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Detection of Serum Antibodies to CagA and VacA and of Serum Neutralizing Activity for Vacuolating Cytotoxin in Patients with *Helicobacter pylori*-Induced Gastritis

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Thirty patients with dyspepsia, with histological diagnosis of gastritis, and with endoscopic diagnosis of peptic ulcer disease (PUD) ($n = 13$) or nonulcer dyspepsia (NUD) ($n = 17$) were admitted to the study. *Helicobacter pylori* vacuolating cytotoxin-producing strains (Tox^+) were isolated from 14 (46.7%) patients, whereas non-cytotoxin-producing (Tox^-) *H. pylori* strains were isolated from the remaining patients. Of 30 patients studied, 20 (66.7%) had serum cytotoxin neutralizing activity in vitro. Fourteen patients with Tox^+ *H. pylori* strains showed serum cytotoxin neutralizing activity and serum immunoglobulin G (IgG) and IgA antibodies reactive with both 87-kDa *H. pylori* vacuolating cytotoxin (VacA) and 128-kDa cytotoxin-associated gene product (CagA) by immunoblotting using native enriched preparations of VacA and CagA proteins from *H. pylori* culture supernatants as the antigens. A 94-kDa antigen cross-reacting with the 87-kDa VacA protein could be demonstrated in culture supernatant with immune sera from humans and animals. All patients ($n = 10$) lacking serum neutralizing activity were also negative for IgG or IgA against VacA antigen, whereas 6 of the 10 patients showed IgG serum antibody responses against CagA antigen. The prevalence of antibodies to VacA and CagA antigens was significantly ($P < 0.001$) higher in patients with gastritis (20 and 26 patients for VacA and CagA, respectively, of 30 patients) than in *H. pylori* culture-negative controls (0 of 27 for both VacA and CagA) and in randomly selected blood donors (17 and 21 for VacA and CagA, respectively, of 120 subjects). All patients with PUD had antibodies to CagA, whereas 13 of 17 (76.5%) patients with NUD had anti-CagA antibodies. Serum IgG antibodies to VacA were present in 9 (69.2%) patients with PUD of 13 patients and in 11 (64.7%) patients with NUD of 17 patients. Anti-CagA antibodies seemed to correlate better with PUD than anti-VacA antibodies.

Helicobacter pylori has been established as a major cause of chronic active gastritis and peptic ulcer disease (PUD), also associated with gastric cancer (2, 26). However, the clinical diagnosis of *Helicobacter* infection may be difficult due to the absence of either specific symptoms or specific endoscopic findings. In addition, the results of cultures, histological tests, and urease test on biopsies may be highly variable depending on the size and number of the specimens obtained. Therefore, immunological tests may be critical for the reliable diagnosis of *H. pylori* infections.

Several serological studies using enzyme-linked immunosorbent assay (ELISA) or Western immunoblotting (WB) have been performed to detect antibodies against *H. pylori*. Most of the available data have been obtained by ELISA for serum immunoglobulins against *H. pylori* (17, 22, 24), where whole bacterial cells have been used as the antigen. However, the presence of serological cross-reactions between *H. pylori* and other gram-negative bacteria such as *Pseudomonas aeruginosa*, *Campylobacter jejuni*, and *Haemophilus influenzae* (3, 19) has called into question the specificity of the results obtained by ELISAs using the whole bacterial cell as the antigen. More recently, recombinant CagA has been used as an antigen by ELISA for specific serological diagnosis of *H. pylori* infection in patients with PUD and nonulcer dyspepsia (NUD) (7, 13, 16).

Many studies have been also performed by WB technique with whole *H. pylori* cells as the antigen (1, 23, 32, 33). The high number of bands recognized by patients' sera hampered reliable detection of specific *H. pylori*-reactive antigens. Very few studies have been performed with purified or partially purified major *H. pylori* antigens (12). Prominent *H. pylori* antigens detected by WB technique are represented by a 120- to 128-kDa CagA protein (11, 14, 31) and by a protein with vacuolating cytotoxic activity (VacA) (21) that migrates at 87 to 94 kDa under denaturing conditions (9, 35).

