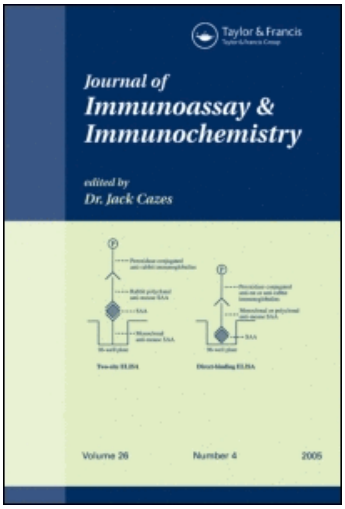

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Serodiagnosis of *Helicobacter pylori* Infection in Korean Patients Using Enzyme-Linked Immunosorbent Assay

S. Y. Kim[a]; J. S. Ahn[a]; Y. J. Ha[a]; H. J. Doh[a]; M. H. Jang[a]; S. I. Chung[a]; H. J. Park[a]
[a] MOGAM Biotechnology Research Institute, Yongin-City, Kyonggi-Do, Korea

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SERODIAGNOSIS OF *HELICOBACTER PYLORI* INFECTION IN KOREAN PATIENTS USING ENZYME-LINKED IMMUNOSORBENT ASSAY

S.Y. Kim, J.S. Ahn, Y.J. Ha, H.J. Doh, M.H. Jang, S.I. Chung, and H.J. Park*
MOGAM Biotechnology Research Institute, 341 Pojung-Ri, Koosung-Myon, Yongin-City, Kyonggi-Do, 449-910, Korea

ABSTRACT

Helicobacter pylori (H. pylori) is a gram-negative spiral bacteria that are associated with gastritis, peptic ulcer and gastric cancer. We have developed enzyme-linked immunosorbent assay (ELISA) that detects serum anti-*H. pylori* immunoglobulin G antibodies using *H. pylori* strains isolated from Korean patients. To assess the sensitivity and specificity of our assay system with different commercial kits, serum samples from 249 Korean patients with a variety of gastrointestinal diseases were tested. Among 249 Korean patients, 178 (71.5%) were positive in culture and/or urease test. The sensitivity and specificity between our assay system and four other commercial kits (Bio-Rad™, DAKO™, ROCHE™, and IPR™) were as follows: 97.8 % and 92%, 94.3% and 53%, 56.5% and 92%, 83.3% and 96%, 58.2% and 92%, respectively. All sera showing discordant immunoassay results between different ELISA kits were confirmed by immunoblot analysis. These results indicate that our assay system showed a highly accurate and reliable results in diagnosis of *H. pylori* infection in Korean patients.

(KEY WORDS : *H. pylori*, gastric disease, ELISA)

* Correspondence : Dr. Hae-Joon Park, MOGAM Biotechnology Research Institute, 341 Pojung-Ri, Koosung-Myon, Yongin-City, Kyonggi-Do, 449-910, Korea (tel: 82-2-741-0611, fax: 82-331-262-6622).

INTRODUCTION

Following the successful isolation of *H. pylori* in 1983 by Warren and Marshall, numerous studies on the role of *H. pylori* on the gastritis, duodenal ulcer, benign gastric ulcer and gastric cancer have been carried out (1-5). *H. pylori* infection causes gastritis and peptic ulcer and is associated with the appearance of gastric cancers and lymphomas. In 1994, National Institute of Health (NIH) in USA announced that *H. pylori* is a causative agent of stomach gastritis and ulcer (6). *H. pylori* infection is very common throughout the world and especially the prevalence rate of *H. pylori* infection is high in Korea and many Korean people become carriers of *H. pylori* from early childhood (7-8).

