Use of a Novel Enzyme Immunoassay Based on Detection of Circulating Antigen in Serum for Diagnosis of Helicobacter pylori Infection

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Recently, noninvasive diagnostic tests for Helicobacter pylori infection have gained in significance. We have developed a sensitive and specific noninvasive immunoassay based on the detection of an H. pylori circulating antigen (HpCA) in sera from H. pylori-infected individuals. Monospecific antibody and Western blot analyses were used to demonstrate the presence of the target antigen in H. pylori cell lysate and serum samples. A novel enzyme-linked immunosorbent assay (ELISA) was developed for the detection of HpCA in serum. Endoscopic biopsy specimens from the gastric antra of 221 individuals (143 males and 78 females) with dyspeptic symptoms were evaluated for H. pylori infection, with culture used as a "gold standard" for diagnosis. The target H. pylori antigen was identified at 58 kDa. HpCA has been detected by ELISA with high degrees of sensitivity, specificity, and efficiency (>90%), and ELISA results show no significant difference (P > 0.05) from results of H. pylori culture of gastric biopsy specimens. The test’s positive and negative predictive values were also high (95 and 86%, respectively). In conclusion, a sensitive and specific immunoassay was developed for the detection of HpCA in human serum. This test can be applied for noninvasive laboratory and field diagnoses of H. pylori infection.

Helicobacter pylori is a common bacterial infection in humans that is responsible for a variety of gastroduodenal pathologies, peptic and gastric ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (7, 7a, 14, 25). Several tests can be used to diagnose H. pylori infection; the selection of the appropriate test depends on the clinical setting (12, 18). H. pylori infection can be diagnosed by tests requiring upper gastrointestinal endoscopy for the retrieval of a gastric biopsy specimen (microbiological culture, histological examination, and rapid urease tests). These methods have high sensitivities and specificities (10), yet the invasiveness and expense of direct observation of the organism have led to a search for valid and reliable noninvasive alternatives (33). During recent years, noninvasive diagnostic tests for H. pylori infection have gained in significance (29). Although PCR, a powerful method known for its high sensitivity, can detect low numbers of H. pylori and has been used to follow up eradication therapy, PCR requires specialized laboratory facilities and is not generally available as a routine diagnostic test (13). The urea breath test has been the most widely used accurate noninvasive test, both in the pretreatment examination of infected individuals and for early-posttreatment follow-up, and meets the requirements for such a test (9). However, the performance of the test has been associated with some disadvantages. Although it is less costly than endoscopy, the urea breath test requires a specialized technician and expensive instrumentation that is not available in routine clinical laboratories (a scintillation counter or a mass spectrometer) for analyzing and handling the radioactive isotope in a specific way; also, patients may be hesitant to ingest radioactive test material (31). Enzyme immunoassays have been used to detect H. pylori infection in human secretions, such as feces, urine, and saliva (14). Such assays are attractive in comparison with other noninvasive methods because they are simple, inexpensive, and less of a burden for the patient (1, 15). Here, we have identified an H. pylori antigen in the sera from infected individuals and described the development of an antigen detection enzyme-linked immunosorbent assay (ELISA) suitable for the laboratory diagnosis of and the screening of large populations for H. pylori infection.

