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Serologic Detection of Infection with *cagA*$^+$ *Helicobacter pylori* Strains

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Approximately 60% of *Helicobacter pylori* isolates possess the *cagA* gene and express its 120- to 140-kDa product (CagA). In this study, the *cagA* gene was detected in *H. pylori* isolates from 26 (81.3%) of 32 patients with duodenal ulcers (DU), 17 (68.0%) of 25 patients with gastric ulcers, and 23 (59.0%) of 39 patients with nonulcer dyspepsia (NUD). By Western blotting (immunoblotting) with antiserum to CagA, in vitro CagA expression was demonstrated for 95.5% of *cagA*$^+$ strains compared with 0% of strains lacking *cagA*. Sera from patients infected with *cagA*$^+$ strains ($n = 66$) reacted with recombinant CagA in an enzyme-linked immunosorbent assay to a significantly greater extent than either sera from patients infected with strains lacking *cagA* ($n = 30$) or sera from uninfected persons ($n = 25$) ($P < 0.001$). A strain lacking *cagA* was isolated from eight patients who had serum immunoglobulin G antibodies to CagA, which suggests that these patients were infected with multiple strains. Serum immunoglobulin G antibodies to CagA were present in 87.5, 76.0, and 56.4% of patients with DU, gastric ulcers, and NUD, respectively (odds ratio, 5.41; 95% confidence interval, 1.44 to 24.72; $P = 0.004$ [DU versus NUD]). These data demonstrate an association between infection with *cagA*$^+$ *H. pylori* and the presence of duodenal ulceration and indicate that serologic testing is a sensitive method for detecting infection with *cagA*$^+$ strains.

Helicobacter pylori organisms are gram-negative bacteria that infect the gastric mucosa of 20 to 80% of humans throughout the world (2, 35). The prevalence of *H. pylori* infection varies depending upon the age, the geographic location, and the socioeconomic status of the subjects studied (35). In most infected persons, *H. pylori* infection is well tolerated, with few or no symptoms for decades (13). However, infection with this organism is a significant risk factor for the development of peptic ulceration (23, 26) and adenocarcinoma of the distal stomach (22, 24). The lifetime risk for development of peptic ulcer disease in *H. pylori*-infected persons is estimated to be greater than 10% (32). The eradication of *H. pylori* results in a marked reduction in the rate of recurrence of duodenal or gastric ulcers (18–20, 30).

The basis for the development of peptic ulcer disease in only a subset of *H. pylori*-infected persons is poorly understood. Several host factors, include smoking, blood type O, and male gender, are known to predispose individuals to the development of peptic ulceration. In addition, there may be specific virulence determinants of *H. pylori* strains that influence the outcome of infection. Approximately 50% of *H. pylori* strains produce vacuolating cytotoxin activity in vitro (5, 9), and infection with cytotoxin-producing strains has been associated with the presence of peptic ulceration (14, 17, 29, 36). Another potential virulence determinant of *H. pylori* is the *cagA* product. The *cagA* gene, which encodes a family of high-molecular-mass proteins (120 to 140 kDa), is present in approximately 60% of *H. pylori* isolates and is the first *H. pylori* gene to be identified that is not present in all strains (4, 38). Importantly, serologic responses to CagA have been detected in *H. pylori*-infected persons with peptic ulcer disease more frequently than in infected persons with gastritis alone (4, 8, 11, 40). However, it is not known whether this association reflects primarily the characteristics of the infecting *H. pylori* strains or whether there may be nonspecific humoral immune activation in ulcer patients that accounts for this association. Therefore, we sought to determine whether serum antibodies to CagA accurately reflect the characteristics of infecting *H. pylori* strains and to test the hypothesis that *H. pylori* isolates from patients with peptic ulcer disease possess the *cagA* gene more frequently than isolates from patients with nonulcer dyspepsia.

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MATERIALS AND METHODS

Study population. All 96 *H. pylori*-infected patients studied were symptomatic adults who underwent gastroduodenoscopy and gastric biopsies in the Nouvelle Clinique de la Basilique, Brussels, Belgium, between 1989 and 1993. The patients were classified at the time of endoscopy as having duodenal ulcers ($n = 26$), gastric ulcers ($n = 25$), or no evidence of mucosal ulceration ($n = 39$). Six other patients had no ulceration at the time that the *H. pylori* isolate was cultured but had a past history of endoscopically diagnosed duodenal ulcers; thus, 32 patients were considered to have duodenal ulcer disease. Patients with gastric or duodenal ulcers associated with the chronic intake of nonsteroidal anti-inflammatory agents were excluded from the study (34). Serum samples were obtained from each patient on the day of the endoscopic procedure. Sera from 25 previously described patients (7) who were not infected with *H. pylori* as assessed by histologic examination of antral biopsies, CLO test, and serologic assay were studied as controls.