Several invasive and noninvasive diagnostic tests have been used for detection of *H. pylori* infections (9). Invasive tests require invasive procedures such as gastroendoscopy. When a gastroscopy is performed and biopsies are taken, histological and culture investigation allow accurate detection of *H. pylori* (10). The current "gold standard" tests for diagnosing *H. pylori* infection involve histological staining and/or culture of antral biopsy specimens (11). However, these tests are not suitable for screening of large patients because the sampling method is high cost, time-consuming, and uncomfortable to the patient,. A possible noninvasive alternative is ^{14}C-urea breath test (12). This test reliably detects an *H. pylori* infection but has the disadvantages of inconvenience, high cost in materials,

and the radiation expose associated with the use of ^{14}C-urea. Recently, polymerase chain reaction (PCR) techniques for detection of *H. pylori* in gastric biopsies or feces have been received the most attention but are not yet applicable to routine diagnosis (13).

The noninvasive methods used include the serological detection of antibodies to *H. pylori* by techniques such as enzyme-linked immunosorbent assay (ELISA), complement fixation, immunoblot, and immunochromatography (14-15). Among these methods, ELISA is a simple, quick, reproducible, and low cost technique that permits immunoglobulin class-specific determinations (16). Also, serological tests can be used for follow-up of progression of the diseases, long-term follow up in treatment studies, and screening for *H. pylori* in individuals patients. Recently, various commercial kits have been used for serological diagnosis of *H. pylori* infection, but data on their diagnostic accuracy and direct comparisons between the tests are lacking (17). The only way to assess correctly *H. pylori* serology is to evaluate it with the same serum specimens, from well-defined group of patients.

In this study, we developed ELISA system for the detection of *H. pylori* antibodies in serum using whole-cell sonicates of *H. pylori* strains isolated Korean patients. The sensitivity and specificity of our ELISA system were evaluated and compared with other commercially available ELISA kits in symptomatic Korean patients.

MATERIALS AND METHODS

Materials

ELISA plates (96 well, flat bottom, high binding capacity, #4-69914) were purchased from Nunc (Roskilde, Denmark). Horseradish peroxidase - conjugated goat anti-human immunoglobulin G was obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Tryptic soy agar and columbia blood agar base were obtained from Difco Laboratories (Detroit, MI). Unless otherwise mentioned, all reagents were from Sigma Chemical Co. (St. Louis, MO).

Subjects

The patients group consisted of a series of 249 consecutive Korean patients undergoing upper gastrointestinal endoscopy at Dankuk University hospital in Korea. The patients had a variety of symptoms relating to the upper gastrointestinal disorders. Of 249 patients, 162 were males and 87 were females. The mean age was 47 years, ranging 16 to 70 years. In each endoscopy, two separate antral biopsies were taken and determined the presence of *H. pylori* by culture and urease test, respectively. The patients were classified as *H. pylori*-positive if culture, urease test, or both were positive. A blood sample was taken from each patient immediately before the endoscopy procedure, and sera were stored at - 20℃ until assayed. *H. pylori* negative 50 sera were obtained from Dr. Hazell in the University of New South Wales, Australia. *H. pylori* negative subjects were all

negative for culture, urease, and histology test. The 40 healthy Korean children sera (age range, 6 month to 3 years) were used in order to determine the cut-off value of our assay system.

H. pylori strains and Culture Conditions

Reference *H. pylori* strains were obtained from ATCC (ATTC 43504, 43579, 43629; Rockville, MD). Two *H. pylori* strains were isolated from Korean patients which have superficial gastritis and gastric ulcer symptom, respectively. *H. pylori* strains were grown on a blood agar plate containing tryptic soy agar and columbia blood agar base supplemented with 7 % sheep blood and a antibiotic supplement consisted of vancomycin (10μg/ml), colistin (300unit/ml) and amphotericin B (2.5μg/ml). Bacteria were cultured under microanaerobic condition (10% CO_2, 95% relative humidity) at 37°C for 2-3 days. The bacterial colonies were identified as *H. pylori* on the basis of the oxidase, catalase and urease reaction.

