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Designing an ELISA Technique for *H.pylori* Antibody Detection Using Water Extracted Antigens

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Abstract

H.pylori infection stimulates immune responses. These responses at the mucosal level are predominantly of IgA types, while circulating antibodies against this microorganism are predominantly IgG classes. IgM antibodies are rarely found and seem to be non-specific for this bacterium. In this research, water extract antigen, from three strains of *H.pylori* (isolated from patients with gastritis, duodenal ulcer and normal human) was investigated for the detection of serum IgG antibodies against *H.pylori* by an indirect ELISA technique. Antibody titers against *H.pylori* were measured in 72 patients of whom 64 cases were *H.pylori* positive and 8 cases were *H.pylori* negative (confirmed by culture and urease tests). In this test, those titers that were more than 1/6400 indicated the rising of IgG titers and serum positive, being in testee, and the titers, which were equal or less than 1/6400 indicated the serum negative, being in individuals. Our ELISA results indicated that between 64 *H.pylori* positive individuals, 61 cases were serum positive and between 8 *H.pylori* negative patients, 5 individuals were serum negative; thus, specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) of the test were, 62.5%, 95.31%, 95.31%, 62.5%, respectively. The high level of sensitivity is because of using 3 different strains for preparing of antigens. But the reasons of low specificity are probably using of semi purified antigen.

Keywords: *ELISA,* H.pylori*, Serological diagnosis, Antibody Detection, Iran*

Introduction

Helicobacter pylori has now been established as the major cause of active chronic gastritis in humans and are strongly associated with duodenitis and duodenal ulceration. *H.pylori* has also frequently been found in patients with gastric ulceration, gastric cancer and Barrett's esophagitis. Gastroscopy is necessary to obtain biopsy material for histological and microbiological examination. Cultivation and identification of *H.pylori* is time- consuming and cumbersome, and therefore serological methods should be developed (1-4).

H.pylori infection stimulates immune responses. These responses at the mucosal level are predominantly of IgA types (5, 6), while circulating antibodies to this microorganism are mostly of IgG class (5, 7-9). IgM antibodies are rarely found and seem to be non-specific for this bacterium (5, 8, 10). Serum antibodies against *H.pylori* have been measured by several techniques, such as agglutination and complement fixation. The indirect enzyme-linked Immunosorbent assay (ELISA) has been used most commonly (2, 8, 11).

The antigen preparations in these assays have usually been crude antigens, such as whole *H.pylori* cells, acid glycine- extracted bacteria or sonicated extracts of *H.pylori* (8, 9, 12-14). Only a few studies have been done with puri-

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fied antigens, such as the urease antigen and the 120-kDa protein (8, 15).

Materials and Methods

Patients and serum samples Serum samples and biopsies were obtained from 72 consecutive dyspeptic patients admitted to two hospitals (Imam Khomeini in Karaj and Taleghani in Tehran) for esophagogastroduodenoscopy.

Endoscopy and biopsies Two-biopsy materials were obtained from the pyloric antrum (2-3 cm around the pyloric sphincter). The instruments were disinfected between uses. The endoscopic findings were classified in 4 groups.
Normal endoscopic findings,
Gastritis and duodenitis,
Ulcers including gastric, prepyloric and duodenal ulcers,
Miscellaneous including esophagitis, gastric cancer or precancer, menetriers disease non-tropic sprue or hiatus hernia.

Morphology of Bacteria in biopsies Biopsies were crushed between two slides. Slides were fixed and were stained with carbol fushin and Gram stain to record morphology and the presence of *Helicobacter*- like organisms (Fig. 1).

Urease test of biopsies One of the biopsies was inoculated to urease agar (Christiansen urea agar base) slant tube by streaking the surface of the slant and stabbing the butt of the tube all the way to the bottom, immediately were incubated at 37º C for 24 h (the tubes were checked every 30 min). Positive reaction was indicated when tube color changed to pink.

Microbiology Within three hours after endoscopy the biopsies were seeded on Brucella blood agar with 7% sheep blood and amphotricin B (2 mg/l), trimetoprim (10 mg/l) and vancomycin (10 mg/ml), under microaerophillic conditions (5-10% CO2, that was prepared by candle jar or CO2 incubator) and 70-80% moisture at 37º C for three to six d. *H.pylori* was identified as small translucent colonies (Fig. 2) of motile Gram- negative curved rods, which were oxidase catalase and urease positive. Patients were considered as *H.pylori*- positive either if *H.pylori* was cultured from the biopsies or if urease test of biopsies was positive and as *H.pylori*-negative if both the culture for *H.pylori* and urease test were negative.

