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Detection of *Helicobacter Pylori* Antigen in Stool by Enzyme Immunoassay

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Invasive techniques for diagnosis of *Helicobacter pylori* (*H. pylori*) infection require an endoscopic examination which is expensive and inconvenient and may cause complications. Stool cultures for *H. pylori* or a direct detection of *H. pylori* antigen in stools by PCR are expensive, tedious, and have a low sensitivity. We recently used an enzyme immunoassay (EIA) to detect *H. pylori* antigen in stool specimens. A total of 41 patients were seen at Inha University Hospital, Inchon, Korea between September and October 1998. There were 26 men and 15 women who had an average age of 37.6 years which ranged from 5 to 71 years in the present study. All of these patients came to the hospital complaining of an upper abdominal discomfort and were subjected to endoscopy and biopsies. Fifteen had a gastric ulcer, 13 had a duodenal ulcer, 1 had an early gastric cancer, and there were 12 chronic gastritis patients as shown by endoscopy. The biopsy specimens were examined by histology, CLO™ test, and cultures and these results were used as gold standards. Stool specimens were tested for the *H. pylori* antigen by EIA. A dual wavelength cut-off of 0.100 that was recommended by the manufacturer gave a good performance (87.1% sensitivity, 100% specificity, 100% positive predictive value, 71.4% negative predictive value, and a 90.2% efficiency). But the adjusted cut-off value using the receiver operating characteristic curve improved the performance of the test (using the cut-off value of 0.024, the sensitivity, specificity, PPV, NPV, and efficiency were 100%, 90.0%, 96.9%, 100%, and 97.6% respectively). Re-evaluation of the cut-off value may be needed for Korean patients. This technique is non-invasive, rapid, easy-to-use, and shows good performance characteristics for diagnosis of *H. pylori* infections. Therefore, this technique may be a substitute for gastric endoscopy especially in children and some patients who are unable to tolerate an endoscopic examination and it may be substituted for a serologic test in epidemiological research.

Key Words: *Helicobacter pylori*, stool antigen, EIA, ROC curve, HpSA™

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INTRODUCTION

H. pylori plays a major role in the etiology of peptic ulcer disease and is a probable initiating factor in gastric carcinoma and MALToma.[1-3] Peptic ulcer disease carries a significant morbidity and mortality. Because the eradication of *H. pylori* leads to a cure of the peptic ulcer disease, all *H. pylori*-positive ulcer patients should be treated using antimicrobial regimens. The diagnosis of a *H. pylori* infection is currently established by histology, a rapid urease result, and culture tests using endoscopic biopsy specimens or by serologic and a urea breath test (UBT). However, endoscopic biopsy is an invasive procedure and has the potential for a sampling error because of the heterogenous localization of the *H. pylori* in the stomach. A serologic examination does not necessarily reflect the current status of the infection.[4-5] UBT requires expensive equipment.[6] Attempts to isolate *H. pylori* from human stool samples by various methods have shown incon-

sistent and conflicting results. Cultures of *H. pylori* from feces are tremendously difficult.[7] PCR was tried to detect *H. pylori* DNA from stools,[8] but using PCR on fecal samples can easily give false-negative results because there are a variety of inhibiting chemicals in the stools.[9,10] A new enzyme immunoassay method that uses polyclonal anti-*H. pylori* captures antibody and detects *H. pylori* antigen in stool was developed recently, A receiver operating characteristic (ROC) curve analysis can be used to test in situations where a diagnostic test which yields a numerical test result is being evaluated against an independent "gold standard" or "true" diagnosis of whether the disease is present or absent.

On the basis of the ROC curve, the area under the ROC curve (AURC) can be calculated, which is a measure of the diagnostic performance of the test. In the present study, we evaluated the diagnostic utility of the newly developed EIA method by a comparison with established methods and reevaluated the appropriate cut-off value by using the ROC curve.