Preparation of the Antigen

The cultured cells were collected from plates by scrapper. These cells were centrifuged at 5,000 g for 10 min and resulting pellet washed a further three times in 0.15M phosphate-buffered saline (PBS) [pH 7.2]. After harvesting and washing, cells were disrupted using Sonifier® cell disruptor Model 450 fitted with a microtip (Branson Ultrasonics Co., Danbury, Connecticut, USA.) at 6 kHz (50% duty) for 10 min. The sonicated antigens were filtered with 0.22 μm Millipore™ filter. The protein concentration was

determined using the bicinchoninic acid (BCA) method. Each *H. pylori* antigen were diluted to 1 mg/ml with 0.15M PBS. The whole-cell sonicates mixed with two clinical isolates were used as antigens in ELISA.

Indirect ELISA

The plates were coated with 200 μl/well of sonicated *H. pylori* antigens at 4°C overnight. The antigen concentration ranged from 0.3 μg/ml to 40 μg/ml in 0.1 M carbonate buffer, pH 8.3. After removal of unbound antigens, the wells were blocked with 250 μl of 1% bovine serum albumin (BSA) in 0.15M PBS at room temperature for 2 hrs. The sera and control samples were serially diluted to PBS containing 20% normal goat serum and 0.1% Tween 20, and 200 μl of this cocktail was added to the antigen-coated wells. The first incubation was carried out at 37°C for 1hr. Plates were washed five times with PBS containing 0.1% of Tween 20 (PBST), followed by incubation with horseradish peroxidase-conjugated anti-human immunoglobulin G in PBS containing 10% normal goat serum and 0.1% Tween 20 at 37°C for 1 hr. After washing five times with PBST, 200 μl of 3,3',5,5'-tetramethylbenzidine (TMB) solution in dimethylsulfoxide (DMSO), containing 0.01% H_2O_2 were added and incubated for 30 min at room temperature. The reaction was stopped by the addition of 100 μl of 1.6 N sulfuric acid, and the color development was measured in a plate reader (SLT Labinstrument, Salzburg, Austria) set at 450 nm. All assays were done in duplicate. The cut-off value was set as mean optical density

plus 3 intervals of standard deviation when 40 healthy children sera under 3 years old were tested. Cut-off absorbance of 0.4 appeared to give good discrimination between *H. pylori*- infected patients and normal healthy children.

Inhibition test

Tests for possible cross-reactivity of anti-*H. pylori* specific IgG antibodies with antigens of other enteropathogenic bacterial species were done using a serum pool from *H. pylori* -infected patients that had high absorbance value in our assay system. The pooled serum was absorbed with whole-cell sonicates of *Escherichia coli, C. jejuni* or *H. pylori* at 37℃ for 45 minutes. Antibodies to the bacterial suspension were removed by centrifugation at 12,000 x g for 5 minutes. After saving 100 $\mu\ell$ aliquot for ELISA determination, the supernatant was reabsorbed five times. An unabsorbed serum control was exposed to the same incubation and centrifugation conditions.

Commercial ELISA kits

Among the several *H. pylori* antibody detection kits, four commercial kits were selected for the comparative evaluation. These kits were manufactured by Bio-RAD (GAP-IgG), Roche (Cobas-core second generation anti-*H. pylori* EIA), DAKO (*H. pylori* test), and IPR (*H. pylori* IgG ELISA). The kits were identified by manufacturer name throughout this report. Each test was carried out, and its results were interpreted, according to the

manufacturer's instructions. All tests except *H. pylori* IgG ELISA kit (IPR) used a grey-zone region, which was an indeterminate results with respect to *H. pylori* infection. The test results of specimens within the grey zones were excluded from interpretation of sensitivity and specificity.

SDS-PAGE and Immunoblot

50 μg of antigen per lane was electrophoresed in sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membrane according to the method described previously (18). The nitrocelullose strips were incubated with 1 : 500 diluted serum for 16 hr at room temperature. Sera were diluted with 5 % skim milk in PBS. After washing with PBST, the strips were incubated with 1:1,000 diluted horseradish peroxidase-conjugated goat anti-human immunoglobulin G and then was developed with 4-chloro-1-naphthol (430 μg/ml) solution containing H_2O_2.

