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***In situ* expression of *cagA* and risk of gastroduodenal disease in *Helicobacter pylori* infected children**

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Abstract

Gastroduodenal disease is more common among adults and children with *cagA*+ *H. pylori* infection, but disease severity varies among those infected with *cagA*+ strains. We examined whether *cagA in situ* expression can predict disease manifestations among *H. pylori* infected children. Fifty-one children were selected from 805 patients with abdominal symptoms who underwent esophago-gastroduodenoscopy with gastric biopsies. Endoscopic and histologic gastritis were scored and *H. pylori* colonization was quantified by Genta stain and *in situ* hybridization expression of *16S rRNA* and *cagA*. Endoscopy was either normal (n=14) or demonstrated nodularity (n=18), gastric ulcer (GU, n=8) or duodenal ulcer (DU, n=11). *H. pylori* was present in 7, 18, 6, and 10 children, respectively. Expression of *16S rRNA* and *cagA* were significantly higher in children with ulcer compared to normal. The fraction of *H. pylori* bacteria expressing *cagA in situ* was higher in children with ulcer compared to those with endoscopic nodularity (p<0.05). Thus, *cagA in situ* expression is increased in *H. pylori* infected children with peptic ulcers and may play a role in the pathogenesis of peptic ulcer disease during childhood. Determination of *in situ* expression of *cagA* complements traditional isolation and *in vitro* testing of single colony isolates.

Keywords

H. pylori; gastric ulcer; duodenal ulcer; nodularity; gastritis; *cagA* gene expression; virulence factor

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No potential, perceived, or real conflict of interest exist

• JR and MG designed and performed the study, analyzed the data and revised the manuscript; CSM mentored JR and MG, supervised performance and analysis of *in situ* hybridization studies, and revised the manuscript; HL designed and validated the probes used for *in situ* hybridization studies, and revised the manuscript; CO performed the statistical analysis and revised the manuscript; ERP reviewed and graded histopathology sections and revised the manuscript; CS helped in the analysis of the clinical data and revised the manuscript; and AD closely supervised and participated in the design and performance of all the steps of the study, and revised the manuscript.

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INTRODUCTION

Peptic ulcer disease (PUD) is strongly associated with *H. pylori* infection in adults and in children.^{1, 2} Of note, virtually all infected subjects have various degrees of gastritis but only 10% of *H. pylori* positive individuals develop PUD. This observation is attributed to a combination of environmental factors, host predisposition, *H. pylori* density, and the virulence of some *H. pylori* strains.³⁻⁶

The virulence factor that has received the most attention is the cytotoxin-associated gene A (*cagA*), which encodes the CagA protein and is implicated in the increased risk of PUD. CagA is translocated into the host cell, where it is phosphorylated, binds to SHP-2 tyrosine phosphatase, leading to cytokine production by the host cell, phosphorylation-dependent cytoskeletal rearrangement, and transcriptional activation of host's targets.⁷ The cellular effects of CagA may explain why patients infected with CagA⁺ strains usually have a higher inflammatory response and production of pro-inflammatory cytokines.^{4, 8, 9} In turn, these gastric mucosal responses induce peptic ulcer disease (PUD), precancerous lesions and cancer in adults.¹ The CagA protein also represents a marker for a genomic fragment called the *cag*-pathogenicity associated island (*cag* PAI), which encodes at least 27 proteins that are believed to have pathogenic properties.¹⁰

CagA is strongly immunogenic and high antibody titers are present in subjects infected by *cagA*⁺ strains. As a result, measuring plasma concentrations of anti-CagA IgG by ELISA has allowed testing for *cagA*⁺ infection in large epidemiological studies.¹¹ The ELISA method or direct PCR characterization of the strains isolated from children demonstrated a significant association between *cagA*⁺ *H. pylori* infections and PUD in children,¹²⁻¹⁵ although the association did not reach a level of statistical significance in some studies.^{16, 17} The cause of this variability is unknown, but may be related to differences in methodology, study populations, and/or bacterial strains.⁷

Here, we explored the possibility that *in situ cagA* expression is a critical determinant of disease outcome, i.e. inflammation or serious mucosal damage and PUD. To test this hypothesis, we used an *in situ* hybridization approach to quantify the expression of *H. pylori 16S rRNA* and *cagA* in the gastric mucosa of children with normal endoscopies, nodularity, gastric ulcer (GU), or duodenal ulcer (DU). We observed that bacterial density was increased in patients with GU and DU compared to children with normal endoscopy and that the fraction of *H. pylori* bacteria expressing *cagA* was increased in children with PUD.

