

Diagnosis of helicobacter pylori infection by ELISA stool antigen and comparison with the other diagnostic methods

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Abstract

Purpose: The object of this study is to design a simple and sensitive enzyme immune assay method for detecting *Helicobacter pylori* antigens in stool.

Materials/Methods: Stool samples were collected from 116 adults who were undergoing endoscopic examinations and stomach biopsies and for whom histology and rapid urease tests were performed. A monoclonal antibody was used as the capturing antibody and a polyclonal antibody of rabbit origin conjugated with a peroxidase enzyme as the tracer.

Results: In the histology and rapid urease tests, 21 of the 116 patients (18.1%) had positive results. *H. pylori* antigens were detected by the designed method in 19 of 21 cases (a sensitivity of 91%). Also, all of the 95 cases with negative results in the histology and rapid urease tests were negative for the stool antigen test (a specificity of 100%). For comparison, the sensitivity and specificity of the rapid immuno-chromatography test by the Certest Company were 95% and 99%, respectively. The total correlations between the results of the designed ELISA test with the results of the ra-

pid test and the ELISA test of the Astra Company were 96% and 80%, respectively.

Conclusions: This non-invasive and economical method for the detection of *H. pylori* antigens in stool can be considered as an alternative test that provides comparable reliability and validity to the histology and rapid urease tests for the detection of *H. pylori* infections.

Key words: *Helicobacter pylori*; Enzyme linked immune sorbent assay (ELISA); Stool

Introduction

H. pylori is one of the most prevalent bacterial pathogens in humans. The incidence of infection in various countries ranges from 10% to 80%, and the average is 50%. Infection of the digestive tract by *H. pylori* is among the most widespread, chronic infections in humans (1), and the relationship between *H. pylori* infection and stomach ulcers, duodenal ulcers, and stomach adenocarcinoma has been established (2). Identification of this infection is performed by invasive and non-invasive methods. The invasive method involves endoscopy, after which other tests, such as rapid urease, histo-

pathology, culturing, and Polymerase Chain Reaction (PCR), are used for identification purposes. The non-invasive methods include the respiratory urea test and serologic tests for detecting antibodies and antigens in feces (3). The utilization of invasive methods and endoscopy is limited in some patients, such as children and old patients. The respiratory urea test may be not applicable for children and pregnant women (4, 5). Therefore, it is essential to develop a simple and sensitive method that can be applied to all age groups with results comparable to those of the conventional techniques. The present study is devoted to developing a method for detecting *H. pylori* antigens in stool by the means of monoclonal and polyclonal antibodies.

Materials and Methods

Samples

The samples used in this study consisted of 116 stool samples that were collected from patients who were undergoing endoscopic examinations at the Digestion Research Center at the Shariati Hospital in Tehran. Histological and rapid urease tests were performed for each of the samples.

Stool Antigen Test for *Helicobacter pylori*

In order to investigate the bands of *H. pylori* antigens, they were studied applying sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method, and the bands were compared with a molecular weight marker. A 12% gel with a diameter of 0.75 mm was subjected to electrophoresis in a vertical manner with a constant voltage of 60 volts for two hours.

Immuno-blotting for studying the best Antibody Pair

Immuno-blotting was conducted using the extracted antigens by the acid-glycine method. The SDS-PAGE method was performed on a gel with a 10% concentration. 20 micrograms of antigen were added to each well and electrophoresis

was performed. The antigen was then transferred to nitrocellulose paper and kept frozen at -20°C until the test time.

For immuno-blotting, the antibody with a concentration of 5 $\mu\text{g/ml}$ was diluted with a saline phosphate buffer solution that was supplemented with 1% Bovine Serum Albumin (BSA), and, then, each antibody solution was added to a strip of nitrocellulose paper. In order to control the band presence, a mixture of several sera (pooled sera) that contained the antibody against *H. pylori* was diluted by phosphate buffer at a ratio of 1:100. After two hours of incubation at 37°C , washing was performed using a saline phosphate buffer solution with a 0.05% concentration of Polysorbate 20 (Tween 20). The utilization of enzyme conjugate as a tracer was dependent on the type of antibody.

Stool Antigen Test

Coating was conducted using mouse monoclonal antibody number 2 at different concentrations in a carbonate buffer with $\text{pH} = 9.6$ at 4°C and at room temperature for 24 hours. Subsequently, the plates were washed with phosphate buffer containing Tween 20, and the wells were blocked for one hour at room temperature by a blocker solution containing 1% BSA and carbohydrate. Afterwards, the contents of the well were decanted and kept at room temperature for six hours until they were completely dry. These plates were maintained at $2-8^{\circ}\text{C}$ in a foil that contained a desiccant until the experiment was to be performed. Rabbit polyclonal antibody number 4 was utilized as a tracer.