We now report on the detection of specific serum immunoglobulin G (IgG) and IgA responses against VacA and CagA of *H. pylori* in 30 patients with gastritis and in control subjects by WB technique, using as antigens enriched preparations from *H. pylori* broth culture supernatant that allow simple and clear detection of major *H. pylori* antigens. The relationships between serum antibodies against the VacA and CagA antigens and serum neutralizing activity of vacuolating cytotoxin have also been analyzed in both patients and controls.

MATERIALS AND METHODS

Patients and clinical specimens. Thirty patients (14 men and 16 females; mean age, 46 years; age range, 22 to 75 years) who underwent gastroduodenoscopy for dyspepsia and were positive for *H. pylori* by both culture and histology were admitted to the study. Gastric specimens were cultured for *H. pylori*, as previously described (29). Briefly, samples were homogenized and cultured on Columbia agar base (Oxoid, Milan, Italy) enriched with 7% horse blood and Dent's

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selective supplement (Oxoid). The cultures were incubated in a microaerophilic atmosphere at 37°C in GasPak jars and Campy Pack II envelopes (BBL Microbiology Systems, Cockeysville, Md.), and isolates were identified as *H. pylori* by gram stain morphology and by positive urease, oxidase, and catalase tests. Histological sections of formalin-fixed biopsy specimens were stained with hematoxylin-eosin to evaluate the morphology and the presence of *Helicobacter*-like organisms. A serum sample was obtained by routine venipuncture for serological experiments.

In addition, 27 subjects (14 men and 13 females; mean age, 53 years; age range, 25 to 76 years) with chronic idiopathic dyspepsia negative for *H. pylori* by both culture and histology were included in the study (*H. pylori*-negative controls). These subjects were part of another study group (30), selected from patients with dyspepsia referred to our hospital between September 1991 and November 1992. Finally, sera were also obtained from 120 blood donors to evaluate *H. pylori* serology.

Concentration of *H. pylori* broth culture supernatant. *H. pylori* strains were cultured at 37°C in brucella broth (Difco Laboratories, Detroit, Mich.) containing 5% fetal bovine serum, IsoVitaleX enrichment (BBL Microbiology Systems), Skirrow's selective supplement (Oxoid), and amphotericin B (Fungizone) (2.0 μg/ml). Broth cultures were incubated in an atmosphere of 7.5% CO_2–7.5% H_2–5% O_2–80% N_2 for 48 h. Cultures were centrifuged at 7,000 × *g* for 15 min, and the cell-free supernatants were sterilized by passage through a 0.22-μm-pore-size filter and concentrated 15-fold with Centriprep-100 ultrafiltration units (Amicon, Beverly, Mass.).

Assay for cytotoxicity. Concentrated supernatants from *H. pylori* strains were incubated with HeLa cells in twofold dilutions from 1:5 to 1:160. Cell vacuolization was assessed by light microscopy after 48 h of incubation. Wells in which 50% or more cells were vacuolated were defined as showing cytotoxic effect.

Neutralization of *H. pylori* cytotoxin activity. Sera were heated to 56°C and diluted with Eagle's minimal essential medium. Twofold-diluted sera (from 1:10 to 1:160) were incubated for 1 h at 37°C with an equal volume of concentrated *H. pylori* type strain CCUG 17874 (Culture Collection of the University of Göteborg, Göteborg, Sweden) culture supernatant. Adherent HeLa cells were incubated for 18 h at 37°C in 96-well plates with 50-μl mixtures of serum and *H. pylori* plus 50 μl of Eagle's minimal essential medium. The neutralization titer was defined as the highest dilution of a serum sample completely neutralizing vacuolization, assessed by light microscopy.

SDS-PAGE and WB analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (20) using 7.0% acrylamide gels. The WB procedure of Towbin et al. (28) was performed as described previously (5). Briefly, after electrophoretic transfer, the blots were incubated for 12 h at room temperature with human, rabbit, or mouse sera diluted 1:100 or 1:1,000 in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20. Antigen-antibody complexes were detected with anti-species peroxidase-labeled antibodies (DAKO, Copenhagen, Denmark) diluted 1:500 in phosphate-buffered saline and with 4-chloro-1-naphtol (Bio-Rad, Hercules, Calif.) as the enzyme substrate.