MATERIALS AND METHODS

Study Population and treatment

During a 4-year period, 805 children underwent diagnostic upper endoscopy procedure in the Department of Pediatric Gastroenterology and Nutrition, Walter Reed Army Medical Center. A review of these 805 procedures showed that 41 cases had PUD and/or were *H. pylori* positive by CLO test and/or ELISA. Four of the *H. pylori* positive subjects had normal endoscopy. To increase the sample size in the normal endoscopy group and improve statistical power for comparisons among groups as defined by endoscopic findings, we then selected the 10 most recently endoscoped children who were age-matched, had no esophageal or gastroduodenal endoscopic abnormality, and were *H. pylori* negative by CLO test and H&E stains, plus ELISA if available. None of the patients had had burns or head injury.

Mean age was 12.2 years (range: 3 to 18), 61% were male, and ethnic distribution included 53% Caucasians, 27% African-Americans, 14% Hispanics, and 6% Asians. The indications

for endoscopy included abdominal pain (n=38, 74%), nausea and vomiting (n=33, 65%), upper gastrointestinal bleeding (n=8, 16%), weight loss (n=4, 8%) and heartburn (n=8, 16%). One patient was asymptomatic and evaluation was prompted by intrafamilial *H. pylori* infection and positive serology.

Upper Gastrointestinal Endoscopies

After obtaining informed consent, patients underwent diagnostic upper gastrointestinal endoscopy under conscious sedation or general anesthesia. At least two antral biopsies were obtained. Endoscopic findings were graded by reviewing the reports and photographs by a single investigator (JRR) and scored as normal, antral nodularity, gastric ulcer, or duodenal ulcer. Ulcers were 2–4 mm diameter single ulcers, but the exact size of each ulcer was not recorded. Following the endoscopy, all *H. pylori* infected patients received a triple therapy consisting of omeprazole (1 mg/kg), amoxicillin (40 mg/kg) and clarithromycin (10 mg/kg) twice a day for 10 days. Metronidazole was substituted for amoxicillin in allergic patients. Approval was obtained from the Institutional Review Boards of Walter Reed Army Medical Center and the Uniformed Services University of the Health Sciences.

Histology

Archived antral biopsies from these 51 children were serially sectioned (5 μ m) and H&E-stained, Genta-stained,¹⁸ and processed for fluorescence *in situ* hybridization (FISH). The FISH method is highly specific for *H. pylori* 16S rRNA as it uses a probe (biotin-labeled 5'-TACCTCT CCCACACTCT AGAATAGTAGT TTCAAATGC-3') that is 100% homologous with over 150 *H. pylori* strains published in the GenBank database.¹⁹ Similarly, the FISH *cagA* probe (5'-CTG CAA AAG ATT GTT TGG CAG A-3'-NH₂ digoxigenin labeled)²⁰ completely matched the *cagA* sequence of 128 *H. pylori* strains published in the GenBank database, with only one mismatch between the probe and the *cagA* sequence of 34 additional strains. FISH can detect a sequence that is different from the probe by one bp, and our data strongly indicate that we can detect the vast majority of *H. pylori* strains that express *cagA*.²⁰ The Genta silver stain method clearly outlines the presence of *H. pylori*, but it is not specific for this bacterium as it identifies virtually all Gram positive and negative bacteria, as well as fungi, many viral inclusions (e.g. cytomegalovirus) and parasites in larvae stage.

Chronic and acute inflammation and *H. pylori* density were scored according to the Updated Sydney System.²¹ A pediatric pathologist (ERP) blinded to the endoscopy, Genta, and FISH results performed the histologic assessment of gastritis.