MATERIALS AND METHODS

Clinical specimens. A total of 221 individual serum samples were collected in the Gastro-Enterology and Surgery Center, Mansoura University, Mansoura, Egypt. The individuals were 143 males aged 14 to 74 years (mean, 40.83 ± 11.72 years; median, 42 years) and 78 females aged 16 to 75 years (mean, 41.69 ± 13.81 years; median, 39.5 years). All sera were stored at −20°C until used. All patients underwent upper gastroduodenal endoscopy, and multiple gastric biopsy specimens were taken from the antrum and then processed for microbiological culture of H. pylori. The endoscopy showed 69 gastritis cases (31.2%), 21 gastric erosion cases (9.5%), 8 gastric ulcer cases (3.6%), 17 duodenitis cases (7.7%), 4 duodenal erosion cases (1.8%), 32 duodenal ulcer cases (14.5%), and 70 normal endoscopic mucosa cases (31.7%). The Ethical Committee of the Gastro-Enterology and Surgery Center, Mansoura University, approved the present study. Informed consent was obtained from all participants, and they were fully informed of the diagnostic procedures involved and nature of the disease. Most of these subjects had received no antimicrobial therapies during the previous 3 months.
Microbiological culture. *H. pylori* was cultured by rubbing the gastric biopsy specimens of Egyptian patients onto Columbia agar plates supplemented with lysed horse blood (5%) and Skirrow's supplement containing vancomycin, trimethoprim lactate, cefsulodin, and amphotericin B (Oxoid, Basingstoke, United Kingdom). Agar plates were incubated at 37°C for 4 to 7 days in a microaerophilic atmosphere (5% O₂, 10% CO₂, 85% N₂, and 99% relative humidity) provided by a CO₂ incubator (Heraeus Instruments, Berlin, Germany). The microorganism was identified as *H. pylori* by the standard methods, on the basis of colony morphology, Gram staining, and the production of urease, catalase, and oxidase enzymes.

Preparation of *H. pylori* cell lysate. Bacterial cells were harvested, washed three times in phosphate-buffered saline (PBS; pH 7.2), and disrupted by sonication three times at 4°C for 15 s each time at 47 kHz with a Branson ultrasonic cleaner (B-1200 E-1; Branson Ultrasonics Corporation, Danbury, Conn.). After centrifugation at 10,000 rpm (J2-HS; Beckman Instruments, Inc., Fullerton, Calif.) for 10 min at 4°C, the protein content of the supernatant solution was determined with the use of bovine serum albumin as a standard (17). The supernatant was split into aliquots and stored at −20°C until used.

Production of anti-*H. pylori* antibody. A group of three New Zealand rabbits were immunized subcutaneously at three different inoculation sites with 500 μg of *H. pylori* cell lysate diluted (by volume) with Freund's complete adjuvant. Another group of three New Zealand rabbits were immunized with 500 μg of the purified 58-kDa *H. pylori* antigen diluted (by volume) with Freund's complete adjuvant. On day 15, the rabbits were immunized again with the same dose of antigen emulsified with incomplete Freund's adjuvant. On day 28, rabbits were immunized with one more dose of antigen with incomplete adjuvant, and they were sacrificed 4 days later. Blood samples were collected from all rabbits on day 0 and at 28 and 32 days after immunization, and sera were separated. The reactivity of the collected sera was tested against serial concentrations of *H. pylori* lysate and the purified 58-kDa antigen by using an indirect ELISA and anti-rabbit immunoglobulin G (IgG) alkaline phosphatase conjugate (Sigma). Incubation with the enzyme substrate produced a color in proportion to the amount of anti-*H. pylori* IgG antibodies present.

SDS-PAGE and gel electroelution. Various samples (see Results) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), at 50 μl, using vertical slabs of 12 or 16% polyacrylamide (16). Molecular weight standards (Sigma) were run in parallel. After staining with Coomassie blue R-250, the band of interest (58 kDa) was cut, and the antigen was electroeluted from the polyacrylamide gel (2). The protein content of the electroeluted antigen was determined, and the samples were stored at −20°C until used. Initially, the purity of the electroeluted antigen was assessed using SDS-PAGE and Coomassie blue staining. Then, serum samples (diluted 1:100 in 0.2% (wt/vol) nonfat milk in carbonate-bicarbonate buffer) were washed, 50 μl of anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:350 in TBS. After being washed, the blots were followed by incubation for 2 h with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Incubation with the enzyme substrate produced a color in proportion to the amount of anti-*H. pylori* IgG antibodies present.

