We describe a solid-phase immunochromatographic serologic test, FlexSure® HP, to detect IgG antibodies against Helicobacter pylori. H. pylori colonizes the stomach and proximal duodenum, cause ulcer disease and mucosa-associated lymphoid tissue lymphoma, and have a role in the development of other disorders, including gastric adenocarcinoma. FlexSure HP consists of a test strip, conjugate pad, and absorbent pad, in a novel reverse-flow chromatography format. In these studies, FlexSure HP was demonstrated to be specific for IgG antibodies against H. pylori. The reactive cutoff of the test was consistent with [13C]urea breath test and commercially available ELISAs. FlexSure HP had 94% sensitivity, 88% specificity, and 91% accuracy relative to [13C]urea breath test; and 95% sensitivity, 94% specificity, and 95% overall agreement relative to high-molecular-mass cell-associated protein enzyme immunoassay (HM-CAP EIA). FlexSure HP is a simple-to-perform, visually read test requiring no specialized training, equipment, or instrumentation, and yields rapid, accurate, qualitative results.

Changes in healthcare delivery are increasing the requirement for more cost-effective, timely diagnostic tests that can be performed at the point of care, such as at the patient’s bedside, in the clinic, or in the physician’s office. In response to these market needs, we have developed a novel, solid-phase immunochromatographic test format (FlexSure®) with broad applications to point-of-care-based diagnostic tests. The new test format and method have been used to develop a rapid test (FlexSure HP) designed to detect IgG antibodies against Helicobacter pylori in human serum.

H. pylori, a Gram-negative, spiral, flagellated bacterium that colonizes the mucosal lining of the stomach and proximal duodenum, was discovered by Warren and Marshall in 1984 [1]. H. pylori has since been recognized as a primary causative agent in the development of gastric and duodenal ulcers [2], chronic active gastritis [3], primary gastric B-cell lymphoma [4], and gastric adenocarcinoma [5]. Dixon’s metaanalysis of ulcer patients yielded a 93% H. pylori infection rate for those with duodenal ulcers and 80% for those with gastric ulcers [2]. Treatment of patients with H. pylori infections with combinations of H2 blockers (i.e., ranitidine) or proton pump inhibitors (i.e., omeprazole), bismuth, and antibiotics (i.e., amoxicillin, metronidazole, tetracycline, clarithromycin) has resulted in cure rates of up to 90% [6–8]. If H. pylori infection is eradicated, the reinfection rate for disease is <0.5%/year [9, 10]. Patients treated with bismuth and H2 blockers alone experienced ulcer recurrence rates of 95% for duodenal ulcer and 74% for gastric ulcer [11]. In 1994, the NIH Consensus Conference on H. pylori in Peptic Ulcer Disease concluded that H. pylori infection caused peptic ulcer disease and recommended that all patients diagnosed with H. pylori and peptic ulcers be treated for the infection with antimicrobial drugs [12].

Several validated methods are used to diagnose H. pylori infection. Until recently, the preferred method of diagnosis was endoscopy and biopsy coupled with a rapid urease test, culture, and (or) histology. These methods require specialized equipment, trained personnel, hours to days to obtain test results, and cost between $200 and $1200 per patient. With the observation that H. pylori infection is usually associated with a systemic immune response, immunoassays that detect IgG antibodies to H. pylori provide a cost-effective alternative to endoscopic biopsy for the initial diagnosis of H. pylori infection.

In this communication we describe the functional char-
characteristics and clinical performance of FlexSure HP, a new rapid test for the detection of IgG antibodies against *H. pylori* in serum. FlexSure HP is a simple, qualitative test that can be performed safely and easily in <5 min in the physician’s office or at the point of care.