Intrinsic labeling and autoradiography. *H. pylori* CCUG 17874 was grown at 37°C in brucella broth (Difco) containing 5% fetal bovine serum in the presence of 5 μCi of ^{14}C-labeled amino acid mixture (Amersham, Amersham, United Kingdom) per ml. After 48 h of incubation, the cell-free supernatants were concentrated and sterilized as reported above. The preparations were then electrophoresed, and fluorograms of ^{14}C-labeled polypeptides were obtained by standard procedures as previously reported (4).

Immune sera from animals. The antibodies used in this study were polyclonal rabbit antiserum raised against the purified CagA protein and rabbit antiserum against VacA recombinant protein (35) obtained by R. Covacci and D. Burroni (Immunobiological Research Institute, Siena, Italy). Mouse immune ascitic fluids containing polyclonal antibodies were obtained by immunizing BALB/c mice with jack bean urease type VII (Sigma, St. Louis, Mo.) or with whole-cell preparations of *H. pylori* CCUG 17874 strain by the previously described technique (6). Briefly, 0.8-ml volumes of immunogen (100 μg of protein) emulsified 1:9 (vol/vol) in complete Freund's adjuvant were injected intraperitoneally into 8- to 12-week-old mice on days 0, 7, 14, and 21. Ascitic fluid samples were collected on day 30 by peritoneal paracentesis.

PCR. The oligonucleotides used as PCR primers previously described by Xiang et al. (35) were derived from the sequences of the *cagA* and *vacA* genes, respectively. Briefly, the *H. pylori cagA* primers 5′-AGTAAGGAGAAACAA TGA and 5′-AATAAGCCTTAGAGTCTTTTTGGAAATC amplify a 1,350-bp DNA fragment, whereas *vacA* primers 5′-GCTTCTCTTACCACCAATGC and 5′-TGTCAGGGTTGTTCACCATG amplify a 1,160-bp DNA segment (35). The PCR mixtures contained 50 mM KCl, 10 mM Tris, 200 μM deoxynucleoside triphosphates, 30 pmol of each primer, 0.1 μg of bovine serum albumin, 2.5 U of Amplitaq (Perkin-Elmer, Norwalk, Conn.), and 10 ng of DNA from each bacterial strain studied. Amplifications were performed on a PCR 9600 thermocycler (Perkin-Elmer) as follows: 30 cycles, with 1 cycle consisting of 45 s at 94°C, 45 s at 50°C (58°C for *vacA* sequence), and 45 s at 72°C, and then an extension step at 72°C. Five-microliter samples of the PCR product were electrophoresed on a 1.5% agarose gel (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with 1× Tris-acetate-EDTA buffer containing 1 μg of ethidium bromide per ml. The gels were examined by transillumination and photographed. A *vacA*- and *cagA*-positive (CCUG 17874) and a *cagA*-negative and *vacA*-positive (HPG21) *H. pylori* strain were used as controls in PCR experiments.

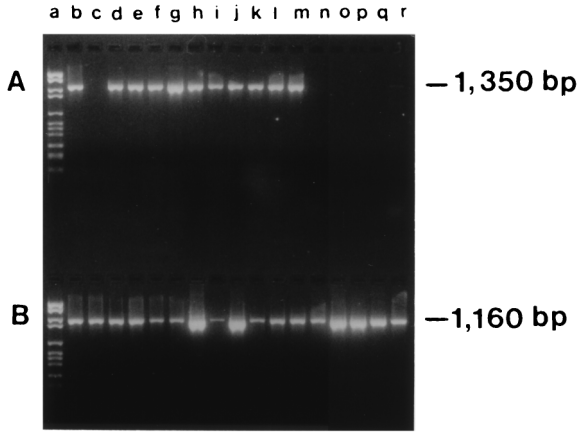

FIG. 1. Amplification of *H. pylori* DNA. Bacterial DNA was extracted from *H. pylori* isolates and from reference strains *H. pylori* CCUG 17874 and HPG21. The PCR protocol amplified 1,350-bp products of *H. pylori cagA* gene (A) and 1,160-bp products of *vacA* gene (B). Lane b, *H. pylori* CCUG strain; lane c, *cagA*-negative HPG21 strain; lanes d to m, 10 *cagA*-positive isolates; lanes n to r, five isolates which gave no PCR product when amplified with primers for *cagA* gene. Lane a contains marker DNAs. Size markers for the expected products appear on the right. PCR products were visualized with UV light following staining with ethidium bromide.