Genta and FISH quantification.¹⁹

In brief, an Eclipse E800 Nikon microscope was used to examine the sections at 100X, 400X and 1000X. Morphometric quantification of *H. pylori* and gene expression was performed with fluorescence at 400X using an intraocular 27-mm grid (Klarmann Rulings, Inc.) and a fully functional super high pressure mercury lamp (Hg100W) (Ushio Electric Inc.) as reported.¹⁹ Bacterial density was counted without knowledge of the clinical diagnosis or endoscopic appearance and using constant fluorescence exposure. Isolated bacteria and clusters visualized in the lumen of the stomach and gastric glands were counted. Bacteria with characteristic morphology of *H. pylori* after Genta, 16S rRNA expression (red fluorescence by avidin Texas red, TR), *cagA* expression (green fluorescence per by avidin-fluorescein isothiocyanate, FITC), and co-localization of 16S rRNA and *cagA* (yellow fluorescence), corresponding to merged pictures, were observed and quantified at 400X (Figure 1). Three randomly selected fields of view were counted and the number of bacteria or clusters of bacteria per 10⁴ μ m³ (i.e. per 50- μ m by 40- μ m region of a 5- μ m-thick formalin-fixed section) was calculated (means \pm SEM).¹⁹ The expression of *cagA* relative to 16S rRNA expression was calculated as the quotient of these two parameters. Positive, negative, and non specific binding controls were performed as

described.²⁰ In addition, images were digitized using a charge-coupled device color camera (model HV-C20C20M; Hitachi) interfaced with a Sun X-20 workstation. Of note, Genta-stain quantification gives a general quantification of bacterial infection whereas *in situ*-hybridization is specific for *H. pylori* genes.

***H. pylori* positivity**

Among the 51 children, 41 patients were found to be *H. pylori* positive by Genta stain and/or fluorescence *in situ* hybridization (FISH) (see below). Three of them were from the group of 10 children with normal endoscopy who tested *H. pylori* negative by CLO and H&E and were found to be *H. pylori* positive by Genta stain and/or fluorescence *in situ* hybridization (FISH).

Data Analysis

Analysis of Variance (ANOVA) analysis was used to determine the overall significance of differences between groups; if significant differences were present, then post-hoc Tukey's tests were performed. The first ANOVA model included all subjects regardless of *H. pylori* status, in order to describe the association between endoscopy findings and gastritis in a general population of children receiving diagnostic upper endoscopies. A second ANOVA excluded *H. pylori* negative subjects, none of whom had gastritis, in order to explore whether any associations identified in the first ANOVA model could be explained by differences in *H. pylori* infection in the different groups.

Pearson's *r* was used to assess correlation between inflammatory indices, bacterial colonization and *cagA* gene expression. Fisher's exact test was used to compare the prevalence of intestinal metaplasia in the different groups; $p < 0.05$ was considered statistically significant.

RESULTS

Endoscopy findings and *H. pylori* status

Children were grouped according to findings at endoscopy (Table 1). The age, gender, ethnicity, and subjective symptoms of the patients were not significantly different among the endoscopic groups.

Histopathology (Figures 1 and 2)

Clusters of *H. pylori* were observed by Genta stain and FISH in 7/14 children with normal endoscopies, in 18/18 children with nodularity, in 10/11 DU and in 6/8 GU. Bacteria were adherent to the foveolar and epithelial surface of antrum, with occasional localization in superficial tortuous pits, outlining the contour of the convoluted surfaces of the epithelial cells. Some *H. pylori* were in close association with the apical part of columnar mucus secreting cells, in the areas where mucigen secretory granules are released by exocytosis. Bacteria were also abundant in the lumen of dilated pits.

Among all 51 children, acute gastritis scores were significantly higher ($p < 0.05$) in patients with nodularity compared to those with normal endoscopy but not compared to children with GU and DU. In addition, chronic gastritis scores were significantly higher in children with nodularity compared to those with normal endoscopy and GU, but not compared to those with DU (Table 1). In order to study the effect of *H. pylori* density and *cagA* expression on endoscopic appearance and gastritis, we then calculated gastritis scores only in *H. pylori* infected children (Table 2). We observed that chronic and acute gastritis scores were not significantly different among the groups, although acute gastritis scores were twice as high in patients with nodularity, GU, and DU compared to those with normal endoscopy.

Quantification of bacterial density in *H. pylori* infected children (Table 2)

Densities of *H. pylori* and *cagA*⁺ expression by FISH were significantly higher in the patients with GU and DU compared to *H. pylori* infected patients with normal endoscopy. In addition, the density of *cagA*⁺ bacteria in patients with nodularity was twice as high than in those with normal endoscopy (N.S.) and four times higher in those PUD ($p < 0.05$). Interestingly, the proportion of *H. pylori* expressing *cagA* was significantly higher in the GU and DU groups compared to the nodularity group, thus suggesting that PUD is more likely to appear in children infected by *H. pylori* with higher level of *in situ cagA* expression. Acute and chronic gastritis positively and significantly ($P < 0.05$) correlated with bacterial density by Genta ($r = 0.64$ and 0.56 , respectively), *16S rRNA* expression ($r = 0.64$ and 0.51) and *cagA* expression ($r = 0.45$ and 0.33).