Sample Preparation

The collected stool samples were kept frozen at -20°C . Before the test, the samples were thawed and thoroughly mixed so that the probable antigens could locate all over the stool sample constantly. In the next stage, 200 μl of the triple buffers, i.e., 0.05 M saline phosphate buffer, saline phosphate buffer containing 0.1% triton X-100, and 1.5 M glycine buffer with $\text{pH} = 7.2$ as a diluent, were added to a 5 mm diameter piece of completely premixed stool that was further mixed thoroughly

using a vortex mixer. Subsequently, the samples were centrifuged at 5000 rpm for 10 minutes. The supernatant was transferred to a 1.5-ml Eppendorf tube that was then used for the ELISA test. This sample was kept in the freezer at -20 °C until it was needed in the experiment.

Performing the Test

Six 100- μ l volumes that contained different concentrations of the *H. pylori* antigenic soup of the supernatant of stool samples diluted by extracting solutions were prepared. The concentrations of them were 0, 0.1, 0.5, 1, 2.5, and 5 μ g/ml, and these solutions were added to each of the wells coated with monoclonal antibody.

Then, 50 μ l of the anti-*Helicobacter pylori* polyclonal antibody conjugated with peroxidase enzyme were added to each well. The mixture was incubated for two hours in a water bath at 37 °C. After incubation, the contents of the wells were removed, washed five times with 300 μ l of washing buffer and then removed completely. Subsequently, 100 μ l of a substrate chromogen solution containing tetramethyl benzidine and hydrogen peroxide was added to all the wells, and they were incubated at room temperature for 15 minutes. The reaction was terminated using 100 μ l of 1 N HCl, followed by reading the Optical Density by Asys ELISA reader at wavelengths of 450 nm and 630 nm as reference filters.

Investigation of fecal inhibitive effect

To study the fecal inhibitive effect, a certain amount of antigen was added to the feces supernatant of several negative and positive samples, in which the final concentration of antigen was 5.2 μ g/ml. The resultant samples were tested along with the negative and positive samples without adding antigen, for antigen concentration.

Study of Cross-reaction

A microbial suspension with a concentration of 1×10^8 per ml containing the following bacteria

was added to the negative and positive samples; then, the samples were analyzed and compared to the samples prior to adding the bacterial suspension. The bacteria included: *Klebsiella pneumoniae*, *Salmonella* group B, *Shigella flexneri*, *Shigella sonnei*, *Citrobacter freundii*, *Citrobacter diversus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*.

Analytic Sensitivity of the Assay

Using serial dilution of the antigen with phosphate buffer, the standard curve was drawn, and the amount of antigen that had an optical density (OD) greater than the cut-off value was defined as the assay detection limit.

Investigation of Assay Imprecision

Assaying was conducted several times on negative, weak positive, and strong positive samples. Averages, standard deviations, and coefficients of variation (CV%) were calculated.

Comparative Test

In order to compare the results of the new method with other methods, we used an immuno-chromatography kit from the Certest and the ELISA kit from the Astra Companies. Both were used according to the manufacturers' instructions.

Results

The results of the SDS-PAGE method on the antigenic soup and staining with Coomassie Brilliant Blue revealed that this antigenic soup contains most of the bacterial antigens.

The results of immuno-blotting of existing antibodies indicated that only two antibodies, i.e., number 2 and number 4, can identify most significant antigens of the *H. pylori*. Antibody number 5 only identified antigens in the range of 60 to 70 kD; it is noticeable that most antigens of *H. pylori*

that have cross-reactions are located in this region. Furthermore, antibodies number 1 and number 3 can identify a few antigens.

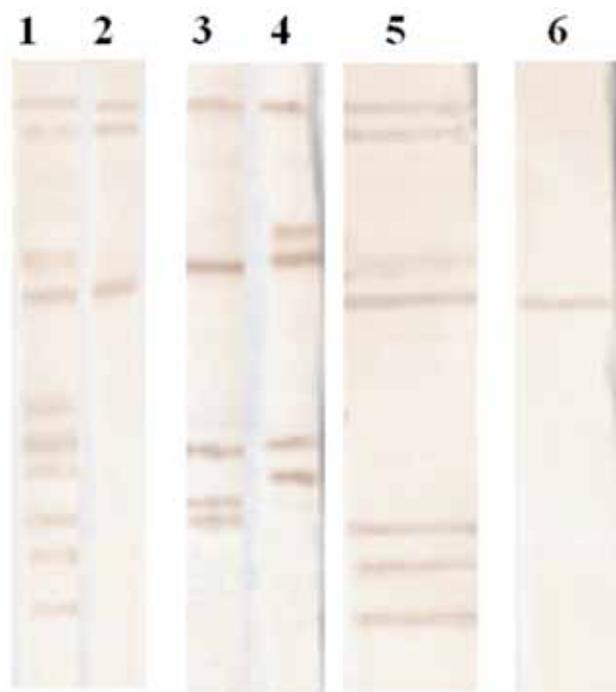


Fig. 1. Immuno-blotting of anti-*Helicobacter pylori* antibodies

Row 1: anti-*H. pylori* serum; 2: monoclonal antibody number 1; 3: monoclonal antibody number 2; 4: polyclonal antibody number 3 of rabbit origin; 5: polyclonal antibody number 4 of rabbit origin; Row 6: monoclonal antibody number 5.