RESULTS AND DISCUSSION

Prevalence of *H. pylori* infection in gastrointestinal disease

In each gastoscopy of total 249 Korean patients, two separate antral biopsies were obtained and evaluated *H. pylori* infection by culture and/or urease test. A patient was classified as *H. pylori* positive if culture, urease, or both examination revealed the presence of the *H. pylori*.. A urease biopsy test only was not considered sufficient for diagnosis because of the possible false-positive result originated from other urease-producing bacteria. Of 249 patients, 178 patients (71.5 %) were positive (Table. 1). In 161 of

TABLE 1. Detection rate of *H. pylori* infection according to clinical diagnosis.

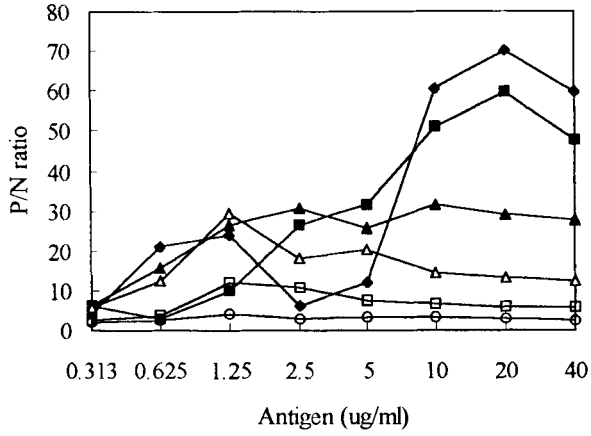
Clinical diagnosis	No. of cases	No. of *H. pylori* infection (%)
Superficial gastritis	145	100 (69.0)
Gastric ulcer	43	29 (67.4)
Duodenal ulcer	41	34 (82.9)
Gastric cancer	20	15 (75.0)
Total	249	178 (71.5)

178 *H. pylori*- positive patients, both culture and urease test were positive. In 17 patients, culture alone was positive. The prevalence rate of *H. pylori* infection in related to various symptoms was 69 % (100/145) in gastritis patients , 67.4 % (29/43) in benign gastric ulcer, 82.9 % (34/41) in duodenal ulcer, and 75 % (15/20) in gastric cancer patients. This results indicated that there was a strong association between the *H. pylori* infection and gastric diseases and also was consistent with the previous report (19).

Optimization of assay format for indirect ELISA

To detect serum IgG antibody to *H. pylori* in Korean patients, we developed the ELISA system. The mixture of whole-cell sonicates of two clinical isolates from Korean patients was used as antigen in our assay system. The optimal assay format with antigen concentration and conjugate and serum dilution fold were investigated. As shown in Figure 1.A, the effect of various coating antigen and conjugate concentration was evaluated. The color reaction was expressed as the ratio of *H. pylori* antibody positive and negative serum (P/N). Increasing the amount of coating antigen

A.



B.