Statistical analysis. Prevalence rates were compared by χ^2 test.

RESULTS

Thirty patients who underwent gastroduodenoscopy for dyspepsia were admitted to the study. Endoscopic diagnoses were PUD (n = 13) and NUD (n = 17). Gastric tissue specimens from both antral and fundus mucosae were used for histological examination and *H. pylori* culture. The histological diagnoses were chronic active gastritis (n = 21) and chronic gastritis (n = 9). All patients were *H. pylori* positive by culture. Supernatants from 14 (46.7%) of 30 *H. pylori* isolates produced vacuolization of HeLa cells, whereas the remaining 16 did not. The supernatant dilution that produced vacuolization ranged from 1:5 to ≥1:160. Specific sequences of *vacA* and *cagA* genes of *H. pylori* isolates were searched for by PCR: all the strains were *vacA* positive, whereas only 25 of 30 strains possessed the *cagA* gene (Fig. 1). The relationships between genetic properties and in vitro vacuolating cytotoxin activity of *H. pylori* strains isolated from 30 patients with gastritis are shown in Table 1.

Serum neutralizing activity (serum titers ranging from 1:10 to 1:160) was present in 20 (66.7%) of the 30 *H. pylori* culture-positive patients. Ten (33.3%) patients did not present either serum neutralizing activity or a Tox^+ *H. pylori* strain. In addition to the above patients, 27 *H. pylori*-negative control subjects were studied for the presence of serum cytotoxin neutralizing activity: all sera were negative.

We next sought to determine by WB whether the presence of serum neutralizing activity was associated with the presence of serum IgG and IgA responses to CagA and VacA, using concentrated supernatant from *H. pylori* type strain CCUG 17874 broth culture as the antigen. Some experiments had been previously done to partially characterize the antigenic preparation used in WB. Radiolabeling of *H. pylori* CCUG strain grown in broth culture in the presence of ^{14}C-labeled amino acid mixture allowed us to verify whether isotope-labeled *H. pylori*-derived products were present in the concentrated supernatant used as the antigen in WB and to compare

TABLE 1. Serological, phenotypic, and genetic properties of *H. pylori* isolates in 30 patients with gastritis and endoscopically defined gastroduodenal pathology

Total no. of patients	No. of patients with gastritis and:		Serology[a]					Phenotypic and genetic properties of *H. pylori* isolates[a]		
	PUD	NUD	Serum neutralizing activity	Anti-VacA		Anti-CagA		Tox^{+b}	*vacA*	*cagA*
				IgG	IgA	IgG	IgA			
14	6	8	+	+	+	+	+	+	+	+
2	2	0	+	+	+	+	+	−	+	+
1	0	1	+	+	+	+	+	−	+	−
3	1	2	+	+	−	+	−	−	+	+
6	4	2	−	−	−	+	−	−	+	+
4	0	4	−	−	−	−	−	−	+	−

[a] Symbols: +, possesses the activity, antibody, or gene; −, does not possess the activity, antibody, or gene.
[b] Tox^{+}, in vitro cytotoxin production.

the protein profiles of isotope-labeled bacteria. Several radiolabeled bands were present in the gel run with whole-bacterium preparations, whereas only two major radiolabeled bands of 87 and 40 kDa and two minor bands of 94 and 128 kDa were observed in the gel performed with concentrated supernatant preparation (Fig. 2).

Concentrated supernatant from *H. pylori* broth culture was first tested with rabbit polyclonal monospecific antibodies raised against the purified CagA and VacA proteins (34). Rabbit anti-CagA immune serum recognized the 128-kDa CagA band, whereas anti-VacA immune serum recognized both a major 87-kDa band (VacA) and a less intensely stained 94-kDa band, which proved to be antigenically related to the 87-kDa protein (Fig. 3B).