Investigating the appropriate buffer for the extraction of antigens from stool

There is no advantage to use the phosphate buffer containing triton X-100 and glycine buffer compared to phosphate buffer alone. (Table 1).

Investigating the inhibitive effect of fecal material on assays

The data indicated that no interfering inhibitor existed in the samples to result in false negative values (Table 2). This was obtained by adding some amount of antigen to stool so that the final concentration of antigen in the supernatant of the stool samples was 2.5 $\mu\text{g/ml}$, comparing the OD antigen with the same concentration in the phosphate buffer as the diluent for the feces sample, and calculating the recovery level of antigen.

Investigating the Cross-reaction

After addition of the microbial suspension, no increase was observed in the ODs of the negative samples. Furthermore, no interfering effect was noticed in the positive samples subsequent to the addition of the microbial suspension.

Table 1. Results of tests of different buffers for extracting antigens from feces

	Glycine buffer OD unit	Phosphate buffer containing triton OD unit	Phosphate buffer OD unit
Positive sample	1.7	1.9	2.2
Negative sample	0.05	0.02	0.03
Negative sample containing 5 μg of antigen	1.6	1.7	2.0
Antigen sample in buffers	2.3	2.4	2.7
Only buffers	0.04	0.02	0.03

Table 2. Results of the investigating the interfering effect of stool composition on antigen assay

	Recovery mean (%)	OD unit of stool samples after adding 2.5 μg antigen	OD unit of phosphate buffer after adding 2.5 μg antigen	OD unit of native samples
Negative sample 1	93.5	1.31	1.4	0.03
Negative sample 2	91.4	1.28	1.4	0.025
Negative sample 3	95.7	1.34	1.4	0.017

The mean OD of the samples that were negative in the histology tests and the rapid urease tests was 0.04 with standard deviation of 0.02. The cut-off OD was calculated adding three SD to the mean OD of the negative samples, which yielded the value of 0.10. All samples with OD values greater than 0.10 were considered positive.

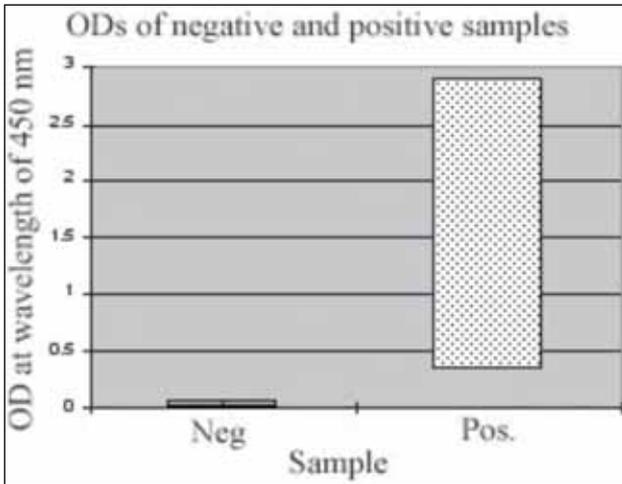


Fig. 2. Comparison of the ODs of negative and positive samples using the designed ELISA method

Among 116 samples obtained from patients undergoing endoscopic examinations, 21 cases had positive results in the histology and rapid urease tests. Nineteen of these 21 samples (90%) were also positive in the stool antigen test. Among the 95 samples with negative results in the histology and rapid urease tests, all (100%) were negative in the stool antigen test. According these results, the sensitivity and specificity of the designed assay method were calculatedly 90 and 100%, respectively. The positive and negative declarative values of the designed assay were 100% and 98%, respectively. The sensitivity and specificity of the rapid kit produced by the Certest Company were 95% and 99%, respectively. Furthermore, the correlations of the designed assay for all samples with the rapid kit and with the ELISA kit were 96% and 80% respectively (Table 3).

Analytical Sensitivity of Assay

Using serial dilutions of antigen in phosphate buffer and according to the cut-off value, the lowest recognizable concentration using this assay was determined to be less than 0.1 µg/ml of antigen.