MATERIALS AND METHODS

The *H. pylori* status was evaluated in 41 consecutive patients (26 male, 15 female; with a mean age 37.6 years (with a range of 5 - 71 years)) who attended our endoscopy department complaining of an upper abdominal discomfort in the period between September and October 1998. Those who agreed to participate in the study cooperated by submitting their fresh stool specimens. Those patients who had used bismuth-containing compounds or antibiotics in the preceding 2 months or a proton pump inhibitor in the preceding month were excluded from the study. Patients who had undergone a gastric surgery were also excluded. There were 15 patients who had a gastric ulcer, 13 who had a duodenal ulcer, 12 who had a gastritis, and 1 who had an early gastric cancer (Table 1).

Table 1. Patient Characteristics

Total	41
Sex	
Male	26
Female	15
Age in years (mean ± S.D.)	5 - 71 (37.6 ± 19.3)
Disease	
Gastric ulcer	15
Duodenal ulcer	13
Gastritis	12
Early gastric cancer	1

Diagnostic methods

A culture, CLO™ test (Delta West, Bentley, Western Australia), and histologic examination were performed using endoscopic biopsy specimens that were obtained from the antrum and the body of the stomach. In the histologic examination, a pathologist who was blind to the patient's *H. pylori* status examined the hematoxylin-eosin and the alcian yellow-toluidine blue[11] stained slides.

Standard for determination of the *H. pylori* infection status

Patients who had positive results on the culture test or in both of two other methods (CLO™, histologic tests) were classified as *H. pylori* infected. Patients who had negative results in all three tests were classified as being non-infected. Patients who could not be categorized as being infected or non-infected were classified as being indeterminate (Table 2).

Table 2. Criteria for Determination of *H. pylori* Infection Status

	Results				
Culture	+	-	-	-	-
Histology	any	+	+	-	-
CLO	any	+	-	+	-
Criteria	infected	infected	indeterminate		non-infected

+, positive; -, negative.

EIA test for detection of *H. pylori* antigen in stools

Stool specimens were received in an airtight transport container and stored at -70℃ until tested.

An enzyme immunoassay (Premier Platinum HpSA™; Meridian Diagnostics Inc., Cincinnati, OH, USA) utilizing an immunoaffinity- purified polyclonal anti-*H. pylori* rabbit antibody adsorbed to microwells was carried out according to the manufacturer's instructions. Diluted patient samples and a peroxidase conjugated polyclonal antibody were added to the wells and incubated for one hour at room temperature, then a wash was performed to remove unbound material. The substrate was added and incubated for ten minutes at room temperature. In the presence of bound *H. pylori* antigens, a color develops. A stop solution was added and the absorbance was read at 450/630 nm by a spectrophotometer. The cut-off values recommended by manufacturer were as follows: it was negative when the optical densities of the dual wavelengths ($OD_{450/630}$) were less than 0.100, and it was positive when the $OD_{450/630}$ were equal or greater than 0.120, and the other values were equivocal. Equivocal results, according to the manufacturer's instructions, should be repeated. In this study, equivocal results were not repeated but used for the calculation of a new cut-off value using the ROC curve.

Statistical analysis

The sensitivity, specificity, positive (PPV) and negative (NPV) predictive values were calculated using standard methods. All of the data was analyzed using a statistical program (GraphROC for Windows, Version 2.0). The ROC curve was obtained by integrating varying sensitivity and specificity data and plotting the curve of sensitivity versus 1-specificity for all the cut-points of the test.[12] The AURC was expressed by a Wilcoxon (W) value, which is a relative measure of the performance characteristics of the test. The W value varied between 0.5 (this shows no apparent accuracy) and 1.0 (this is a perfect accuracy) as the ROC curve moves toward the left and top boundaries of the ROC graph. The standard error of the W values (SE[W]) was also calculated. This is an objective measure of the performance characteristics and expresses the degree of resolution of the data and their accordance with the "fitted" ROC curve.[13] To establish the best cut-off value (optical density at 450/630 nm) in our patient group, we recalculated the cut-off value by a ROC analysis by using OD values according to the *H. pylori* positivity expression using standard methods.