**Materials and Methods**

**FlexSure HP Test**

FlexSure HP involves the principle of reverse-flow immunochromatography to detect human IgG antibodies against *H. pylori* in serum (Fig. 1). The test card is opened so it lies flat on the work surface. Two drops of FlexSure HP buffer are added to the pink conjugate pad to rehydrate a goat anti-human IgG colloidal gold conjugate. One drop of serum (~45 µL) is then added to the blue sample pad at the bottom of the test strip with a transfer pipette. The serum mobilizes the blue dye and they migrate together up the test strip through a zone of immobilized *H. pylori* antigen to the limit line printed on the test strip. This allows specific IgG antibodies to *H. pylori*, if present, to bind to the antigen. When the sample reaches the limit line printed on the test strip, the device is closed, bringing the conjugate and the absorbent pads into contact with the top and bottom of the test strip, respectively; this initiates reverse flow on the immunochromatographic strip. As the conjugate migrates down the test strip, serum and unbound antibodies are washed from the test strip by the liquid front of the conjugate. If antibodies to *H. pylori* are present, only the control line will be visible. The test results are visually interpreted 4 min after closing the test card. One pink line in the control region indicates a negative test result; two pink lines (test and control) indicate a positive test. Although positive tests may appear before 4 min, the operator must wait 4 min to confirm a negative test result.

**High-Molecular-Mass Cell-Associated Protein Enzyme Immunoassay (HM-CAP EIA)**

HM-CAP EIA (Enteric Products) is a microwell ELISA method for the detection of IgG serum antibodies to *H. pylori* in serum.² Aliquots (5 µL) of each serum sample were diluted with 500 µL of wash buffer. Next, 100 µL of each diluted sample was transferred to the appropriate well of the microtiter plate. Two wells were run for each sample. In addition, the kit controls were run according to the manufacturer’s instructions; two wells each were run with the negative, low-positive, and high-positive calibrators. The plates were incubated for 20 min at room temperature in a 95% humidity chamber. The plates were emptied, washed three times with 350 µL/well of wash buffer, and then 100 µL of 1× conjugate was added to each well and incubated for 20 min in a 95% humidity chamber. The plates were emptied, washed three times with 350 µL/well of wash buffer, and 100 µL of 1× substrate was placed in each well. The plates were incubated for 10 min at room temperature, and then 100 µL of stop solution was added to each well and the absorbance for each well was measured at 450 nm with a BioTek plate reader. DeltaSoft II software was used to generate a calibration curve with the serum calibrators expressed in ELISA value (EV) units. The absorbance values for each sample were subsequently converted to EVs by using the calibration curve. Samples with EVs <1.8 were negative, EVs 1.8–2.2 were indeterminate (inclusive), and EVs >2.2 were positive.

**Cross-Reactivity**

The cross-reactivity test method was adopted from Perez-Perez et al. [13]. Bacterial suspensions grown in standard cultures were harvested, washed, and centrifuged to a pellet. The pellet of bacteria was resuspended in an aliquot of previously characterized undiluted serum. The bacterial suspensions were centrifuged to a pellet and the serum supernatant was transferred to a tube with a fresh pellet of bacteria. The procedure was repeated until the aliquot of serum had been treated with five successive pellets of bacteria. All serum samples for evaluation were processed in this manner. After each of the five adsorption cycles for each bacterial strain, a small volume (<100 µL) of serum was removed for testing with FlexSure HP and HM-CAP EIA. Test results for each sample were compared with the untreated serum. The following bacterial species were used: *Campylobacter jejuni* (four strains), *C. fetus* (three strains), *C. coli* (three strains), *Escherichia coli* (four strains), *H. mustela* (one strain), or *H. pylori* (five positive control strains). If antibodies in a

² Nonstandard abbreviations: HM-CAP, high-molecular-mass cell-associated protein; EIA, enzyme immunoassay; and EV, ELISA value.
serum sample cross-reacted with the HM-CAP antigens and proteins in specific bacterial strains used in the adsorption, the antibody titer would be reduced in the sample by the adsorption step. This would be indicated by a lower EV for the HM-CAP EIA test and (or) a negative FlexSure HP test result (if the amount of anti-\textit{H. pylori} antibodies had been reduced below the threshold of the FlexSure HP test). If antibodies in a serum sample did not cross-react with the HM-CAP antigen and the specific bacterial strain used in the adsorption, the antibody titer would remain the same. Within experimental variation, no change in the EV or the FlexSure HP test would be observed, when compared with the untreated serum.