Since *H. pylori* urease subunit polypeptide sizes (66 and 29.5 kDa) add up to an apparent molecular mass of 95.5 kDa (18), which is very close to the 94-kDa mass of the antigen identified by rabbit anti-VacA immune serum, we ruled out any cross-reactivity of the immune serum by using mouse antiurease immune ascitic fluid. As expected, the antiurease preparation recognized only two bands of 66 and 29 kDa, when probed with *H. pylori* cell extract (data not shown). When concentrated

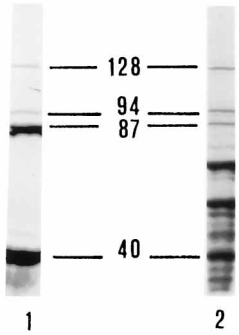

FIG. 2. SDS-PAGE protein profile of *H. pylori* CCUG 17874 strain grown in broth culture in the presence of ^{14}C-labeled amino acid mixture. Concentrated culture supernatant (lane 1) and whole-cell sonicate of *H. pylori* (lane 2) were applied to a 7% acrylamide gel. Bands of 40, 87, 94, and 128 kDa were clearly evident in concentrated supernatant. Several other bands were observed in whole-cell sonicate preparations. The apparent molecular masses (in kilodaltons) of the principal bands are indicated.

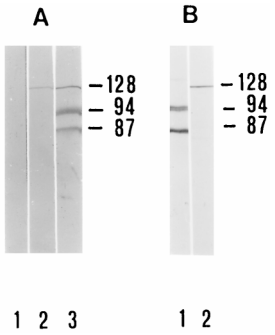

FIG. 3. Reactivities of *H. pylori* major antigens with immune sera from humans (A) and animals (B) by WB. (A) The following three patterns of reactivity were observed when 30 serum samples from patients with gastritis were tested with *H. pylori* concentrated supernatant: negative (lane 1); positive for one band of 128 kDa (lane 2); and positive for three bands of 128, 94, and 87 kDa (lane 3). (B) Rabbit immune serum raised against purified VacA protein recognized two bands of 87 and 94 kDa (lane 1), whereas rabbit immune serum raised against CagA recognized a 128-kDa band (lane 2).

supernatant from *H. pylori* broth culture was tested with sera from patients with gastritis and positive for *H. pylori* by culture of gastric biopsy, 20 of 30 serum samples recognized three bands with apparent molecular masses of 128, 94, and 87 kDa, 6 serum samples recognized only the band of 128 kDa, whereas 4 serum samples were not reactive (Fig. 3A). The relationships between serum neutralizing activity, serum IgG and IgA against *H. pylori* VacA and CagA antigens, and in vitro cytotoxin production (Tox^{+}) by *H. pylori* isolates in patients with gastritis and with defined gastroduodenal pathology (NUD and PUD) are shown in Table 1. Twenty patients with serum neutralizing activity against vacuolating cytotoxin showed specific IgG against both VacA and CagA antigens, whereas 17 (85%) of 20 were also positive for serum IgA against the same antigens. It is interesting to point out that sera reactive with the 87-kDa band also recognized the 94-kDa band which is antigenically related to the 87-kDa protein. Of 10 patients with gastritis lacking serum neutralizing activity for vacuolating cytotoxin, 6 had serum IgG against CagA, whereas 4 were negative. Of 13 patients with PUD, 9 (69.2%) had serum neutralizing activity and serum antibodies against VacA, whereas all patients were positive for antibodies against CagA. Of 17 patients with NUD, 11 (64.7%) had serum neutralizing activity and serum antibodies against VacA, whereas 13 (76.5%) were positive for antibodies against CagA. Four patients with NUD were serologically negative for *H. pylori*. The prevalences of serum antibodies directed against both VacA and CagA antigens in patients with gastritis, *H. pylori*-negative controls, and blood donors are reported in Table 2. The prevalence of anti-VacA and anti-CagA antibodies was significantly ($P < 0.001$) higher in patients with gastritis than in the other subjects. Cytotoxin neutralizing activity was studied in 60 random serum samples from 103 blood donors and in 27 *H. pylori*-negative control subjects: all samples were found negative.

DISCUSSION

The cytotoxin-associated protein CagA and the vacuolating cytotoxin VacA, which are thought to be the major *H. pylori* virulence factors involved in the pathogenesis of *H. pylori* diseases (15, 27), are produced by only a subset of clinical *H. pylori* isolates, defined as *H. pylori* type I (35). Bacterial strains which do not express CagA and cytotoxin form a group termed *H. pylori* type II (35). Only type I *H. pylori* strains have been

TABLE 2. Prevalence of anti-VacA and anti-CagA serum IgG and IgA antibodies in patients with gastritis and in control subjects

Study population (*n*)	No. (%) of subjects with antibody			
	Anti-VacA IgG	Anti-VacA IgA	Anti-CagA IgG	Anti-CagA IgA
Patients with gastritis (30)	20 (66.7)	17 (56.7)	26 (86.6)	17 (56.7)
H. pylori-negative control subjects (27)	0	0	0	0
Randomly selected blood donors (120)	17 (14.2)	15 (12.5)	21 (17.5)	15 (12.5)

associated with the most-severe gastroduodenal pathologies in humans (8, 33).