Culture of *H. pylori* isolates. Gastric biopsy specimens were homogenized, inoculated onto selective charcoal agar medium (BCCA) (16) and 10% horse blood agar plates, and then incubated for 7 days at 37°C under microaerobic conditions. A single colony was picked from each primary culture plate and identified as *H. pylori* on the basis of typical colony morphology, characteristic appearance on Gram staining, and positive urease, oxidase, and catalase tests. The single colony was subsequently passaged and frozen at −70°C in brucella broth containing 15% glycerol prior to subsequent studies.

Characterization of *H. pylori* isolates. To determine whether *H. pylori* isolates contained the *cagA* gene, each strain underwent colony hybridization with a ^{32}P-labelled *cagA* probe, as described previously (38) except that washes were more stringent (0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] containing 0.5% sodium dodecyl sulfate [SDS] at 65°C). The probe used in these studies was a 1.4-kb *Bam*HI-*Eco*RI restriction fragment of plasmid pMC3, which contains bp 845 to 2240 of the 3,543-bp *cagA* gene (38). *H. pylori* 60190 and

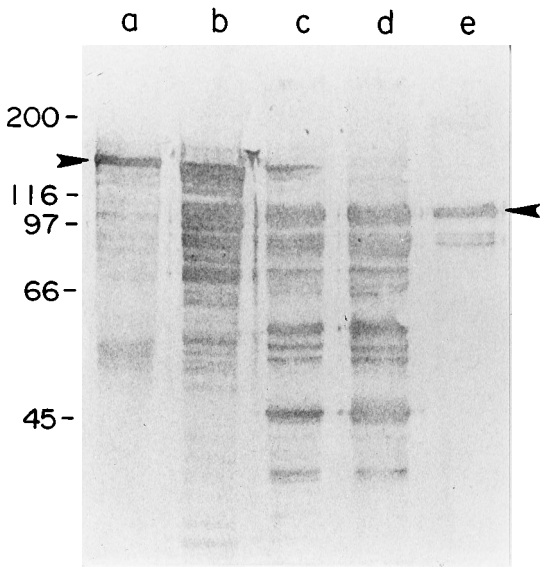

FIG. 1. Purification of rCagA from *E. coli* DH5α containing pEM3. Sequential preparations in the purification of recombinant CagA were electrophoresed on a 7% acrylamide gel and, after transfer to nitrocellulose paper, were immunoblotted with rabbit anti-CagA serum. Lane a, *H. pylori* 84-183 whole cells; lane b, *E. coli* DH5α whole cells containing pEM3; lane c, *E. coli* DH5α containing pEM3, following sonication, centrifugation, and precipitation of the supernatant with 50% ammonium sulfate; lane d, sample from lane c after subsequent anion-exchange chromatography; lane e, sample from lane d after subsequent gel filtration chromatography. Numbers on the left indicate molecular masses in kilodaltons. The 128-kDa *cagA* product (lane a) and its largest degradation product in *E. coli* (lane e) are indicated by arrowheads.

Escherichia coli XL1Blue were tested as positive and negative controls, respectively.