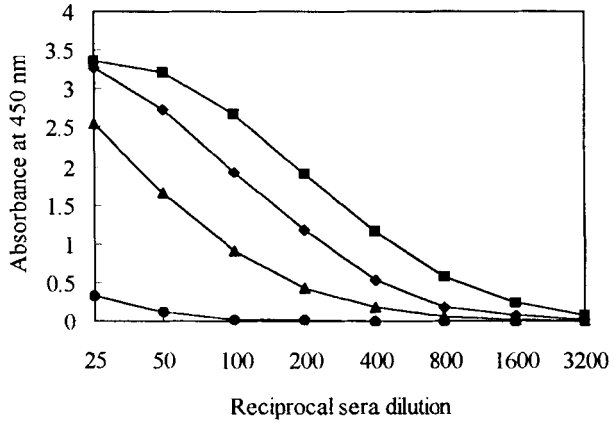


FIGURE 1. Reactivity and titration curves of anti-*H. pylori* positive and negative sera in an indirect ELISA. A. Antisera diluted 1:100 was tested with various antigen and conjugate concentration. Added amount of conjugate : 3.13(■), 6.25 (◆), 12.5 (▲), 25 (△), 50 (□), and 100 (○) ng per ml. B. Three anti-*H. pylori* positive and one negative sera serially diluted were tested with the optimized ELISA. Anti-*H. pylori* antibody positive sera : 1 (■), 2 (◆), and 3 (▲). Anti-*H. pylori* antibody negative sera : 1 (●). The ELISA procedure was described in materials and methods.

increased the P/N ratio in a range from 0.313 μg/ml to 40 μg/ml. Since the maximal P/N ratio was considered to optimal condition, the concentration of coating antigen was employed to 20 μg/ml in further experiment. The color reaction was also dependent upon the concentration of conjugate. Employed conjugate concentration was from 3.13 ng/ml to 100 ng/ml. The absorbance of anti-*H. pylori* antibody negative sera showed nonspecific increase at higher conjugate concentration. Therefore, the optimal P/N ratio was obtained at 6.25 ng/ml of conjugate concentration (Figure 1A).

The relative sensitivity of optimized ELISA system for three different positive sera and one negative serum also was investigated. Serum samples from *H. pylori* infected individuals were serially diluted and reacted with ELISA (Figure 1B). In case of 1:100 dilution, an absorbance of *H. pylori* negative serum was below 0.1 and anti-*H. pylori* antibody positive sera showed high absorbance values. Therefore, sample diluted to 1:100 was employed in further experiment.

Inhibition test

We investigated the cross-reactivity of anti- *H. pylori* IgG antibodies with other enterogenic bacterial species in our ELISA. As shown in Figure 2, preincubation of known positive sera in the *H. pylori* IgG ELISA test with homogenous *H. pylori* sonicates significantly reduced optical density. Absorption of these sera with *C. jejuni* or *E. coli* produced minimal

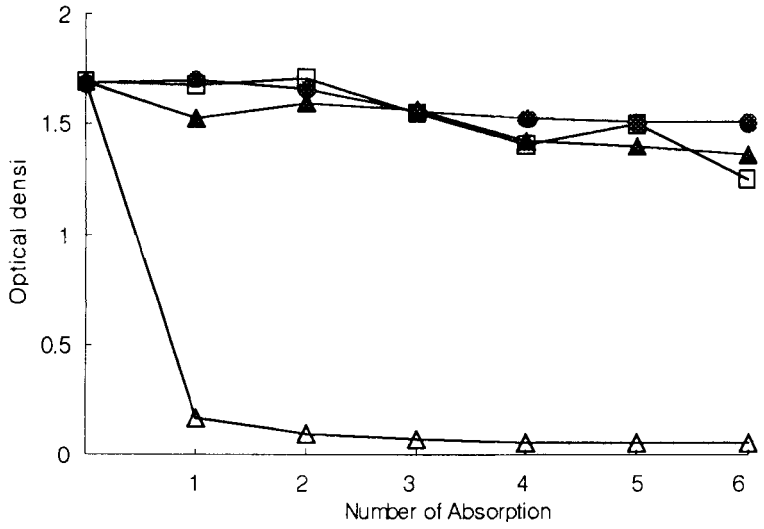


FIGURE 2. Inhibition assay of our MBRI IgG enzyme-linked immunosorbent assay (ELISA) using *H. pylori* sonicate as antigen. Pooled high-titer serum from persons infected with *H. pylori* was absorbed with whole-cell sonicate preparations of *Escherichia coli* (□), *C. jejuni* (▲), *H. pylori* (△) or incubated alone (●) as control.

decreases in optical density. Therefore, there was no marked cross-reactivity with *E. coli* and *C. jejuni*.