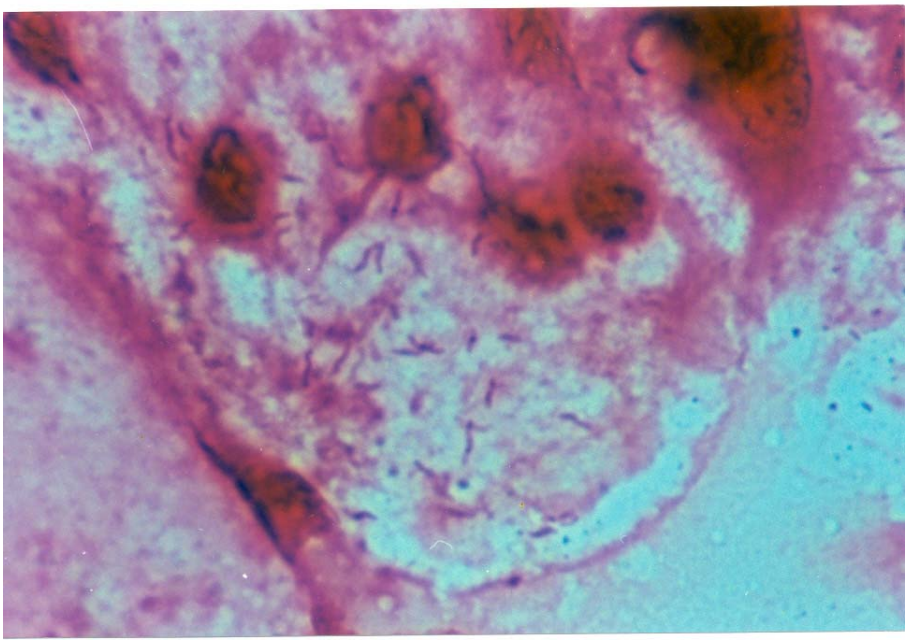

Fig. 1: *H.pylori* bacteria in biopsy smear (carboal fushin staining *1000).

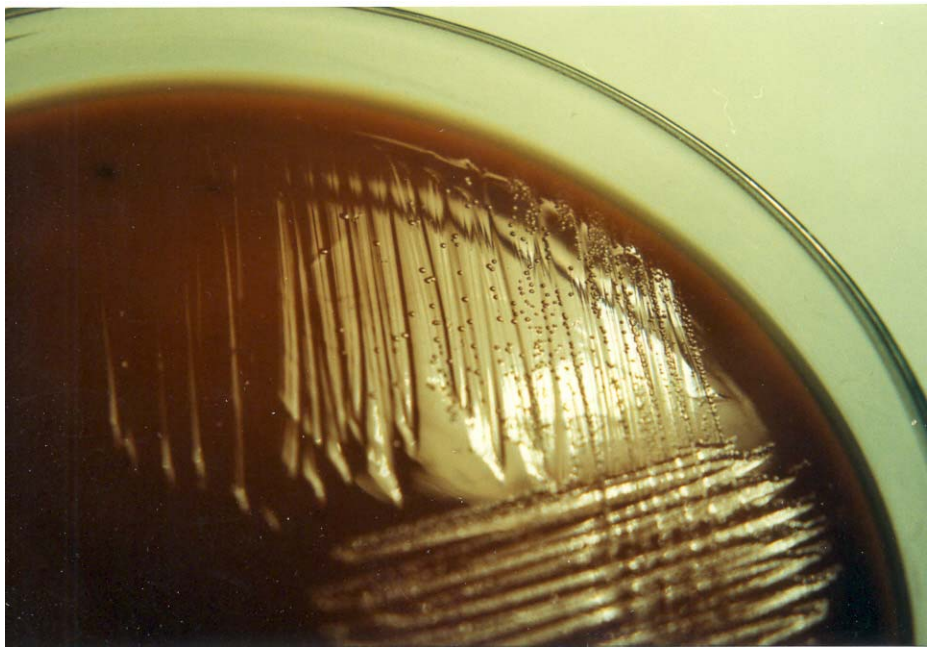

Fig. 2: *H.pylori* colonies in *Brucella* blood agar.

Preparation of water extract antigen from H.pylori Three different isolated *H.Pylori* (isolated from gastritis, duodenal ulcer and without symptom's patients) were grown on *Brucella* blood agar without antibiotic under 5-10% CO2 that prepared by candle jar or CO2 incubator and 70-80% moisture at 37º C for 48 hours. The bacteria were harvested under sterile conditions in phosphate- buffere saline (PBS), pH 7.4.