RESULTS

Forty-one patients were enrolled in this study (26 males, 15 females; the age range was 5 - 71 years with a mean of 37.6). According to the predefined study criteria, 31 of 41 patients (75.6%) were positive for *H. pylori* and none was classified as being indeterminate. All stool samples could be processed by the HpSA test and a definitive result was obtained for all of the patients.

Table 3 shows the HpSA™ test results using a cut-off value of 0.100 ($OD_{450/630}$) in *H. pylori*-positive and *H. pylori*-negative patients. Twenty-seven of the 31 fecal specimens from H. pylori infected patients were positive by EIA suggesting a sensitivity of 87.1% (95% confidence interval [CI], 72.5 - 95.4%). Of the 10 fecal specimens from the noninfected individuals, none was positive by EIA suggesting specificity of 100% (95% CI, 74.1-

Table 3. Results of HpSA™ Enzyme Immunoassay with a Cut-off Value of 0.100 ($OD_{450/630}$) Compared to the *H. pylori* Status

	No. of patients indicated with *H. pylori* status		Total
	Positive	Negative	
HpSA™			
Positive	27	0	27
Negative	4	10	14
Total	31	10	41

Sensitivity 87.1%, specificity 100%, PPV 100%, NPV 71.4%, and efficiency 90.2%. No, Number.

100%), The positive predictive value (PPV) was 100%, the negative predictive value (NPV) was 71.4%, and the efficiency was 90.2%.

There were 4 false-negative cases that had a cut-off value of 0.100 ($OD_{450/630}$). The optical densities of the 4 false-negative specimens as determined by HpSA were just below 0.100. Two patients had intestinal metaplasia endoscopically and one was an 8 year old child (Table 4, Fig. 1).

At a cut-off value of 0.120 the specificity was not changed (100%) (95% CI, 74.1 - 100%), but the sensitivity dropped to 80.6% (95% CI, 65.0 - 91.2%) with a poor NPV (62.5%) (Table 5).

According to the ROC curve, the W value (area under curve) was 0.9871, which was very close to 1 (perfect accuracy), and expressed very good performance characteristics. The SE [W] was only 0.0148, and expressed the high resolution and good accordance with the fitted ROC curve. The most efficient cut-off value that could be recalculated from this ROC curve was 0.024. Using this cut-off value, the sensitivity, specificity, PPV, NPV, and efficiency were 100% (95% CI, 90.8-100%), 90.0% (95% CI, 59.4-99.7%), 96.9%, 100%, and 97.6%, respectively (Table 6, Fig. 2).

Table 4. Characteristics of 4 False-negative Cases with a Cut-off Value of 0.100 ($OD_{450/630}$)

Age	Sex	Endoscopic diagnosis	Intestinal Metaplasia	$OD_{450/630}$
8	F	Gastritis	absent	0.062
39	M	Duodenal ulcer	absent	0.070
45	M	Gastric ulcer	present	0.083
71	F	Gastritis	present	0.035

F, female; M, male.

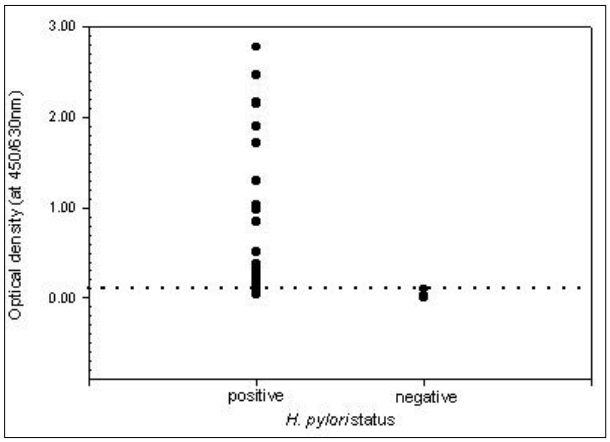

Fig. 1. Optical density at 450/630nm according to *H. pylori* status. Dotted line indicates cut-off level of 0.100.

