ORIGINAL ARTICLE

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Development of an ELISA for Quantitative Detection of Immunoglobulin G (IgG) and IgA Antibodies to *Helicobacter pylori* for Use in Korean Patients with *H. pylori*-Associated Diseases

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Background/Aims: We aimed to develop a quantitative enzyme-linked immunosorbent assay (ELISA) using whole-cell lysates of *Helicobacter pylori* 51 and to investigate its validity. **Methods:** Data from 300 plates were obtained by two different operators. Standard sera were used to make a standard curve to analyze the quantity of anti-*H. pylori* immunoglobulin G (IgG) and IgA antibody. We obtained reproducible data with fewer dilutions of samples by the addition of serially diluted standard serum to each ELISA plate. To evaluate the validity of this ELISA, the 114 *H. pylori*-positive and -negative subjects were stratified into four age groups, i.e., 0 to 4, 5 to 9, 10 to 15, and 20 to 29 years, before testing. **Results:** The mean IgG-antibody titers in *H. pylori*-positive and -negative subjects were 1,766.4 IU/mL and 654.3 IU/mL ($p<0.001$). The mean IgA-antibody titers in *H. pylori*-positive and -negative subjects were 350.1 IU/mL and 193.5 IU/mL ($p<0.001$). Anti-*H. pylori* IgG and IgA titers in the four age groups were higher in *H. pylori*-positive subjects than in *H. pylori*-negative subjects ($p<0.05$). **Conclusions:** Using the current ELISA based on whole-cell lysates of *H. pylori* 51, reliable anti-*H. pylori* antibody titers were obtained regardless of the subject's age. **(Gut Liver 2013;7:437-442)**

Key Words: *Helicobacter pylori*; Enzyme-linked immunosorbent assay; Antibodies

INTRODUCTION

Enzyme-linked immunosorbent assay (ELISA) has long been used to detect immunoglobulin G (IgG) for screening *Helicobacter pylori* infection.[1] Furthermore, the ELISA is an easier and cheaper method to diagnose *H. pylori* infection noninvasively. However, antibody positivity against *H. pylori* could not differentiate between the recent and past infections. Moreover, it could not be used as a follow-up test after chemotherapy of *H. pylori*.

Quantitative evaluation of anti-*H. pylori* antibodies or against *H. pylori* recombinant purified proteins such as cytotoxin-associated gene A (CagA) or urease A has been performed in some human diseases in which *H. pylori* infection might play a role in their pathogenesis.[2-5] However, the clinical significance of high antibody levels against *H. pylori* as determined by a quantitative ELISA has not yet been established. Also, the phenomenon is yet a proven method to predict the severity of gastroduodenal diseases or the density of *H. pylori* colonization.[6]

The usefulness of most commercial ELISA kits is generally limited by the specificity and sensitivity of antigens used for the assays.[7] For instance, ELISA for anti-*H. pylori* IgG in children produced controversial results with various sensitivities and specificities.[8,9]

We reasoned that if quantitative ELISA measurements of anti-*H. pylori* or anti-recombinant purified proteins of *H. pylori* antibodies were performed in Korean patients with *H. pylori*-associated diseases, the whole cell lysates or the sequences of the recombinant proteins must be derived from a strain isolated in Korea. To this end, we aimed to develop a quantitative ELISA using whole cell lysates of *H. pylori* 51 strain isolated from a Korean patient. The whole genome sequence of the strain has already been known, hence, the anti-*H. pylori* IgG and IgA antibodies that will be used to test *H. pylori*-associated diseases has already been determined. Finally, we evaluated the validity of this ELISA by comparing the antibody titers of both serum IgG and IgA between *H. pylori* positive and *H. pylori* negative groups following urease tests and histopathological studies.

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MATERIALS AND METHODS

1. Antigen preparation

H. pylori strain 51 isolated from a patient in Gyeongsang National University Hospital (Jinju, Korea) were cultivated overnight at 38°C under 10% CO_2 and 100% humid atmosphere. For the preparation of whole cell proteins, cells were washed with washing solution (0.1 M phophophate buffer solution [PBS], pH 7.4), resuspended with PBS, and added phenylmethylsulfonyl fluoride. The cells were disrupted by the use of an ultrasonicator (Ultrasonics W-380; Sonics & Materials Inc., Danbury, CT, USA) and stored in deep freezer prior to use.

2. ELISA

To make a standard curve, 96-well flat-bottomed ELISA plates (EIA plate, Costar 3590; Costar, Cornig, NY, USA) were coated overnight with 50 μL per well of purified antigen diluted with coating buffer (10 μg/mL) containing 50 mM sodium carbonate buffer (pH9.6; Bioshop, Burlington, ON, Canada) at 4°C. To initiate the assay, plates were blocked with 150 μL/well of blocking buffer containing 3% bovine serum albumin in PBS with Tween (PBST) (BioShop) for 3 hours at 37°C. Independent triplicate dilutions of standard pooled serum (IgG 1:400, and IgA 1:100) were prepared in dilution buffer (PBST) and 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Diluted sera were added to antigen-coated wells (50 μL/well) and incubated for 1 hour at 37°C. After four washing with washing buffer (PBST), 50 μL of peroxide-conjugated goat antihuman IgG (Bethyl Laboratories Inc., Montgomery, TX, USA; diluted 1:30,000) and IgA (Bethyl Laboratories Inc.; diluted 1:5,000) were added to the wells and incubated at 37°C for 1 hour. The plates were then washed five times with PBST and wells were added with 0.8 mg of *o*-nitrophenyl phosphate (Sigma-Aldrich). The reaction was stopped after 30 minutes at room temperature by adding 25 μL of stopping buffer which contained 2N H_2SO_4. Immediately after this addition, optical density (OD) was measured at 492 nm using a VERSA max microplate reader (Molecular Devices Co., Sunnyvale, CA, USA).