**USER STUDY**
A panel of 50 blind-coded serum samples were sent to eight individuals working in various medical capacities (three physicians, one registered nurse, one licensed practical nurse, one medical technologist, and two clerical staff). Each end-user received only the written product instructions as a basis for performing the FlexSure HP tests. Test results were compared with those obtained by five trained scientists who were very familiar with the FlexSure HP test. The serum samples spanned a range of antibody titers from negative to high positive as determined by HM-CAP EIA.

**WITHIN- AND BETWEEN-SITE REPRODUCIBILITY**
Five serum samples with EVs of 0.2, 1.8, 2.5, 3.5, and 6.1 were prepared in blind-coded sets comprising 10 replicates of each serum for a total of 50 samples. The within-site study was performed by three technicians working independently at the same site. The between-site study was performed by individual technicians working at three geographically separated laboratories. The frequency of positive and negative test results were calculated for each study and reported as percent correct.

**CLINICAL SAMPLES**
Serum samples (n = 551) were obtained as part of an epidemiological study of the prevalence of \textit{H. pylori} infection in the Houston metropolitan area [14] in healthy volunteers without symptoms referable to the upper gastrointestinal tract (n = 354) and from patients undergoing upper gastrointestinal endoscopy for evaluation of dyspepsia (n = 197). Serum samples were stored frozen in aliquots. \textit{H. pylori} infection status was determined in all patients and volunteers by using the $[^{13}\text{C}]$urea breath test [15]. In some patients infection status was confirmed by histology and (or) culture.

All serum samples were coded and forwarded to the clinical trial site (Enteric Products) for testing by FlexSure HP and HM-CAP diagnostic tests. All tests were stored at 4 °C until used. The test kits and clinical trial sera were equilibrated to room temperature before use. The test devices were run according to the manufacturers’ instructions. During testing, laboratory personnel remained blinded with regard to sample identification, \textit{H. pylori} infection status, clinical group, and the results of competing tests. After completion of all testing, the code for \textit{H. pylori} infection status was broken and the data analyzed.

**Results**

**CROSS-REACTIVITY**

The serum pool used in this study had a titer of approximately 6.5 EV. No decrease in the serum titer (reported as EV) was observed for any of the samples adsorbed against non-\textit{H. pylori} strains of bacteria. These results indicated that the non-\textit{H. pylori} bacterial species tested did not cross-react with HM-CAP antigen used in the HM-CAP EIA or the FlexSure HP tests (Table 1), even after five consecutive adsorptions against each strain. One low titer (EV) and negative FlexSure HP test result were recorded for the fourth adsorption of one of the \textit{C. jejuni} strains. This was attributed to experimental error because the serum sample following the fifth adsorption had an EV equivalent to the untreated serum sample and a positive FlexSure HP test result. The decline in EV observed with the non-\textit{H. pylori} strains of bacteria used in the study could have been caused either by adsorption of \textit{H. pylori}-specific antibodies from the serum pool or dilution of the serum sample as it was passed through subsequent adsorptions.

As expected, serum samples adsorbed with different strains of \textit{H. pylori} had lower titers (EVs) with each successive adsorption and resulted in negative FlexSure HP test results. Of the five strains of \textit{H. pylori} used in the study, the serum pool was negative by FlexSure HP in two strains following the first adsorption, in two other strains following the second adsorption, and in the fifth strain following the fourth. The final EVs for the serum samples adsorbed with \textit{H. pylori} ranged between 0.7 and 3.5. The apparent false-negative FlexSure HP test run on the sample with an EV of 3.5 may be attributed to the presence of antibody–antigen complexes remaining in the serum after centrifugation that were detected by ELISA but not the rapid test. \textit{H. mustalae} showed only slight cross-reactivity, which resulted in a decline in serum titer from 6.5 EV to 4.9 EV.