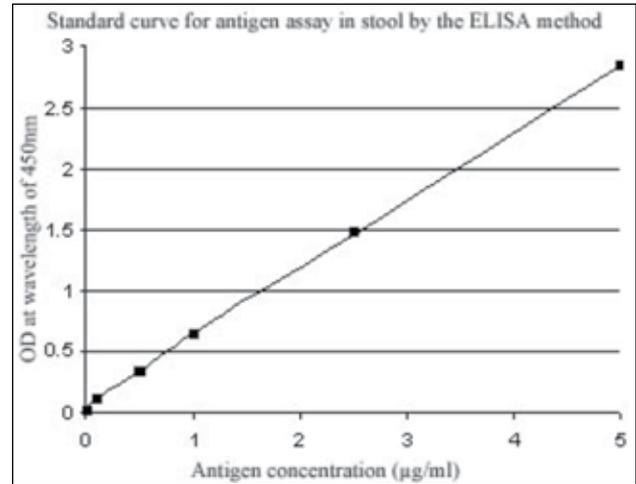


Fig. 3. Standard curve for antigen assay in stool by the ELISA method

According to the results obtained, this assay has an acceptable level of imprecision that is comparable to levels observed with other existing kits (Table 3).

Discussion

Regarding the wide-spread incidence of Helicobacter pylori infections in developing countries, including Iran, the correct and proper identification of this infection and the extermination of this bacterium can change the normal period of the disease through decreasing the rate of its recurrence in treated patients.

Histological evaluation is considered to be the gold standard for identifying H. pylori bacteria. Nevertheless, it suffers from limitations, such as need for an endoscopic examination, which is unpleasant for patients; in addition, biopsy sam-

Table 3. Results of imprecision tests of the antigen assay in feces by the ELISA method

	Mean OD	Standard deviation	Coefficient of Variation (CV), %
Negative sample	0.07	0.009	12.8
Weak positive sample	0.56	0.004	7.8
Strong positive sample	1.92	0.8	4.2

ples may not be adequate for identification purposes. Culturing is another invasive method that has a limited role in the identification of infections due to difficulty in growing the bacteria and their slow rate of growth in the culture medium (8). Polymerase Chain Reaction (PCR) is considered as a method with desirable sensitivity and specificity. However, it is expensive and may not be used in all laboratories. It is not applicable in case of treatment, and also produces false positive results sometimes. The rapid urease test, on the other hand, is a fast and available technique that is applied to biopsy samples; nevertheless, it has low specificity, and the results it produces are influenced by antibiotics, bismuth compounds, or proton-pump inhibitors. Among the non-invasive methods, serology is a simple and fast option that is also inexpensive. It is practical for use in regions with low incidences of *H. pylori* infections, and it should be performed and considered as the second priority of diagnosis tests, due to its low predictive value (9).

A non-invasive, simple, inexpensive, and easily applicable method will be very useful in solving the existing problem of identifying *H. pylori* infection. Taking advantage of assaying *H. pylori* antigens in stool by the ELISA method is an appropriate solution for this problem (10).

In 2003, Andrews et al. studied 111 adult dyspepsia patients under endoscopy and post-biopsy, and they compared the results regarding the presence of *H. pylori* antigens in stool samples using three types of ELISA kits. Cultures, histopathological tests, and rapid urease tests were conducted as the gold standard for investigating infection status. The three kits used were Premier Platinum, Femtolab, and Diapro. In the first kit, a polyclonal antibody is used, while the other two kits make use of monoclonal antibody. The three mentioned kits had sensitivity values of 63.6%, 88%, and 56%, respectively. Also, their specificity values were 92.6%, 97.6%, and 97.6%, respectively (11). We acquired better results by using a monoclonal antibody with an incubation time of two hours at 37 °C.

In 2004, A.S. Chisholm et al. surveyed 112 patients using the Premier Platinum HpSA ELISA kit and obtained sensitivity and specificity values of 91% and 96%, respectively. Additionally, in the-

ir research, they utilized the ImmunoCard STAT! HpSA kit and reported sensitivity and specificity values of 91% and 95%, respectively. They considered histological methods and culturing as the gold standard (12).

In 2005, E.M. Gulcan and his colleagues performed a study on 80 patients under endoscopy by the HpSA ELISA method (Premier Platinum kit) and obtained sensitivity and specificity values of 99% and 96%, respectively (13).

In our research, the sensitivity and specificity of the designed kit were determined to be 90% and 100% and the test is accomplished in 2.25 hours. We compared our assay with ImmunoCard HpSA kit from the Certest Company and the ELISA kit from the Astra Company. For the ImmunoCard kit, the resulting sensitivity and specificity values were 95% and 99% respectively; whereas the values for the ELISA kit were 85% and 90%, respectively. It should also be noted that the histological and rapid urease tests were considered as gold standard in the present study.

Conclusions

This non-invasive and economical method for the detection of *H. pylori* antigens in stool can be considered as an alternative test that provides comparable reliability and validity to the histology and rapid urease tests for the detection of *H. pylori* infections.

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