**FIG. 1. Western blot analysis of *H. pylori* cell lysate and the 58-kDa purified antigen.** Lane 1, immunostaining with nonimmunizing-rabbit serum; lane 2, immunostaining with polyclonal rabbit IgG to *H. pylori* cell lysate; lane 3, immunostaining with rabbit IgG-monospecific antibody to the 58-kDa purified antigen. A highly reactive band was identified at 58 kDa by using rabbit IgG anti-*H. pylori* lysate and the anti-58-kDa antigen. Molecular mass markers (bands are not shown but are indicated by arrows) include phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55.0 kDa), ovalbumin (42.7 kDa), aldolase (40 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

**RESULTS**

Reactivity of the developed anti-*H. pylori* antibodies. Sera collected from the rabbits immunized with the *H. pylori* lysate reacted strongly with several bands of various molecular weights on Western blots. However, a broad band at 58 kDa in *H. pylori* lysate showed particularly high reactivity. The 58-kDa *H. pylori* antigen was isolated and purified from *H. pylori* lysate by using a gel electrophoresis technique. Antibodies specific to the 58-kDa *H. pylori* antigen were produced in rabbits immunized with the purified *H. pylori* antigen. In a Western blot, the developed IgG antibodies appeared to react only with an antigen of 58 kDa in whole *H. pylori* cell lysate and purified antigen (Fig. 1). Sera collected from these rabbits at zero time as control sera were not specifically reactive with that band (Fig. 1). In addition, sera collected from immunized rabbits showed increased levels of specific IgG antibodies (P < 0.05) towards *H. pylori* lysate and the purified antigen in comparison (range, 0.135 to 0.377) of a group of 24 serum samples from noninfected healthy individuals ± 3 standard deviations [i.e., 0.257 ± (3 × 0.077) = 0.398]. The mean absorbance value of a group of 32 *H. pylori*-positive individuals was 0.751 (range, 0.411 to 1.250).

Characterization of the purified *H. pylori* antigen. To determine some of the target antigen’s chemical characteristics, samples of the antigen were treated with protease and other chemical reagents and then tested by ELISA to see if these treatments affected the active epitope as described by Attallah et al. (2). Lysate and bovine serum albumin were tested in parallel, as positive and negative controls, respectively.

Statistical analysis. Standard methods were used to calculate sensitivity, specificity, efficiency, and positive and negative predictive values. All parameters were transferred to an IBM PC-A XT-compatible computer for analysis using statistical analysis program package Instat Software for Science, version 2.3 (Graphpad Software, Inc., San Diego, Calif.). The Mann-Whitney U test was used to compare the means of two distributions. Fisher’s exact test was used to compare the differences between two proportions. P values (two-tailed test) of < 0.05 were considered significant.
A novel ELISA based on the detection of the target HpCA in serum was developed for the diagnosis of *H. pylori* infection. The mean absorbance values of the serum samples were 0.751 (range, 0.411 to 1.250; all positive) for 32 *H. pylori*-positive subjects and 0.271 (range, 0.135 to 0.377; all negative) for 24 *H. pylori*-negative subjects (*P* < 0.001, Mann-Whitney U test). To evaluate the clinical application of the test, endoscopic biopsy specimens from the gastric antra of 221 individuals were evaluated for *H. pylori* infection by using culture as the gold standard for diagnosis. A total of 144 individuals were diagnosed as *H. pylori* infected and 77 were diagnosed as noninfected by microbiological culture. No significant differences (*P* > 0.05, Fisher’s exact test) were shown between the results of circulating-antigen detection by ELISA and *H. pylori* culture of gastric biopsy samples. The sensitivity, specificity, efficiency, and predictive values of positive and a negative results for the newly developed ELISA in comparison to those of microbiological culture are shown in Table 1.

### TABLE 1. ELISA detection of a 58-kDa HpCA in 221 serum samples of individuals who were confirmed by culture to be infected or not infected with *H. pylori*

<table>
<thead>
<tr>
<th>Result of microbiological culture of gastric biopsy specimen</th>
<th>No. of samples</th>
<th>No. of samples with indicated HpCA ELISA result</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>144</td>
<td>133 (TP)</td>
<td>92</td>
</tr>
<tr>
<td>Negative</td>
<td>77</td>
<td>7 (FP)</td>
<td>9</td>
</tr>
</tbody>
</table>

*The gold standard for the diagnosis of *H. pylori* infection.*

DISCUSSION

Immunodiagnosis of *H. pylori* infection is attractive in comparison with other noninvasive diagnostic methods for the investigation of upper gastrointestinal symptoms. There are a number of different techniques for antibody detection (19, 30). However, antibody detection tests are less useful in children aged below 10 (21) and are not suitable for the follow-up examination of treated patients (15, 22). In addition, the accuracy of these tests is no longer adequate to justify their clinical use on clinical or economic grounds (29).