Discussion

The major finding of the present study is that *in situ* transcription of *16S rRNA* and *cagA* is significantly increased in children with DU and GU compared to those with normal endoscopy and to those with endoscopic nodularity. In addition, a significantly greater proportion of *H. pylori* colonizing the stomach mucosa of children with PUD express *cagA in situ* compared to those with nodularity (table 2; $p < 0.05$), similar to our earlier observation in adult patients with gastric adenocarcinoma compared to controls.²⁰ Finally, both acute and chronic gastritis directly correlated with *in situ cagA* expression ($p < 0.05$) as previously reported with gastric *H. pylori* density assessed by quantitative culture and histology.⁴ These findings suggest that the relative fraction of *H. pylori* strains expressing *cagA in situ* determine in part whether a child develops PUD or not, although it has no significant effect on histologic gastritis. If these observations are confirmed Therefore, the test could be used to potentially predict the likelihood that a given patient has a higher risk of developing PUD and its complications, and it would justify verification of *H. pylori* eradication following antibiotic treatment in those patients. However, establishment of a cutoff indicating that an ulcer is present does requires a larger study. In addition, such a study should include a follow-up of children with normal endoscopy to determine whether carriage of strains with high % *cagA*⁺ *H. pylori* increases the ulcer risk, even if no ulcer is present at the time the biopsy was taken.

Importantly, these observations reflect *in situ* transcription of the *cagA* gene at the site of mucosal damage, an effect that is present only if the colonizing strain carries the *cagA* gene. Therefore, the present findings are in agreement with most studies showing that the risk of developing PUD is higher in pediatric and adult patients who are CagA seropositive or carry CagA⁺ strains.^{14, 15, 22, 23} They also agree with earlier observations made in adults with PUD using quantitative cultures and quantitative PCR.^{4, 24}

Our observations may also help to reconcile the absence of statistical association between PUD and carriage of *cagA* positive strains in two other studies^{16, 25} since *in vitro* testing for the presence of *cagA* in isolates may underestimate *in vivo* transcription as shown for the expression of *iceA1* and *vacA* virulence genes in biopsies of patients.²⁶ Similarly, transcription of *cagA* was increased in gastric biopsies harvested 10 days after inoculation of a *H. pylori* strain to Mongolian gerbils compared to *in vitro* testing of cultured isolates, and this increase appeared to be related to exposure to low pH.²⁷ The latter observation is of prominent relevance to *cagA* transcription in patients with DU since hyperacidity is a hallmark of duodenal ulcer disease. Because the multiple host and bacterial factors that determine the final outcome of *H. pylori* infection are known to vary in different populations, we propose that the direct determination of *cagA* transcription *in situ* provides a measurement of the combined effect of these factors in each of the patients being studied.

Another important observation made in the present study is that the fraction of *H. pylori* expressing *cagA in situ* was significantly higher in the group with PUD than in those with nodularity, although the absolute bacterial density was similar in the two groups (Table 2). Nodular gastritis is frequent in infected children and exceptional in uninfected individuals.¹⁷ The finding that *cagA* is expressed by a greater proportion of the *H. pylori* that colonize the stomach of children with PUD compared to those with nodular gastritis is relevant since *cagA*-positive *H. pylori* have been found to induce higher levels of DNA degradation than *cagA*-negative mutants *in vitro*.²⁸ and a relative increase in CagA expression may be responsible in part for defective repair in the gastroduodenal mucosa. However, the observation that the % of *cagA*+ strains was not significantly different in the normal and nodularity groups suggests that nodularity, in contrast to PUD, is not related to the % of *cagA*+ *H. pylori*.

Unexpectedly, three of the normal controls were found to be *H. pylori* positive by Genta and *in situ* hybridization despite the fact that CLO and H&E had been negative. This observation suggests that the sensitivity of usual *H. pylori* testing may be insufficient in pediatric gastroenterology..

One limitation of the present study was its retrospective nature, and the fact that frozen biopsies were not available for corroboration of *in situ* findings with quantitative RT-PCR to measure *cagA* RNA expression and/or with *in vitro* isolation and culture of colonizing *H. pylori* strains. We propose that these disadvantages are compensated for by the fact that the present approach can be used in archival material and that endoscopic observations, pathological analysis, and determination of *H. pylori* and *cagA* density were made by three different observers who were uninformed of the findings made by the other participants in the study.