**Table 1. Anti-\textit{H. pylori} \textit{IgG} antibody activity of serum samples after adsorption with whole cells of \textit{C. jejuni}, \textit{C. fetus}, \textit{C. coli}, \textit{E. coli}, \textit{H. mustalae}, and \textit{H. pylori}.**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>No. of strains</th>
<th>EV</th>
<th>FlexSure HP test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{C. jejuni}</td>
<td>4</td>
<td>5.5 to 5.7</td>
<td>4 of 4 positive</td>
</tr>
<tr>
<td>\textit{C. fetus}</td>
<td>3</td>
<td>5.1 to 5.9</td>
<td>3 of 3 positive</td>
</tr>
<tr>
<td>\textit{C. coli}</td>
<td>3</td>
<td>6.2 to 6.6</td>
<td>3 of 3 positive</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>4</td>
<td>6.0 to 6.1</td>
<td>4 of 4 positive</td>
</tr>
<tr>
<td>\textit{H. mustalae}</td>
<td>1</td>
<td>4.9</td>
<td>1 of 1 positive</td>
</tr>
<tr>
<td>\textit{H. pylori}</td>
<td>5</td>
<td>0.7 to 3.5</td>
<td>5 of 5 negative</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>6.3</td>
<td>1 of 1 positive</td>
</tr>
</tbody>
</table>
CLINICAL SAMPLES

The sample population of patients and healthy volunteers was selected to provide an approximately equal distribution between [13C]urea breath test-positive and-negative samples. The patients and volunteers included African Americans, Caucasians, Hispanics, and Asians between the ages of 4 and 82 (mean age 44 ± 17 years), 36% female and 64% male. All patients (n = 197) had undergone upper gastrointestinal clinical evaluation for dyspepsia with primary diagnosis by endoscopy. In addition to endoscopy, biopsies were obtained from 75 of the patients to confirm diagnosis. Diagnoses included 44 patients with gastric ulcers, 140 with duodenal ulcers, 6 with pernicious anemia, 3 with nonulcer dyspepsia, 3 with ulcers from nonsteroidal antiinflammatory drugs, and 1 with esophagitis. None of the volunteers (n = 354) reported symptoms associated with upper gastrointestinal disease and received no other follow-up. All subjects provided serum samples and consented to the [13C]urea breath test [15]. The sensitivity, specificity, and overall agreement of FlexSure HP with [13C]urea breath test or HM-CAP EIA were determined.

Analysis of 551 serum samples comparing FlexSure HP test with [13C]urea breath test (reference method) yielded a sensitivity of 94.4%, specificity of 87.6%, and an overall agreement of 91.1% (Table 2). The predictive value for a positive test in this population of patients and volunteers was 89.1%; the predictive value of a negative FlexSure HP test result was 93.5%. There were 49 instances where the FlexSure HP test did not agree with the [13C]urea breath test: 16 FlexSure HP false-negative and 33 FlexSure HP false-positive tests. The discordant samples were run on HM-CAP EIA to determine if antibodies to H. pylori were detectable by another immunoassay.

Of the 33 FlexSure HP-positive/[13C]urea breath test-negative patients, 18 serum samples were positive by HM-CAP EIA. Although these serum samples contained antibodies to H. pylori, the patients may not have had active infections or bacterial loads sufficient to yield positive [13C]urea breath tests. Three of the 16 FlexSure HP-negative/[13C]urea breath test-positive samples were negative by HM-CAP EIA, indicating false serologic test results for FlexSure HP.

Table 2. Sensitivity, specificity, and accuracy of FlexSure HP in a total test population, asymptomatic volunteers, and patients with [13C]urea breath test as the reference test for the presence or absence of H. pylori.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive predictive value</th>
<th>Negative predictive value</th>
<th>Overall agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population</td>
<td>551</td>
<td>94.4%</td>
<td>87.6%</td>
<td>(270/296)</td>
<td>(232/265)</td>
<td>91.1%</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>354</td>
<td>93.3%</td>
<td>90.0%</td>
<td>(98/105)</td>
<td>(227/249)</td>
<td>91.0%</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>197</td>
<td>94.9%</td>
<td>Insufficient</td>
<td>(169/178)</td>
<td>data</td>
<td>91.4%</td>
</tr>
</tbody>
</table>

As stated above, the subjects used in this study consisted of both patients and healthy volunteers. In the population of symptomatic patients (n = 197), FlexSure HP yielded positive serologic test results in 169 of 178 patients positive with the [13C]urea breath test for a sensitivity of 94.9%. There were too few symptomatic patients to determine the specificity of FlexSure HP. Asymptomatic volunteers (n = 354) were recruited for the study to provide sufficient numbers of [13C]urea breath test-negative subjects to estimate the specificity of FlexSure HP. FlexSure HP and [13C]urea breath tests yielded concordant positive test results on 98 of 106 breath test-positive and concordant negative tests on 224 of 249 breath test-negative volunteers. The sensitivity, specificity, and overall agreement of FlexSure HP were 93.3%, 90.0%, and 91.0%, respectively.