Serology tests

In order to assess the correlation between culture and serology, we compared the our MBRI anti-*H. pylori* IgG ELISA with four different commercial kits. A summary of the endoscopic findings reference to culture and serology is presented in Table 2. Of the culture-positive gastritis subjects, 98% was positive in the MBRI *H. pylori* IgG test, 93% positive in Bio-Rad™ test, 83% positive in ROCHE™, 57% positive in

TABLE 2. Summary of endoscopic findings : culture and serology results.

Endoscopic Finding	No. of subjects culture-positive	No. of subjects seropositive / total (%) by :				
		MBRI *H. pylori* IgG	Bio-Rad™ GAP-IgG	Roche™ Cobas core	DAKO™ *H. pylori* IgG	IPR™ *H. pylori* IgG
Superficial gastritis	100	98/100 (98)	57/61 (93)	20/24 (83)	16/28 (57)	19/36 (53)
Gastric ulcer	29	28/29 (97)	23/24 (96)	9/11 (82)	6/11 (57)	5/10 (50)
Duodenal ulcer	34	34/34 (100)	28/30 (93)	15/18 (83)	8/15 (53)	10/15 (67)
Gastric cancer	15	14/15 (93)	8/8 (100)	6/7 (86)	5/8 (63)	5/6 (83)

TABLE 3. Sensitivities and specificities of *H. pylori* antibody determinations.

Assay kit	Serological results for subjects with :							
	H. pylori present[a]				*H. pylori* absent[b]			
	No. +	No. +/-[c]	No. -	Sensitivity	No. +	No. +/-	No. -	Sensitivity
MBRI *H. pylori* IgG	174	NA	4	97.8 %	4	NA	46	92.0 %
Bio-Rad™ GAP-IgG	95	21	7	94.3 %	23	7	19	53.0 %
Roche™ cobas core	50	0	10	83.3 %	3	0	47	96.0 %
DAKO™ *H. pylori* IgG	32	3	27	56.5 %	5	0	45	92.0 %
IPR™ *H. pylori* IgG	39	NA	28	58.2 %	4	NA	46	92.0 %

[a]Positive by culture and/or urease

[b]Negative by culture, urease and histological investigation

[c]Grey zone. NA, not applicable.

DAKO™, and 53% positive in IPR™ kit. Upper three kits showed relatively high correlation results with culture data. On the other hand, the results of DAKO™ and IPR™ kits were poor. In case of gastric ulcer, duodenal ulcer, and gastric cancer, the same patterns were found.

The sensitivity and specificity of all ELISA kits, presented in Table 3, were calculated by using the cut-off level given by the manufacturer. The sensitivity and specificity of each test (our ELISA system, Bio-Rad™, DAKO™, ROCHE™, and IPR™) were as follows : 97.8% and 92%, 94.3% and 53%, 56.5% and 92%, 83.3% and 96%, 58.2% and 92%, respectively. From these results, our MBRI ELISA system showed the best correlation between *H. pylori* infection and seropositivity and followed by Bio-Rad™ kit and ROCHE™ kit. DAKO™ and IPR™ kit showed the low sensitivity (about 50%). In case of Bio-Rad™ GAP, Roche™ cobas core, and DAKO™ *H. pylori* kit, the grey zone was used. The percentage of sera whose fell into grey zone ranged to 0% for Roche™ kit, 2.7% for DAKO™ kit and 16.3% for Bio-Rad™ kit, respectively. This area of uncertainty appeared to be a problem for serological tests and is an area where further attention is required. The specificities were similar in all tested kits except Bio-Rad™ GAP-IgG kit.