In conclusion, the present study demonstrates that the *in situ cagA* gene expression is increased in *H. pylori* positive children with gastric and duodenal ulcer, which may play an important role in the pathogenesis of *H. pylori* related peptic ulcer during childhood. The present observations may help understand why only a fraction of CagA+ patients have DU, since patients who are *cagA* positive by PCR may have a relatively low expression level of the virulence gene *in situ*. These results are also consistent with the *in vivo* finding of genetic divergence between subclones of *H. pylori* and observations that most patients are infected by both *cagA*+ and *cagA*- strains.²⁹ We propose that the *in situ* approach is a relatively simple and inexpensive method that provides novel information on *in vivo* transcription of *H. pylori* virulence genes and thereby may complement the information provided by serology studies and traditional isolation and *in vitro* testing of single colony isolates. As such, the present results extend our understanding of PUD pathogenesis by demonstrating the importance of *cagA* expression at the cellular level. If such observations are confirmed, they could be used in clinical practice and permit identification of patients who need post-treatment control of *H. pylori* eradication and verification that *in situ cagA* expression is abolished.

Abbreviations

<i>H. pylori</i>	<i>Helicobacter pylori</i>
<i>cagA</i>	cytotoxin associated gene A
FISH	fluorescence <i>in situ</i> hybridization
PUD	peptic ulcer disease
DU	duodenal ulcer
GU	gastric ulcer

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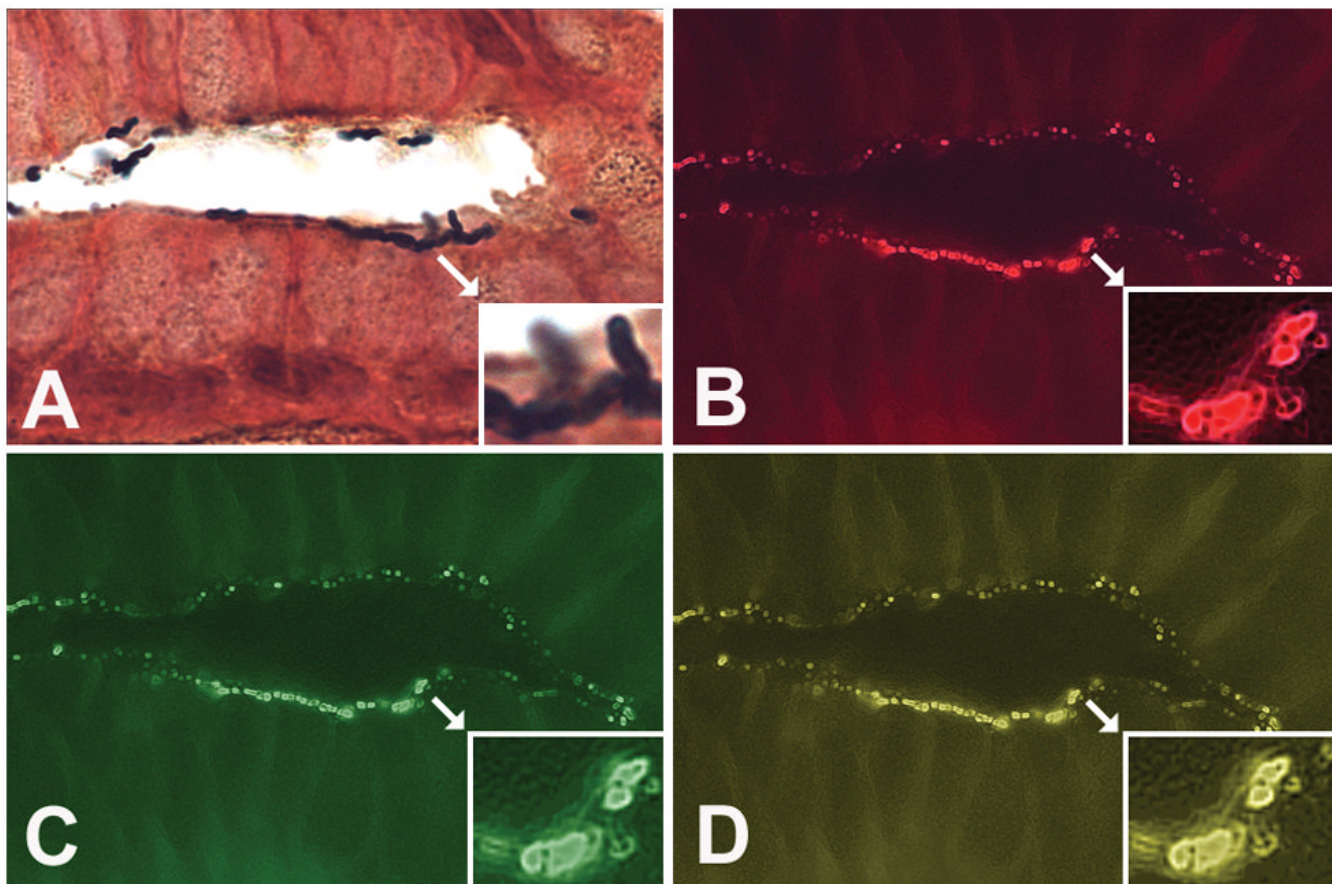