Purification of rCagA by sequential column chromatography. A plasmid (pEM3) containing the entire *cagA* gene from *H. pylori* 84-183 was constructed by digestion and ligation of two plasmids (pMC3 and pYB2) which contained overlapping fragments of the *cagA* gene (38). In brief, the 3.6-kb *cagA*-containing insert in pMC3 was cloned into pGEM7Z to generate pEM1, and then the 2.6-kb *Sac*I-*Bst*EII fragment from pYB2 was cloned into *Sac*I-digested pEM1. A clone with the correct orientation was selected and was named pEM2. A 1-kb *Ssp*I fragment upstream from *cagA* then was deleted to create pEM3 (ATCC 69273). A band approximately 128 kDa in size was detected by immunoblotting *E. coli* DH5α cells containing pEM3, but not pGEM7Z, with human serum known to recognize CagA (not shown). *E. coli* DH5α containing pEM3 was cultured for 16 h in 2XYT medium with 100 μg of carbenicillin per ml. After centrifugation, the cell pellet was resuspended in 20 mM Tris (pH 8.0) containing 10 mM EDTA and 0.1 mM dithiothreitol. The cell suspension was sonicated and then centrifuged for 15 min at 20,000 × *g*. A 50% saturated solution of ammonium sulfate was added to the supernatant, and the solution was centrifuged for 15 min at 20,000 × *g*. The pellet was resuspended in 20 mM Tris (pH 7.6) containing 6 M urea, dialyzed in the same buffer, applied to a MonoQ 5/5 anion-exchange column (Pharmacia), and eluted with the same buffer containing a gradient of 1 M NaCl. The peak containing recombinant CagA (rCagA) then was purified further by passage through a Superose 6 10/50 column in 60 mM Tris (pH 7.6) containing 1% SDS. Immunoblotting of the CagA-containing preparations indicated that the rCagA produced by pEM3 was identical in size to the native CagA of *H. pylori* 84-183 (Fig. 1, lanes a and b). However, apparent degradation of the recombinant 128-kDa protein to an approximately 105-kDa band occurred during the purification process (Fig. 1, lanes c, d, and e).

Detection of CagA expression by *H. pylori* isolates. Antiserum to CagA was prepared by immunizing a New Zealand White rabbit with rCagA that had been purified by sequential column chromatography as described above. The procedure for immunization was as described previously (5). Expression of CagA by *H. pylori* strains was assessed by immunoblotting whole cells with either the rabbit anti-CagA serum or human anti-128-kDa protein serum (38).

ELISA for detection of serum IgG antibodies to CagA. An enzyme-linked immunosorbent assay (ELISA) was performed with approximately 0.1 μg of chromatographically purified rCagA per microtiter well, using ELISA methodology described previously (7, 25). Optimal concentrations of rCagA and dilutions of human sera were determined by checkerboard analysis (not shown). Serum samples from patients were diluted 1:500, and the second antibody was peroxidase-conjugated anti-human immunoglobulin G (IgG) (7, 25). Results are expressed as optical density ratios, which were calculated on the basis of comparison with a panel of six reference sera. A cutoff for seropositivity was defined as 2 standard deviations above the mean reactivity of the sera from 25 uninfected persons.

Statistical methods. Results are expressed as the mean ± standard error of the mean. Distributions of optical densities were compared by using Student's *t* test for independent variables. Proportions were compared by using Fisher's exact test or chi-square analysis.

TABLE 1. Demographic characteristics of the study population of *H. pylori*-infected patients

Classification (*n*)	% Male	Age (yr) (mean ± SEM)	Ethnicity (%)		
			Belgian	Moroccan	Other
Duodenal ulcer (32)[a]	62.5	48.6 ± 3.1	43.7	18.8	37.5
Gastric ulcer (25)	32.0	62.1 ± 3.4	76.0	16.0	8.0
Nonulcer dyspepsia (39)	38.5	52.7 ± 2.7	61.5	15.4	23.1

[a] Includes six patients who had endoscopically confirmed duodenal ulcers in the past.

RESULTS

Characteristics of the study population. The demographic characteristics of the study population are shown in Table 1. Altogether, 53 female and 43 male adults were studied (mean age, 53.8 years; range, 20 to 92 years). The ethnicities of the 96 patients were as follows: Belgian ($n = 57$), Moroccan ($n = 16$), non-Moroccan African ($n = 3$), Italian ($n = 7$), Turkish ($n = 5$), Spanish ($n = 4$), Greek ($n = 2$), and Eastern European ($n = 2$). The patient groups were similar (Table 1), except that patients with gastric ulcers were significantly older than patients with either duodenal ulcers or nonulcer dyspepsia ($P < 0.05$), and patients with duodenal ulcers were more likely to be male.

Detection of *cagA* in *H. pylori* isolates. To determine which *H. pylori* isolates possessed *cagA*, the 96 *H. pylori* isolates underwent colony hybridization with a *cagA* probe under high-stringency conditions. Overall, *cagA* was detected in 66 (68.7%) of the 96 isolates. Seven patients underwent endoscopy on two occasions, and in each of these cases, the *cagA* genotype of the organism isolated remained unchanged. The *cagA* gene was detected in 26 (81.3%) of 32 isolates from patients with current or previous duodenal ulcer disease, compared with 59.0% of isolates from patients with nonulcer dyspepsia (odds ratio [OR], 3.01; 95% confidence interval [95% CI], 0.91 to 10.90; $P = 0.04$) (Table 2). Isolates from the 16