SDS-PAGE and Immunoblot

In order to confirm presence of anti-*H. pylori* antibodies in serum which showed the discordant results between our assay system and four commercial kits, we performed the sodium dodecyl sulfate polyacrylamide gel

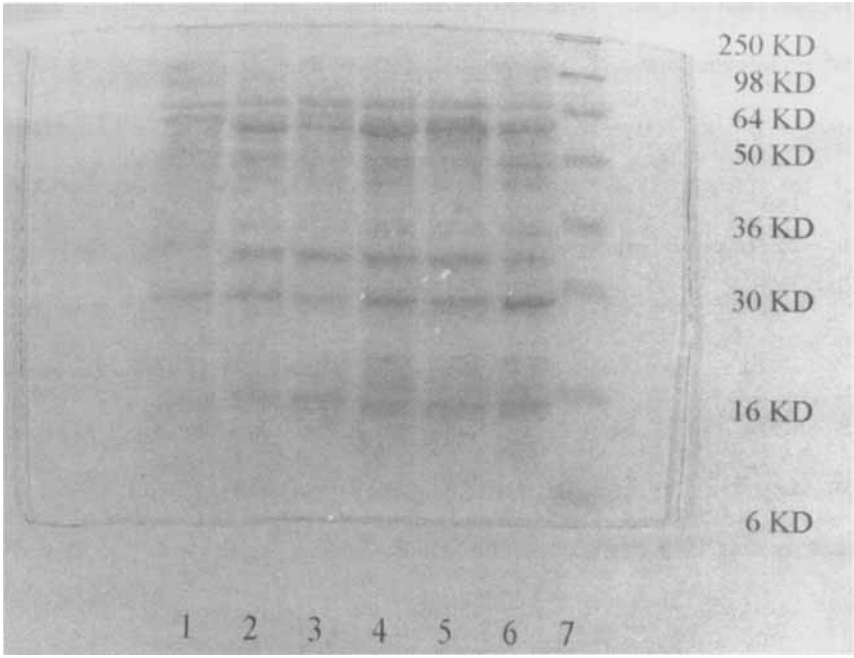


FIGURE 3. SDS-PAGE of several sonicated *H. pylori* strains. Extract of ATCC 43629 (lane 1), 43579 (lane 2), 43504 (lane 3); extract of clinically isolated strains: strain MBRI 2 plus 8 (lane 4), MBRI 8 (lane 5), MBRI 2 (lane 6); molecular weight marker (lane 7). Gel was stained with Coomassie blue R-250.

electrophoresis (SDS-PAGE) and immunoblot analysis. Some sera showing discordant immunoassay results compared with different ELISA kits were tested further by immunoblotting.

The SDS-PAGE analysis were performed on whole-cell sonicates of three reference strains (ATCC 43629, 43579, 43504) and our *H. pylori* strains (MBRI 2 and MBRI 8) (Figure 3). After Coomassie blue staining, all strains exhibited multiple protein bands. The major proteins bands at 64, 58, 50,

33, 29, and 16 KDa were recognized similarly in all strains. However, pattern of minor protein bands was different on four strains (60 to 45 KDa, and 25 to 10 KDa). In agreement with earlier reports (20-21), the results of the present study showed that antigenic variation existed between different *H. pylori* strains.

The immunoblot was performed on four types of *H. pylori* strains (Figure 4). Serum samples with no *H. pylori* IgG antibodies in IPR™ kit (Lane 1) or DAKO™ kit (Lane 2) or Bio-Rad™ and Roche™ kit (Lane 3) or negative for these four kits (Lane 4) or all kits (Lane 5) but with *H. pylori* present in their gastric biopsy specimens by culture and/or urease test were tested. For quality control, *H. pylori* antibody-positive patients sera with high antibody titer (Lane 6), and positive and negative control serum (Lane 7 and 8, respectively) in our assay system were used. The immunoblot analysis confirmed the presence of *H. pylori* IgG antibodies in all discordant sera except lane 5, irrespective of four different *H. pylori* strains. Therefore, four serum showing false negative serological results with four commercial ELISA tests were positive by immunoblotting. The negative sera in all kits (lane 5), but it was positive in culture and/or urease test, was also negative in immunoblot analysis. Although the data were not shown in here, the sera was only raised for IgA antibody. This results were consistent with other report that for unknown reasons a small percentage of *H. pylori*-infected patients failed to mount a systemic IgG response and produced an IgA antibodies response only (22-23). Therefore, Our ELISA results