The *H. pylori* stool antigen enzyme immunoassay has been validated in various regions of the world with comparable sensitivities and specificities (13, 31). However, a potential problem with the *H. pylori* stool antigen test appears to be patient reluctance about stool handling, and this could prove to be a significant obstacle in patient compliance and the acceptability of the test in everyday clinical practice (5, 34). In addition, the accumulating data concerning the use of the test in evaluating treatment remain unconvincing (24).

Soluble bacterial antigens of *H. pylori* on the stomach mucosa can be passively absorbed by pinocytosis and can be transferred into the blood through injured tight junctions and by the absorption of intestinal mucosal epithelial cells (3, 4). In the present study, we were able to detect a specific 58-kDa antigen in *H. pylori* lysate and in serum samples from *H. pylori*-infected individuals. The molecular mass of the serum antigen is analogous to the 58-kDa fragment of the 87-kDa cytotoxin domain of the VacA protein of 42 kDa in some of these samples. Lane 1, *H. pylori* cell lysate as a positive control; lanes 2 to 4, serum samples from three noninfected healthy individuals; lanes 5 to 10, serum samples from six individuals infected with *H. pylori*. Molecular mass bands are not shown but are indicated by arrows.
protein (8, 23, 27), the subunit cellular antigen (59 kDa) of the native H. pylori catalase (26), and the H. pylori catalase gene product (20). However, further investigation of the structure of the target H. pylori serum antigen will be performed.

Based on these encouraging results, we have developed a direct ELISA based on the detection of HpCA in serum samples and suitable for the laboratory diagnosis and screening of large populations for H. pylori infection. The HpCA test has several potential advantages over other techniques for populations studies. No expensive instrumentation or expertise is required to perform a standard ELISA. The advantages of the HpCA test include its ability to detect the presence of H. pylori and its usefulness with individuals for whom endoscopy is difficult to justify (8).

To evaluate the diagnostic performance of the HpCA diagnostic test, we included in the study 221 patients with dyspeptic symptoms who were examined by upper gastrointestinal endoscopy. The infection with H. pylori was judged to be present when a microbiological culture of gastric biopsy specimens was positive. The HpCA test detected the circulating antigen in 92% of sera from H. pylori-infected individuals, with high specificity (91%) among noninfected individuals.

The false-negative results (8%) of our HpCA ELISA may be explained as follows. The H. pylori antigen level among false-negative samples may be too low to be detected by the present assay conditions. The direct ELISA, with which the unknown elements are bound directly to the plate, may suffer from problems with uneven absorption and interference with absorption due to other serum components. Most commercial tests are of the antigen capture variety, which requires an additional antibody; however, with further work, the HpCA test can be improved. H. pylori antigens have been found as components of circulating immune complexes (34), so it may be necessary to dissociate the immune complexes to achieve a higher sensitivity in the immunoassay (6).

The false-positive results (9%) reported by the HpCA test may be due to biopsy sampling errors as a result of the patchy distribution of the infection with H. pylori in the stomach. The HpCA test has higher sensitivity in the immunoassay (6). The false-positive results (9%) reported by the HpCA test may be due to biopsy sampling errors as a result of the patchy distribution of the infection with H. pylori in the stomach. The HpCA test has higher sensitivity in the immunoassay (6). The false-positive results (9%) reported by the HpCA test may be due to biopsy sampling errors as a result of the patchy distribution of the infection with H. pylori in the stomach. The HpCA test has higher sensitivity in the immunoassay (6). The false-positive results (9%) reported by the HpCA test may be due to biopsy sampling errors as a result of the patchy distribution of the infection with H. pylori in the stomach. The HpCA test has higher sensitivity in the immunoassay (6). The false-positive results (9%) reported by the HpCA test may be due to biopsy sampling errors as a result of the patchy distribution of the infection with H. pylori in the stomach. The HpCA test has higher sensitivity in the immunoassay (6).