The harvested cultures were mixed in sterile double distilled water for 25 min. Then this suspension was centrifuged at 23500×g, and the supernatant (after protein determination by lowery method) dialyzed with PBS for an overnight. This dialyzed solution was stored at 70º C, as antigen.

ELISA The antigen concentrations, serum dilutions and dilution of conjugated antibodies were selected preliminary after experiments using different concenterations in each layer. Microtiter plate (NUNC Cat. No. 269787) were coated overnight at room temperature with the antigen preparations at a concentration of 4 microgram protein per ml for each serum sample, uncoated wells served as controls for unspesific plastic binding of serum antibodies. After that the coated miocroplates were blocked with PBS/T/BSA solution. Serum samples for *H.Pylori* antibodies were two-fold diluted from 1/400 to 1/51200. The serum samples (100 microliter) were added to each well of the microtiter plates. The serum samples were tested in duplicate. After 90 min of incubation at room temperature the microtiters plate were washed five times for 90s with 0.1 M PBS and 0.05% Tween 20 (PBS/T) and 100 micro liters of horseradish peroxidase-conjugated rabbit antibodies to human serum antibodies (RAZI Vaccine &Serum Research Institute) diluted 1/300 was added to each well and incubated for 90 min at rt. The plates were washed five times for 90s with PBS/T. The chromogenic reaction was stopped with H2SO4 after 15 min and the optical densities (OD) of the plates were read in a photometer at 492 nm. The antibody amount was expressed as OD values as well as the titer calculated as the reciprocal value of the last positive dilution of the two-fold diluted serum.

We used a positive control and a negative control serum (prepared from a standard *H.pylori* Elisa kit) in each microtitre plate. OD of test serums was compared with that of positive and negative control serum in each microtitre plate.

Determination of cut off value The frequency of titer antibody (Table1) and the curve of IgG titers in infected and uninfected persons (Fig. 3) for determination of cut off value were used.

Cut off value is titers of sera that will have the lowest number of false positive or false negative results.