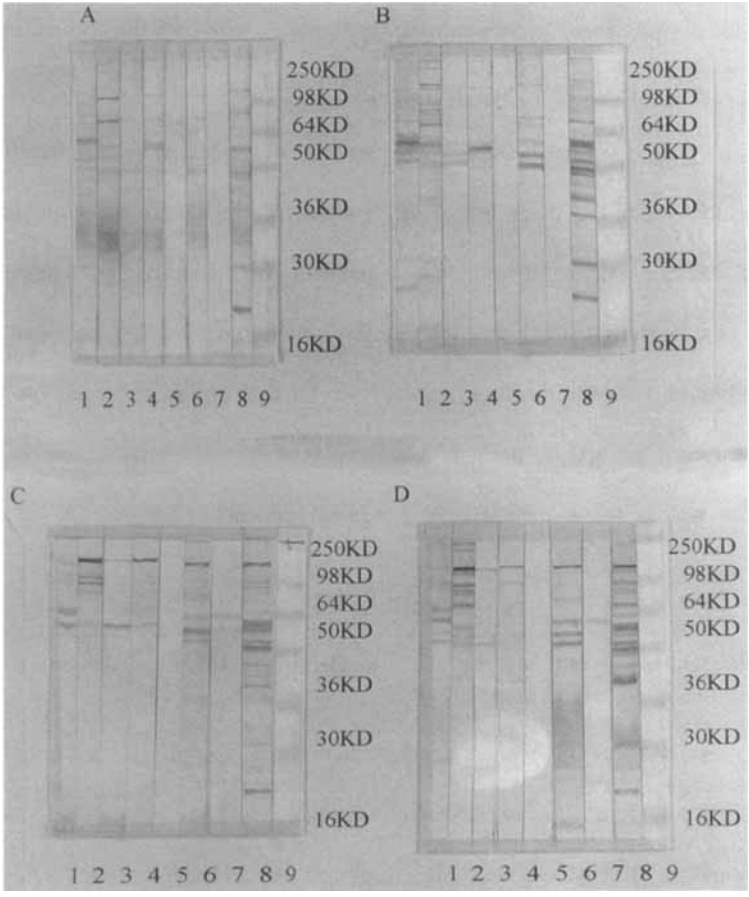


FIGURE 4. *H. pylori* immunoblots of discordant sera among different ELISA kits using sonicated antigens of several strains (A, MBRI 2 plus 8, B, ATCC 43504, C, ATCC 43579, D, ATCC 43629). The strips were incubated with discordant sera diluted 1:500 and developed with horseradish peroxidase conjugated goat anti-human IgG. Negative sera in IPR™ kit (lane 1), DAKO™ kit (lane 2), Bio-Rad™ and Roche™ kit (lane 3), IPR™, DAKO™, and Roche™ kit (lane 4), all four kits (lane 5), sera from *H. pylori*-positive patients (lane 6 & 8), serum from a negative patient (lane 7), molecular weight marker (lane 9).

correlated well with the immunoblot results in regardless of different *H. pylori* strains.

In conclusion, there was a strong association between the detection of *H. pylori* and presence of gastritis, gastric ulcer, duodenal ulcer, and gastric cancer in Korean patients. Our ELISA system showed the higher sensitivity and specificity for the diagnosis of *H. pylori* infection than other commercial kits in Korean upper gastrointestinal patients and was highly reliable in related to bacteriological diagnosis and endoscopic finding, as well as immunoblotting. Therefore, our assay system can be used for monitoring the therapeutic effect of antimicrobial treatment for the eradication of *H. pylori* as well as for screening sera in Korean patients.

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