Figure 1. *H. pylori* colonization in the antral gland of a child with duodenal ulcer in two successive 5- μ m-thick serial sections (Original magnification $\times 1000$). A is a picture of the first serial section and B, C, and D are three pictures of the second serial section with three different epi-fluorescence filters. Because the two serial sections are 5- μ m apart, the *H. pylori* observed in A are not the same as the ones observed in B, C, and D, whereas B, C, and D represent different pictures of the same bacteria. Corresponding inserts show higher magnification of bacterial clusters (white arrows)

A. Genta stain of a gastric gland, first serial section; insert: *H. pylori* colony adherent to superficial epithelial cells.

B, C, and D: FISH of second serial section illustrating *16S rRNA* expression (red fluorescence) (B); *cagA* expression (green fluorescence) (C); and merge of pictures B and C showing co-localization of *16S rRNA* and *cagA* (yellow fluorescence, multiband filter) (D).

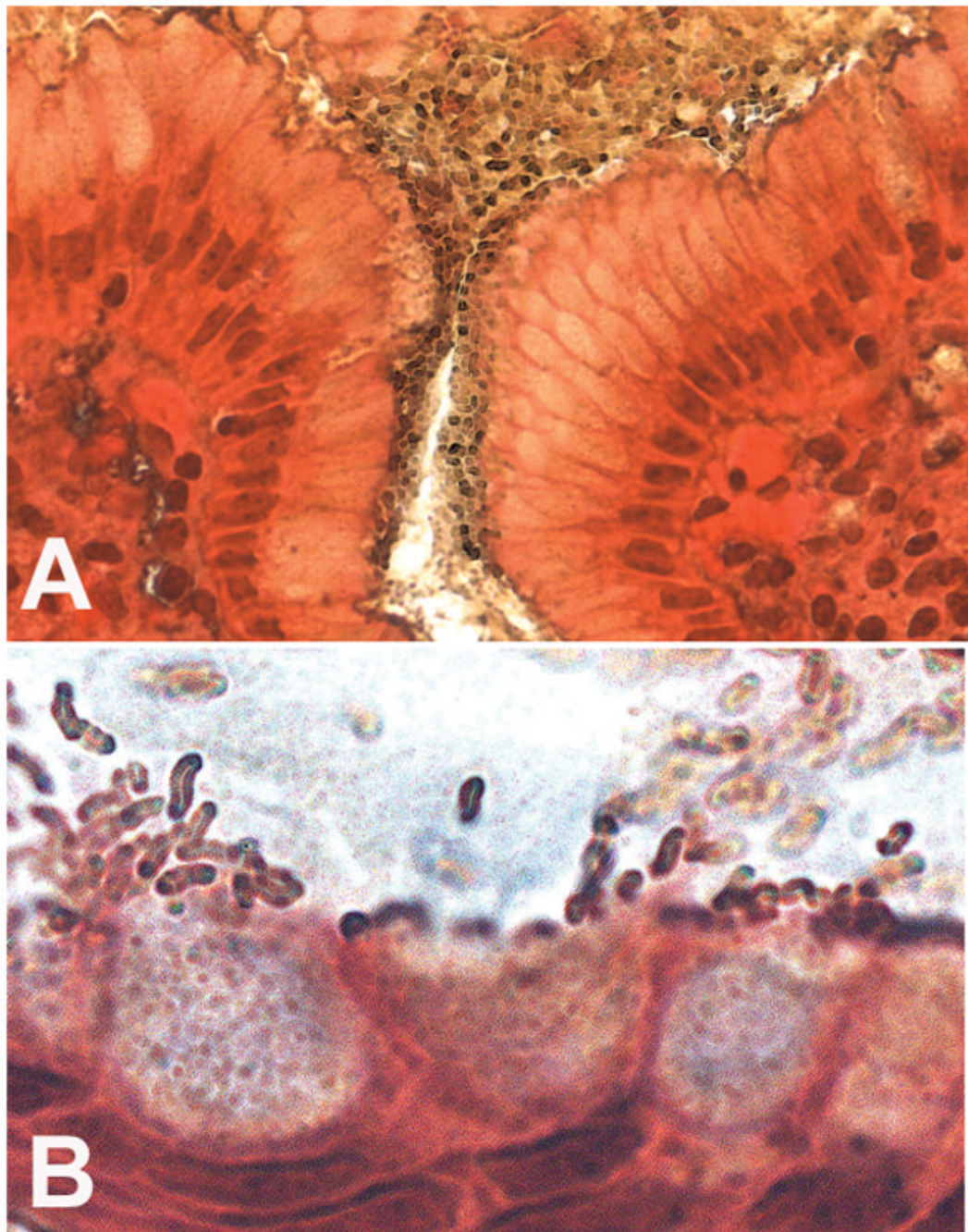


Figure 2. Genta stained paraffin sections of a patient with duodenal ulcer (Original magnification $\times 1,000$)

- A. Illustration of the presence of dense colonies in the adherent mucus that covers the superficial mucosa of the antral lumen and foveola.