TABLE 2. Characterization of *H. pylori* isolates and serum IgG anti-CagA responses in patients with defined gastroduodenal pathology

Classification (*n*)	No. (%)			
	Infected with *cagA*$^+$ isolates[a]	With positive CagA serology	Positive for *cagA* by:	
			Either hybridization or serology	Both hybridization and serology
Nonulcer dyspepsia (39)	23 (59.0)	22 (56.4)	26 (66.7)	19 (48.7)
Gastric ulcer (25)	17 (68.0)	19 (76.0)	20 (80.0)	16 (64.0)
Duodenal ulcer[b] (32)	26 (81.3)[c]	28 (87.5)[c]	28 (87.5)[c]	26 (81.3)[c]

[a] As determined by colony hybridization.
[b] Includes six persons who had endoscopically confirmed duodenal ulcers in the past.
[c] Significantly different ($P < 0.05$) from result for patients with nonulcer dyspepsia.

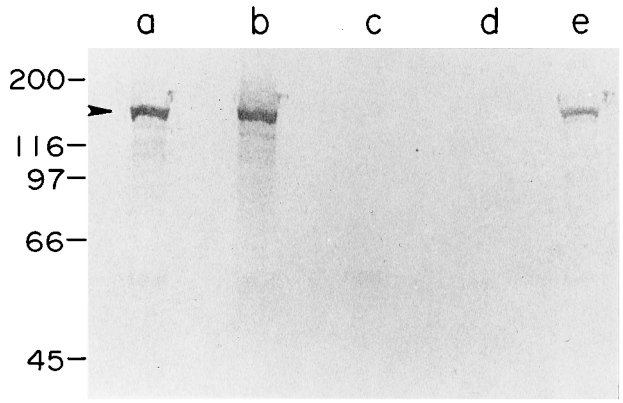

FIG. 2. Immunoblot of *H. pylori* cells with anti-CagA rabbit serum. *H. pylori* cells were electrophoresed on a 7% acrylamide gel, and following transfer to nitrocellulose paper, proteins were immunoblotted with rabbit anti-CagA serum. Lanes a, b, and e, *cagA*$^+$ *H. pylori* strains; lanes c and d, *H. pylori* strains lacking *cagA*. The *cagA* product (arrow) was detected by this antiserum in the three *cagA*$^+$ strains but not in the two strains lacking *cagA*. Numbers on the left indicate molecular masses in kilodaltons.

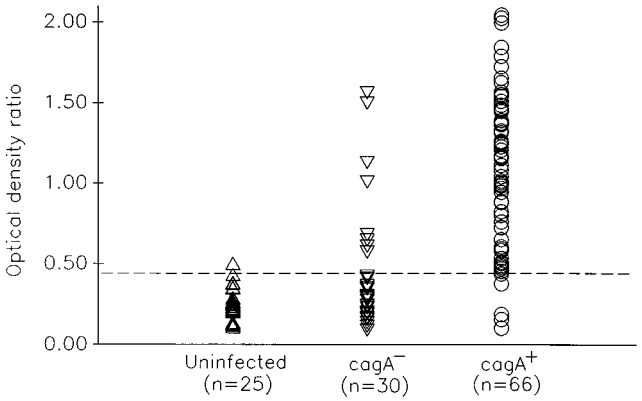

FIG. 3. Presence of serum anti-CagA IgG in *H. pylori*-infected patients and controls. Sera from 96 *H. pylori*-infected patients and 25 uninfected persons were incubated with chromatographically purified rCagA in an ELISA. Sera from the 66 patients from whom *cagA*$^+$ strains were isolated reacted to a significantly greater extent than did either sera from 30 patients from whom strains lacking *cagA* were isolated or sera from 25 uninfected persons (mean optical density ratios, 1.08 ± 0.058, 0.439 ± 0.071, and 0.258 ± 0.019, respectively; $P < 0.001$). A cutoff for seropositivity, defined as 2 standard deviations above the mean optical density ratio produced by the 25 sera from uninfected persons, is indicated by the dashed line.

patients of Moroccan ethnicity were *cagA*$^+$ significantly less frequently than isolates from the 57 Belgian or 23 non-Belgian, non-Moroccan patients (31.3% versus 73.7 and 82.6%, respectively; OR, 7.06; 95% CI, 1.92 to 28.66; $P = 0.001$). Production of the CagA protein was detected by immunoblotting for 63 (95.5%) of the 66 isolates that contained the *cagA* gene but for none of the isolates that lacked the gene (Fig. 2). With one exception, the CagA proteins produced by the *cagA*$^+$ strains ranged in size from approximately 120 to 135 kDa (not shown); one isolate produced a CagA protein 96 kDa in size.