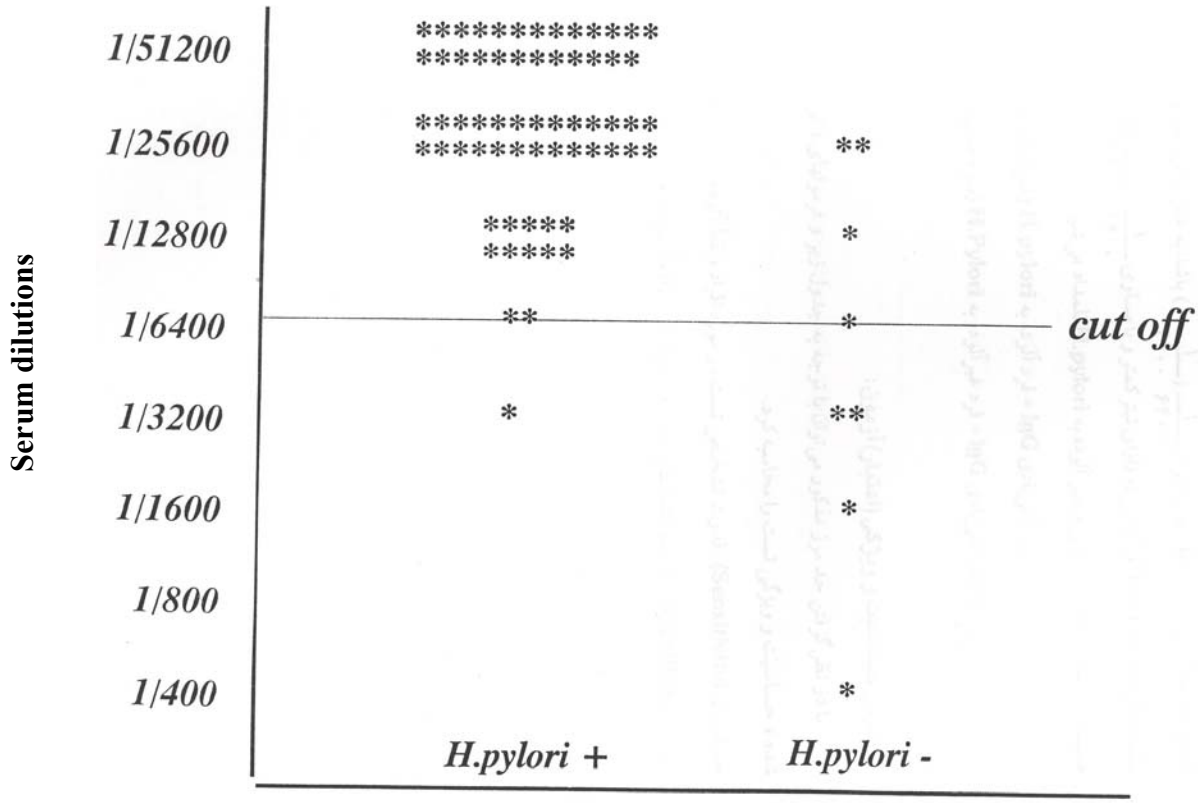

Fig. 3: The curve of IgG titers in infected and uninfected persons

Results

Bacteriological results

Culture was positive in 53 (73.61%) patients and negative in 19 (26.39%) cases.

The result of urease test was positive for 61 (84.72%) and negative for 11(15.28%) patients.

Contamination by H.pylori

64 (88.89%) persons were infected (culture or urease test was positive) and 8 (11.12%) were uninfected.

ELISA results

The frequency of antibodies titers is shown in Table 1 and the curve of IgG titers in infected and uninfected persons is shown in Fig 3.

Depending on the Table 1 and Fig 3 the best cut off was 1/6400 for IgG titers

The specificity, sensitivity, positive and negative predictive value of this study were 62.5%, 95.31%, 95.31% and 62.5%, respectively (Table 2).

Table 1: The frequency table of different antibody titers in our samples

Frequency Ab.titers	No	%
1/400	1	1.38
1/800	0	0
1/1600	1	1.38
1/3200	3	4.17
1/6400	3	4.17
1/12800	11	15.28
1/25600	28	38.89
1/51200	25	34.13
Total	72	100

Table 2: The relationship between serology and *H.Pylori* contamination.

Serology *H.pylori* contamination	+	-	Total
+	61(a)	3(b)	64
-	3(c)	5(d)	8
Total	64	8	72

a= True positive b=False negative c=False positive
d= True negative

Discussion

In this study the discrimination between patients infected with *H.pylori* and uninfected patients were based on either positive culture of *H.pylori* or urease test of biopsies. At least two biopsies were taken from the antrum of the stomach from each patient. This may tend to underestimate the number of *H.pylori*- infected patients in this study.

We found that more than 90% of patients with histologically active chronic gastritis were infected with *H.pylori*. It is, therefore not surprising that the IgG antibody titers were significantly higher (P<0.05) in patients with active chronic gastritis than in patients with normal morphology, as well as in *H.pylori* negative patients (5-10).

Despite these problems in the diagnosis of *H.pylori*, increased IgG antibody titers against water extracted *H.pylori* antigens were found significantly in patients with an ongoing *H.pylori* infection than in patients without it. A considerable overlap of the IgG antibody titers was, however, found between the two groups of patients (infected and uninfected persons). The negative predictive value and specificity in this study are lower than results in other studies (9, 12-14).

The predictive value differs depending on the chosen cut off level. The cut- off level that should be chosen depends on the prevalence of the *H.pylori* infections in the population and the therapeutic consequence of its diagnosis.

In 1989 Bolton et al, used 3 different antigens including glycine extracted (AGE), urease enzyme (UP) and surface antigens (SA) of *H. pylori* in ELISA for detection of IgG titers.

Specificity, sensitivity, P.P.value and N.P.value (positive and negative predictive value) were 82%, 94%, 93%, 79% for AGE, 82%, 92%, 93%, 79% for SA and finally 97%, 90%, 93%, and 96% for UP (10).

The high level of sensitivity (in this study) is because of using 3 different strains for preparaing of antigens. But the reasons of low specificity are probably using of semi purified antigen that has cross reaction with *Campylobacter jejuni* (8,12) or may be existing of false negative results of culture or urease test (because of, using antibiotic by patients, or sampling and handling of biopsy in bad conditions) (8). Therefore, our suggestion is using of pure antigen such as urease antigen for ELISA test.

Incidence of this disease in undeveloped or developing countries is very high (about 85%) and for this reason we could not find a lot of negative (uninfected) patients in our population. With this high frequency of infected persons this small negative control group (8 persons) is enough for determination of specificity and sensitivity of the test (16).

Finally we can say that this kit is a good start for preparing of ELISA kit for *H.pyloi*

infection, but we need additional research but further studies are needed for its developing.

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