- B. Enlargement detail of an antral gland, illustrating the presence of *H. pylori* colonizing the mucus with characteristic denser colonies at an intercellular junction on the left of the picture; note that *H. pylori* density overlying each cell is higher near the tallest epithelial cells, when the mucus is being released, and that some *H. pylori* are adjacent to mucus secretory granules while other bacteria congregate at the apex of the cells.

Table 1Demographics, histology grading, and *H. pylori* prevalence in all patients.

	Normal (n=14)	Nodularity (n=18)	GU (n=8)	DU (n=11)
Mean Age [years (range)]	13.1 (10–17)	12 (4–17)	10.4 (3–17)	12.7 (8.5–17)
Males [N (%)]	7 (50)	10 (53)	5 (63)	9 (82)
<i>H. pylori</i> positive [N (%)]	7 (50)	18 (100)	6(75)	10(91)
Acute Gastritis scores	0.4 ± 0.2	1.7 ± 0.3 ^a	1.1 ± 0.4	1.3 ± 0.4
Chronic Gastritis scores	1.1 ± 0.3	2.6 ± 0.2 ^a	1.3 ± 0.4 ^b	2.2 ± 0.3

^ap<0.05 vs normal *H. pylori* negative and^bp<0.05 vs Nodularity using ANOVA and post hoc Tukey's tests.

Table 2Gastritis scores and *H. pylori* density in *H. pylori* positive children

Endoscopic diagnosis	Normal (n=7)	Nodularity (n=18)	GU (n=6)	DU (n=10)
Acute Gastritis scores	0.7 ± 0.4	1.7 ± 0.3	1.5 ± 0.3	1.4 ± 0.4
Chronic Gastritis scores	2.1 ± 0.3	2.6 ± 0.2	1.7 ± 0.4	2.4 ± 0.2
Genta (N/10 ⁴ μm ³)	5.2 ± 1.0	11.3 ± 1.4 ^a	11.3 ± 1.9	12.8 ± 1.2 ^a
16S rRNA (N/10 ⁴ μm ³)	4.0 ± 1.0	8.4 ± 1.2	11.0 ± 2.1 ^a	10.6 ± 2.3 ^a
cagA+ (N/10 ⁴ μm ³)	2.0 ± 0.3	4.0 ± 0.8	8.2 ± 1.6 ^a	7.9 ± 1.3 ^{a,b}
CagA- (N/10 ⁴ μm ³)	2.0 ± 0.7	4.4 ± 0.6 ^a	2.7 ± 0.6	2.7 ± 0.2
% cagA+ strains	57.7 ± 6.1	45.2 ± 3.6	74.1 ± 2.6 ^b	70.9 ± 4.3 ^b

^a p <0.05 vs. normal group and

^b p <0.05 vs. nodularity group, using ANOVA and post hoc Tukey's tests.