Detection of serum anti-CagA IgG antibodies. To detect antibodies to CagA, sera were tested in an ELISA for reactivity with rCagA purified by sequential column chromatography as described in Materials and Methods. IgG antibodies have been the most specific class of antibodies for the detection of *H. pylori* infection (25), and therefore, we analyzed IgG antibody responses to CagA. We first sought to determine whether there was a correlation between the presence of anti-CagA antibodies in serum and infection with a *cagA*$^+$ *H. pylori* isolate. Sera from the 66 patients from whom *cagA*$^+$ strains were isolated reacted to a significantly greater extent than did either sera from the 30 patients from whom strains lacking *cagA* were isolated or sera from 25 uninfected persons (Fig. 3). On the basis of the cutoff defined in Materials and Methods, 61 (92.4%) of the 66 patients from whom *cagA*$^+$ strains were isolated were seropositive, and 8 (26.7%) of 30 patients from whom strains lacking *cagA* were isolated were seropositive (OR, 33.6; 95% CI, 8.75 to 139.37, $P < 0.001$). Of the three patients infected with *cagA*$^+$ strains that failed to express detectable CagA in vitro, two had antibodies to CagA in serum, suggesting that the loss of CagA expression by these strains could be an artifact related to in vitro passage. As expected on the basis of the colony hybridization data, non-Moroccan patients were seropositive for anti-CagA IgG significantly more frequently than Moroccan patients (77.5 versus 43.7% seropositive, respectively; OR, 4.43; 95% CI, 1.28 to 15.69; $P = 0.012$).

Presence of serum antibodies to CagA in patients with peptic ulcer disease. We next sought to determine whether patients with peptic ulcer disease differed from patients with nonulcer dyspepsia in serologic responses to CagA. On the basis of the cutoff defined above, serum anti-CagA IgG was present in 87.5% of patients with duodenal ulcer disease, compared with 56.4% of patients with nonulcer dyspepsia (OR, 5.41; 95% CI, 1.44 to 24.72; $P = 0.004$) (Table 2). Among patients from whom *cagA*$^+$ strains were isolated, the magnitude of the anti-CagA IgG response was not significantly different for patients with active duodenal ulcers and those with nonulcer dyspepsia (mean optical density ratios, 1.20 ± 0.10 and 1.03 ± 0.11; $p = 0.28$). An analysis of the four duodenal ulcer patients who lacked both bacteriologic and serologic evidence of infection with *cagA*$^+$ strains indicated that these patients were more likely to be of Moroccan ethnicity than the remaining 28 duodenal ulcer patients (75 versus 10.7%; $P = 0.01$). Although patients with duodenal ulcer disease were infected with *cagA*$^+$ strains more often than were patients with gastric ulcers, the differences were not significant in any of the analyses (Table 2).

DISCUSSION

The CagA product of *H. pylori* is an immunodominant antigen, characterized by marked antigenicity despite a relatively low molar representation among cellular proteins (4, 38). Although the function of CagA is not known, *H. pylori* strains can be conveniently typed into two groups based on the presence or absence of the *cagA* gene (4, 38). In this study, we found a strong correlation between the presence of serum antibodies to CagA and the isolation of a *cagA*$^+$ strain from a patient. However, 8 (26.7%) of 30 patients from whom a strain lacking *cagA* was isolated had serum antibodies to CagA. We believe it is unlikely that serologic cross-reactivity is responsible for this phenomenon, because of the uniformly low ELISA values observed with sera from uninfected controls (Fig. 3). One hypothesis is that *H. pylori* strains can spontaneously lose the *cagA* gene during in vitro passage. An alternate explanation is that these eight patients were infected by more than one *H. pylori* strain, a phenomenon that has been observed in several previous studies (1, 15, 28) and that may be more common than previously recognized. The selection of single colonies of *H. pylori* for culture in the current study would have particularly favored incomplete sampling. In contrast, serologic test-

ing permits the detection of antibody responses to all infecting *H. pylori* strains, regardless of their relative concentrations in the stomach, and is likely to be a more sensitive indicator of infection with *cagA*$^+$ strains than testing of single *H. pylori* isolates.