FlexSure HP was compared directly with HM-CAP with 551 frozen serum samples, of which 299 were positive (EV >2.2), 237 were negative (EV <1.8), and 15 were indeterminate (EV 1.8–2.2) by EIA. The indeterminate samples were removed from further data analysis. FlexSure HP test results agreed with HM-CAP EIA on 285 of 299 EIA positive and 222 of 237 EIA negative samples to yield a sensitivity of 95.3% and a specificity of 93.7%. The overall agreement between the two tests was 94.6%.

ROC curve analysis [16] was used to determine the analytical cutoff for FlexSure HP by using the HM-CAP EIA as the standard (Fig. 2). Theoretical EIA cutoffs for positive and negative tests were established at 0.5 EV intervals from 0.0 to 8.5 with the upper limit of the interval set as the cutoff for a “negative” ELISA. The fraction of true-positive and true-negative FlexSure HP tests was calculated for each theoretical EV cutoff. For example, if 0.0 EV cutoff was set as a lower limit for a positive test result, all positive FlexSure HP tests would be considered true positive and all negative FlexSure HP tests would be false positive. As the cutoff is increased, the fraction of true positive tests will decrease to zero and the fraction of negative tests will increase to 1.0. The optimal performance for FlexSure HP (e.g., best combination of sensitivity and specificity) will be on a 45-degree line bisecting the graph.

The EV at which optimal FlexSure HP sensitivity and specificity was observed was 2.0 EV. This coincided with the positive/negative test cutoff previously established...
for HM-CAP EIA with [13C]urea breath test as the gold standard [17].


Fig. 2. Parametric ROC plot of FlexSure HP serum test grouped into intervals of 0.5 EV units.

**Reproducibility**

In both the within- and between-site reproducibility studies, tests results reported by the technicians agreed with the expected result for serum samples with EVs 0.2 (negative), 3.5 (positive), and 6.1 (positive). In addition, all test results with the 1.8-EV serum sample agreed with the expected (negative) for the within-site technicians. Technicians in the between site had one false-positive test result of 60 for the 1.8-EV sample. The within- and between-site technicians each reported 3 of 60 false-negative test results for the 2.5-EV sample. No procedural problems were reported, indicating that the test yielded good reproducibility, particularly around the analytical cutoff.

**User Study**

The ability of personnel in the clinical or physician office laboratory setting to accurately perform FlexSure HP was evaluated by providing a group of eight inexperienced users with education and experience backgrounds ranging from office clerks to physicians with 50 different serum samples each, spanning the range of EVs from 0.2 to 9.1. The accuracy of the inexperienced users was compared with that obtained by four experienced scientists. Each group reported one false-positive test result. The experienced scientists reported no false-negative test results, whereas the inexperienced group reported six false-negative tests of 200. Four of the inexperienced users reported no false-positive or false-negative tests. The results from two groups were independently pooled and a correlation coefficient calculated ($r = 0.9891$; 95% confidence interval = 0.9861–0.9921). The results of the user study indicate that individuals inexperienced in performing FlexSure HP, regardless of their education and professional training, were able to perform the test with ease and accuracy equal to that of trained, experienced technicians.

**Discussion**

The association of *H. pylori* infection with peptic gastric ulcer disease, gastric cancer, and chronic gastritis has required reevaluation of the clinical management of these diseases. The 1994 NIH Consensus Conference on *H. pylori* concluded that all patients with documented *H. pylori* infection and peptic ulcer should receive therapy for the infection [12]. To achieve this objective, physicians must have accurate means at hand to diagnose the presence of *H. pylori* infection. Currently, physicians have a choice of invasive or noninvasive tests.