DISCUSSION

The importance of *H. pylori* in gastrointestinal

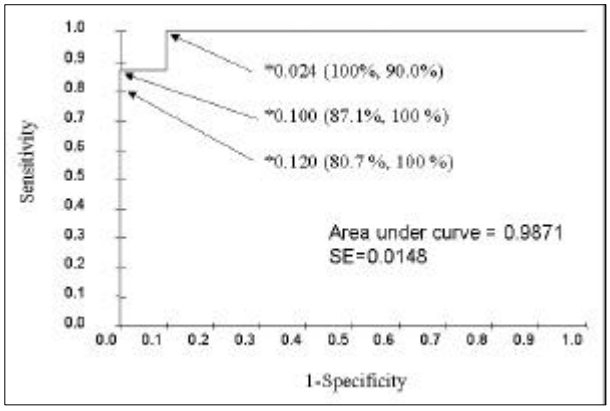

Fig. 2. Receiver operating characteristic curve of the HpSA^{TM} test. W value was 0.9871 ± 0.0148. Based on the ROC curve, the cut-off value of 0.100 recommended by manufacturer shows good specificity (100%) with acceptable sensitivity (87.1%), but the most efficient cut-off value for diagnosis of *H. pylori* was identified as 0.024. * Cut-off value (sensitivity, specificity)

Table 5. Results of HpSA^{TM} Enzyme Immunoassay with a Cut-off Value of 0.120 ($OD_{450/630}$) Compared to the *H. pylori* Status

	No. of patients indicated with *H. pylori* status		Total
	Positive	Negative	
HpSA^{TM}			
Positive	25	0	25
Negative	6	10	16
Total	31	10	41

Sensitivity 80.6%, specificity 100%, PPV 100%, NPV 62.5%, and efficiency 85.4%. No, Number.

Table 6. Results of HpSA™ Enzyme Immunoassay with a Cut-off Value of 0.024 ($OD_{450/630}$) Compared to the *H. pylori* Status

	No. of patients indicated with *H. pylori* status			Total
	Positive		Negative	
HpSA™				
	Positive	31	1	32
	Negative	0	9	9
Total		31	10	41

Sensitivity 100%, specificity 90.0%, PPV 96.9%, NPV 100%, and efficiency 97.6%. No, number.

disease has greatly increased since Marshall and Warren described the presence of Campylobacter-like organisms in the antral mucosa of patients who had gastritis and peptic ulcers.[14,15] The detection of *H. pylori* infection is therefore helpful in the management of patients who have upper abdominal symptoms.

The diagnostic strategies for the determination of *H. pylori* have developed along two lines which are invasive and non-invasive techniques. Direct detection by invasive methods requires that a biopsy must be taken from the upper gastrointestinal tract. The presence of *H. pylori* is then confirmed by a direct microscopic examination, a rapid urease test or culturing of the organism from the biopsy material. This method has the advantage of being able to detect active infections while being specific and has a very high positive predictive value. Disadvantages associated with this approach are the procedure risks and the discomfort of the patient. In addition, *H. pylori* tends to colonize in patches and may be missed totally by a sampling error. Culturing of *H. pylori* from biopsy material tends to be difficult and time consuming.[16-18] The ^{13}C- or ^{14}C-labeled urea breath test (which is based on the detection of ^{13}C- or ^{14}C-labeled CO_2 in expired gas as a result of *H. pylori* urease activity) is a non-invasive method that has a high sensitivity and specificity. But it is expensive, time consuming, requires specialized test equipment, and, in the case of ^{14}C UBT, the radioactivity requires specific handling and storage precautions[19] as well as the high cost of specialized equipment[6] for the detection of ^{14}C or ^{13}C. Also, the UBT may be difficult to perform in infants. The most common non-invasive approach for the detection of *H. pylori* is the serological identification of specific antibodies, because it is simple and inexpensive. However, it suffers from having a low sensitivity and specificity. It is also not a suitable epidemiologic tool for screening for *H. pylori* infection in infants and children in developing countries.[20] It has been reported in Korea that the sensitivity is around 70% and that the specificity between 20 and 90%.[21,22] These rates are lower than those reported from around the world.[23] These tests are somewhat unreliable due to cross reactions with other organisms or due to a "serological scar" that does not necessarily reflect the current status of the infection.[5,24,25] After a successful eradication of the organism, several months are required before the antibody titer falls sufficiently to accurately predict the eradication outcome.[26]