An unexpected finding in this study was that *cagA*$^+$ strains were isolated from Moroccan patients significantly less frequently than from patients of other ethnic groups. Most of the Moroccan adults currently living in Belgium emigrated 20 to 30 years ago, and therefore, many of these patients were probably infected with *H. pylori* strains acquired during childhood in Morocco. The identification of specific populations of *H. pylori* within particular geographic or ethnic groups has not been previously reported. Further studies are required to determine whether Moroccan patients are predominantly infected with strains lacking *cagA* and whether other differences in the *H. pylori* isolates from diverse human populations can be identified.

Previous studies of patients in the United States, Great Britain, and Italy have investigated serum or mucosal antibody responses to CagA and have demonstrated strong associations between the presence of antibodies to CagA and peptic ulcer disease (4, 8, 11, 40). In addition, the presence of serum antibodies to CagA recently has been associated with an increased risk for development of gastric cancer (3, 12). However, *H. pylori* isolates from the patients in these previous studies were not characterized to determine whether the *cagA* gene was present. In this study, using combined serologic and genetic detection methods, we found that patients with duodenal ulcer disease were infected with *cagA*$^+$ strains significantly more frequently than patients with nonulcer dyspepsia. The prevalence of infection with *cagA*$^+$ strains among patients with gastric ulcers was intermediate between the prevalences among patients with nonulcer dyspepsia and with duodenal ulcer disease, a finding consistent with previous serologic data (8). The observed differences in the rates of *cagA*$^+$ *H. pylori* infection among patients with duodenal ulcer and gastric ulcer disease may be related to the known differences in the pathogenesis and epidemiology of these two diseases (31, 33).

The proportion of duodenal ulcer patients in this study who were infected with *cagA*$^+$ strains (87.5% by combined bacteriologic and serologic testing) is lower than that observed in three previous studies, all of which showed anti-CagA seropositivity rates approaching 100% among duodenal ulcer patients (4, 8, 11, 40). Three of the four duodenal ulcer patients in this study who lacked both bacteriologic and serologic evidence of *cagA*$^+$ *H. pylori* infection were of Moroccan ethnicity, which suggests that the association between *cagA*$^+$ *H. pylori* and duodenal ulceration may be stronger among some racial or ethnic groups than others or that there may be additional, unrecognized factors that contributed to duodenal ulceration in the Moroccan patients. Thus, although the present study suggests that duodenal ulceration may occasionally develop in the absence of *cagA*$^+$ *H. pylori* infection, the current and previous data suggest that infection with *cagA*$^+$ *H. pylori* strains is associated with an increased risk for development of duodenal ulcer disease. Peptic ulcer disease does not develop in the majority of nonulcer dyspepsia patients who are infected with *cagA*$^+$ strains, and therefore, it is likely that various host and environmental factors are also important determinants of ulcer pathogenesis.

The basis for the association between *cagA*$^+$ *H. pylori* strains and duodenal ulcer disease is not yet well understood. One possible explanation is that the presence of the *cagA* gene is a marker for vacuolating cytotoxin production by *H. pylori* strains (4, 38, 39). Mucosal ulceration has been produced by the purified cytotoxin in a mouse model (37), which supports this hypothesis. In addition, infection with *cagA*$^+$ *tox*$^+$ isolates is associated with a higher grade of antral polymorphonuclear leukocyte infiltration than that which accompanies infection with strains lacking *cagA* (6, 11, 27). *cagA*$^+$ strains induce greater levels of interleukin-8 production by gastric epithelial cells in vitro than strains lacking *cagA* (10), which may account for the prominent neutrophil infiltration detected in vivo. Thus, *cagA* appears to be a useful marker for identifying *H. pylori* strains that possess several properties associated with increased virulence.

Currently, antimicrobial therapy is recommended only for *H. pylori*-infected patients with peptic ulcer disease (21). However, if the association between *cagA*$^+$ *H. pylori* strains and severe gastroduodenal pathology continues to be confirmed in subsequent studies, it may prove clinically useful to determine whether dyspeptic patients are infected with *cagA*$^+$ strains and to target such patients for antimicrobial therapy. In this event, the availability of a serologic test for detecting infection with *cagA*$^+$ *H. pylori* strains, as described in this study, would reduce the need for endoscopy and gastric biopsy. Prospective clinical studies and cost-benefit analyses will be required to determine whether such a strategy is desirable.

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