Most invasive methods for diagnosis involve endoscopic biopsy followed by histology, rapid urease test, and (or) culture. These tests are nearly 100% specific for diagnosis of *H. pylori* infection, since a positive diagnosis is determined by direct observation of *H. pylori* in tissue sections, detection of urease activity, or culture of *H. pylori* from biopsies.

The most widely used noninvasive tests are immunochemical tests designed to detect the presence of IgG antibodies to *H. pylori* in serum involving either ELISA, immunoblot, or immunochromatographic formats. The other principal noninvasive method is the urea breath test, which requires the patient to ingest [13C]- or [14C]-labeled urea [15, 18]. In the presence of *H. pylori*, bacterial urease decomposes the urea to ammonia and [13CO2] or [14CO2]. The labeled CO2 is subsequently detected in the breath 10 to 60 min after ingestion of the substrate. The [13C]- and [14C]urea breath tests were recently cleared by the FDA.

The simplest and lowest-cost approach for the initial diagnosis of *H. pylori* infection is an accurate and rapid office-based test for the presence of serum IgG antibodies against *H. pylori*. Such tests permit the physicians to begin immediate anti-*H. pylori* treatment for patients with positive test results or prescribe other appropriate medical follow-up for subjects with negative test results. To be effective, physician office tests must be simple to use and interpret, exhibit no cross-reactivity with other bacteria, have no interference with normal blood constituents, have a medically relevant reactive cutoff, and provide high diagnostic specificity and sensitivity.

A key requirement to developing a serology test with high sensitivity and specificity is the antigen preparation used in the test. Soon after Warren and Marshall described this organism in 1984 [1], the correlation between gastritis and serum anti-*H. pylori* antibody was documented [19–22] in complement fixation, bacterial agglutination, and EIAs with crude antigen preparations. First-generation immunoassays had low specificity because they involved crude antigen preparations containing proteins that cross-reacted with antibodies directed against other bacteria (e.g., *C. jejuni*, *C. fetus*, and *E. coli*) [22, 23]. The evolution of antigen preparations used in serum-based tests and the performance of those tests was recently reviewed by Graham et al. [24].

A second-generation antigen described by Evans et al.
[17] was used to develop an ELISA (HM-CAP EIA) with reduced cross-reactivity, 100% specificity, and 99% sensitivity, relative to the urea breath test. They described the antigen preparation as a HM-CAP complex extracted from H. pylori cells with a molecular mass range from 450 000 to 700 000 kDa containing urease activity. We have incorporated this same antigen preparation into FlexSure HP.

FlexSure HP was designed to perform comparably with commercially available ELISAs. The concentrations of HM-CAP antigen and conjugate in FlexSure HP were balanced to obtain the highest clinical utility (i.e., test accuracy) and to show performance characteristics comparable with those of the HM-CAP EIA. ROC curves, which describe the overall performance of a given method independent of a predefined cutoff, were constructed for FlexSure HP with HM-CAP EIA as the standard. The optimum combined sensitivity and specificity was achieved at an EV of 2.0, the same analytical cutoff previously established for the HM-CAP EIA with the [13C]urea breath test as the standard.

FlexSure HP is an easy-to-use, rapid, point-of-care test for the detection of antibodies to H. pylori with performance characteristics essentially equivalent to other immunoassays and the urea breath test, but with results in a fraction of the time. In a clinical evaluation of 569 patients and healthy volunteers, FlexSure HP yielded a relative sensitivity of 95% and relative specificity of 93% when compared directly with HM-CAP EIA, but provided results in 5 min as opposed to 60 min for the EIA. The availability of a rapid, accurate, in-office test like FlexSure HP for the diagnosis of H. pylori infection should simplify the physician’s task of deciding on appropriate treatment strategies for patients presenting with symptoms suggestive of gastritis, peptic ulcer disease, gastric cancer, or mucosa-associated lymphoid tissue lymphoma. Following this practice could reduce the need for invasive endoscopy except for those in whom recurrent or recrudescent infection or cancer is suspected.

References