Serological experiments have shown that antibody titers to the 120- to 128-kDa protein CagA correlate with the severity of the disease and that CagA-producing strains are associated with PUD and gastritis (34). A strong correlation between the presence of IgG antibodies against the 87-kDa protein cytotoxin and cytotoxin-neutralizing activity has been also demonstrated in patients with *H. pylori* infection (10, 11).

The evaluation of phenotypic and genetic properties of *H. pylori* isolates and the serological results obtained in the present study from patients with gastritis showed a good correlation between anti-VacA IgG and serum neutralizing activity, whereas there was no detectable relationship between serology and in vitro cytotoxin production. In fact, of 30 patients with gastritis, only 14 showed an in vitro Tox^+ strain as well as both serum neutralizing activity and serum antibodies against CagA and VacA. Six patients had similar serological responses with apparently Tox^- *H. pylori* isolates from gastric biopsies; in one patient, this response may reflect simultaneous infection with both Tox^+ and Tox^- *H. pylori* strains, since the isolate from this patient was *vacA* positive and *cagA* negative; the remaining five patients may have been infected with *H. pylori* strains producing levels of cytotoxin undetectable in the in vitro assay but high enough to elicit an antibody response in vivo, since the isolates from these patients were indeed *cagA* and *vacA* positive. The fact that there were six patients with serum antibodies against CagA but without both antibodies against VacA and serum neutralizing activity may reflect infection with the *H. pylori* strain possessing the *cagA* gene but with no cytotoxin activity. Finally, four patients lacking serum neutralizing activity and antibodies against both VacA and CagA may reflect infection with the *H. pylori* strain possessing *vacA* but failing to express cytotoxin activity in vitro and very likely in vivo. In addition, the absence of expression of the *vacA* gene and the possible roles of other genes in the control of *vacA* gene expression (possibly *cagA*) also should be considered.

All sera reactive with the 87-kDa protein also reacted with a 94-kDa antigen, present in concentrated supernatant from *H. pylori* broth culture. This antigen, which was radiolabeled when *H. pylori* was grown in the presence of the ^{14}C-labeled amino acid mixture, was antigenically related to the 87-kDa protein, since it was recognized by rabbit polyclonal antiserum raised against VacA protein. The origin and the biological significance, if any, of the 94-kDa band require further investigation. However, it is interesting that the structural gene for cytotoxin, the *vacA* gene, encodes a true secretory protein of 136 kDa (25), whereas the purified protein has an apparent molecular mass of 87 kDa. At present, we do not know whether the active 87-kDa protein is generated by processing of the 136-kDa protein. If this were the case, the 94-kDa protein, antigenically related to the 87-kDa protein, could be derived by the same process.

The prevalence of serum antibodies directed against either VacA or CagA antigens was significantly higher in patients with gastritis than in *H. pylori*-negative controls or in blood donors, a better correlation with gastritis being found when considering anti-CagA antibodies. As far as the association between PUD and NUD with antibodies against CagA and VacA antigens is concerned, our results showed that all patients with PUD had antibodies to CagA, whereas 13 of 17 (76.5%) with NUD had anti-CagA antibodies. Antibodies to VacA antigen were present in 69.2% of patients with PUD and in 64.7% of patients with NUD. Therefore, anti-CagA antibodies seem to correlate better with PUD than anti-VacA antibodies, in agreement with previously reported data (7, 13).

In conclusion, the results suggest that a simple serological test such as the WB described is helpful in identifying subjects infected with *H. pylori* strains producing in vivo major bacterial virulence factors possibly involved in PUD and NUD. The use of a specific anti-*H. pylori* antibody assay, especially for the detection of specific serum IgG against CagA antigen, may help to better select patients for endoscopy and gastric biopsy.

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