Attempts to isolate *H. pylori* from human stool samples by various methods have shown inconsistent and conflicting results. Until recently, no one was able to culture *H. pylori* from the feces of infected patients. However, the microorganism was successfully cultured from the feces of a group of African children.[27] This finding has not yet been confirmed by other laboratories.[28,29] These authors were unable to isolate *H. pylori* from any fecal sample, although *H. pylori* was cultured from control feces spiked with the organism. In fact, the culture of *H. pylori* from feces is tremendously difficult, because the overgrowth of other bacteria can occur due to inevitable delay between defecation and processing of the sample, or the spiral *H. pylori* may change into non-culturable but viable coccoid forms as a result of the toxic fecal environment.[7] PCR was tried in

an attempt to detect the *H. pylori* DNA from the stool because this enables the amplification of minimum quantities of target DNA and provides a very sensitive detection method.[8] However, PCR of fecal samples can also easily give false-negative results because a variety of chemicals in the stool can inhibit the reaction.[9,10] In spite of precautions to avoid the problem of inhibition of PCR with feces, *H. pylori* target DNA could not be detected by van Zwet et al in the stools of *H. pylori* infected patients.[30]

An enzyme immunoassay that detects *H. pylori* antigen in stool offers the advantages of being a simple sampling method and it can be done by a spectrophotometer. This new test utilizes a polyclonal anti-*H. pylori* capture antibody that is adsorbed to microwells and shows good performance characteristics.

The most efficient cut-off value for diagnosis of the *H. pylori* was somewhat lower than that recommended by the manufacturer according to the ROC curve in our patients. With an adjustment of a cut-off value to 0.024, the sensitivity, specificity, PPV, NPV, and efficiency have improved respectively. There were 4 false-negative cases with a cut-off value of 0.100. Colonization with intermittent shedding of bacteria in the stool may explain the false negativity. Because this test are very sensitive to the accuracy of the methodology, reanalysis of the diluted positive samples revealed false-negativity in another study.[31] In the meta-analysis, the weighted mean for the sensitivity was 93.1% and the specificity was 92.8%. Also, there has been no significant difference in the results reported by different investigators between developed and developing countries.[32] However, using a cut-off value of 0.100, our data showed a sensitivity of 87.1%. As a result, the *H. pylori* antigen may be diluted by the bulky volume of the stool in Korean patients who have much more fibers in their diet than Western patients.[33] Korean patients are infected by *H. pylori* at younger ages than Western patients, and more frequently have an intestinal metaplasia that is a hostile environment for the colonization of *H. pylori*.[34] The shedding of bacteria may be lower in these patients. Due to the small number of patients who had intestinal metaplasia in our study, this hypothesis couldn't be proved statistically. Dilution of the antigen or a decrease of shedding because of a hostile environment may explain the false negatives observed with optical densities that are just below the cut-off value. So a downward adjustment of cut-off value may be necessary in Koreans due to differences in race or geographical regions.

This analysis detects *H. pylori* antigen but does not distinguish between live or dead bacteria. Detecting dead bacteria and coccoid forms of *H. pylori* that do not produce urease may cause a false positive result. But in our patients, there were no false positive cases. Long-term retention of *H. pylori* antigen in the colon, especially in patients who have diverticula may cause a false positive result after the eradication of *H. pylori*.

This technique is a highly sensitive and specific, non-invasive tool for the diagnosis of an *H. pylori* infection. It is cost effective and convenient. Therefore, this technique may be a substitute for gastric endoscopy especially in very young pediatric patients and some patients who are unable to tolerate an endoscopic examination and can be a substitute for a serologic test in epidemiological research. Further study is needed to determine whether this test could assess eradication of *H. pylori* after antimicrobial therapies and, if so, which is the optimal time for its application.

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