number of children with duodenal ulcer eval-
uated and by the presence of children from eastern European
and Asian countries, where the prevalence of cagA-positive
strains is high in the general population.

In agreement with the study of Husson et al. [11], who eval-
uated the presence of cagA in *H. pylori* strains isolated from
children without duodenal ulcer, we found that the presence of
the gene was associated with higher gastric inflammation. We
also observed that the presence of degenerative and regenerative
changes was associated with a cagA-positive status. cagA-pos-
itive strains are frequently cytotoxin producers, and cytotoxin
toes tissue damage in the stomachs of mice [25]. Furthermore,
cagA-positive strains induce an enhanced liberation of IL-8,
which possesses potent chemotactic activity for neutrophils and
lymphocytes [26]. Thus, degenerative changes may be the result
of the action of cytotoxin and a more marked inflammation.
From a histologic viewpoint, regenerative change is an expres-
ion of cellular proliferation. Cytokines, free oxygen radicals,
and other factors released by the more intense inflammatory
response after infection with a cagA-positive strain may interact
with cells in the proliferative compartment to increase cell pro-
iferation. Alternatively, the increased cell proliferation may be
a direct compensatory response to the accelerated cell loss and
may be related to an increase in the activity of growth factors
[27]. Thus, we may consider that a cagA-positive strain is more
aggressive, causing more marked destruction and proliferation
of the mucosal epithelium, and these gastric modifications may
aid in the progression to duodenal ulcer.

Another interesting result observed here was that cagA status
was correlated with patient age, being higher in older children.
This was not a result of the colonization of all children with
duodenal ulcer by a cagA-positive strain nor of their older age
in comparison to the control group, because the difference was
also significant when only children without duodenal ulcer were
analyzed. It was previously observed that antibodies against
CagA increase with increasing age up to 15 years [28], which
has been attributed to immaturity of the immune system or to
changes in bacterial antigen presentation, neither of which ac-
counts for our finding, because we did not evaluate cagA status
by determining anti-CagA antibodies but by detecting the gene
from the strain. Thus, in children, susceptibility to colonization
by a cagA-positive strain seems to be linked to age and may be
related to different expressions of gastric mucosa adherence
molecules, which may be modified by age. The fucosylated
blood group antigens Lewis b (Leb) and H-1 (the precursor for
Leb) mediate adherence of *H. pylori* to human gastric epithelial
cells in situ [29]. Ilver et al. [30] confirmed this result, dem-
onstrating that 63 (66%) of 95 clinical isolates of *H. pylori*
bound the Lea antibody. These authors demonstrated that bac-
terial binding to the Lea antibody is coded by the babA2 gene
and is associated with the presence of the cagA gene, although
the association between cagA-positive status and Leα antigen-binding activity is not mechanistic. An interesting and surprising finding, demonstrated by Celik et al. [14], is that children probably have fewer Leα receptors on surface mucous cells, which may explain the fact that susceptibility to colonization with a cagA-positive strain is linked to age.

Although in a first analysis we observed an association between cagA status and sex of the children, no association was present when only children without duodenal ulcer were evaluated. In the first analysis, the result reflected a higher prevalence of boys in the duodenal ulcer group. Men present more frequently with duodenal ulcer than do women, but we cannot provide an explanation for this fact in children. Hormonal differences may act as an ulcerogenic factor in adults but not in children. Duodenal gastric metaplasia, which is also considered a risk factor for duodenal ulcer development, likewise cannot explain our results, because in a recent study of children it was found not to be related to sex [31].

In conclusion, our data demonstrate an association between infection with a cagA-positive strain of H. pylori and duodenal ulcer in children. Infection with a cagA-positive strain was also associated with macroscopic and microscopic alterations of the gastric mucosae and the age of the children.

References