3. Establishing a standard ELISA curve

Sera from 300 patients selected for strong positivity in in-house immunoblot assay[10] were used to prepare a positive reference standard (standard pooled sera). After making the appropriate pool, all standard sera were aliquoted (200 μL of each) and maintained at -80°C until required. Antibody units were assigned to each standard serum following the methods indicated by Miura *et al*.[11] First, one of the aliquots was diluted in 2-fold steps from 1:1,000 to 1:526,000, 1:2,500 to 1:2,560,000, 1:5,000 to 1:5,120,000, and 1:7,500 to 7,680,000 with dilution buffer. These serially diluted sera were applied on an ELISA plate as primary antibodies. Four wells per set of serially diluted standard did not receive any samples or buffer during this step (blank wells), but other ELISA steps (adding blocking buffer, incubating with secondary antibody, adding substrate, and stopping buffer) were performed as those for other wells. These blank wells were used as the negative control wells (reciprocal number of dilution was assigned as 0). The relationship between reciprocal number of the dilution and OD_{492} was approximated by a 4-parameter hyperbolic curve (SOFTmax PRO version 5; Molecular Devices Co., Sunnyvale, CA, USA). If the R^2 of that curve fit was less than 0.997, another standard serum aliquot was thawed and the entire procedure was repeated. Based on the constants of the equation, antibody units were assigned to the standard as the reciprocal of the dilution giving an OD_{492}=1. Once the antibody units of a standard serum were determined, the number was used invariably for all samples tested by ELISA against that standard. Thereafter a reference standard was used on each ELISA plate to make a standard curve. To do this, standard serum aliquots were freshly thawed and two independent serial dilutions (2-fold steps, 10 different dilutions) were prepared starting with a dilution of 20 antibody units. These were applied to the wells on the ELISA plate and four blank wells (assigned zero antibody units) were also designated. The OD values were then fitted to a 4 parameter standard curve (antibody units=C{[(A-OD_{492})/(OD_{492}-D)]^(1/B)}) using SOFTmax PRO version 5 software; Molecular Devices, Sunnyvale, CA, USA). The antibody units in test sera were then calculated from their OD_{492} values using the parameters estimated from this standard curve.

4. Comparison of antibody titers in *H. pylori* positive and *H. pylori* negative patients

To evaluate the validity of this ELISA, sera collected from *H. pylori* positive and *H. pylori* negative patients were tested. *H. pylori* positive patients were those that showed by positive urease test (within 1 hour) and the presence of *H. pylori* in histopathology. *H. pylori* negative patients were defined otherwise. The sera were stratified into four age groups, namely, 0 to 4, 5 to 9, 10 to 15, and 20 to 29 years. The IgG and IgA antibody titers were compared in *H. pylori* positive and *H. pylori* negative groups according to age. The data were analyzed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). Statistically significant differences in antibody titers between *H. pylori* positive and *H. pylori* negative patients were determined using Student t-test. The $p<0.05$ was considered to be statistically significant. The area of under the receiver operating characteristic curve (AUC) of IgG and IgA were established for evaluation of accuracy of this ELISA.

RESULTS

1. Comparison of ELISA antibody units derived from a single dilution versus full dilution curves

Pooled sera were serially diluted and analyzed by ELISA. Two numbers were generated for each test serum. Using a single OD

value which was closest to 1 in each set of diluted sera, the antibody units were calculated referring to a standard curve. Using all OD values from serially diluted sera, the reciprocal dilution giving an OD_{492}=1 calculated based on a 4-parameter fitted hyperbolic curve and the reciprocal dilution giving an OD_{492} of 1 calculated from full dilution curves were significantly correlated (Spearman rank correlation, 1.0; p<0.0001) (Fig. 1). Thus to obtain the antibody units, we reasoned that a single dilution was enough if serially diluted standard serum was tested in the same ELISA plate. The antibody units of IgG and IgA contained in the pooled sera were determined to be 1,200 and 200 IU/mL, respectively.

2. Quality control of standard curves and tested samples

To determine the criteria for acceptable quality of an ELISA plate, data from 300 plates were analyzed by two different operators using sera from whole cell lysate of *H. pylori* strain 51. The proportion of the plates with standard curves having an R^2 less than 0.997 was 4.4%. The mean OD_{492} of blank wells was 0.044 and the standard deviation was 0.019. The R^2 of the standard curve and the OD_{492} of blank wells were independent variables (Fig. 2).

The quality of data obtained with standard sera was examined using two data sets. Standard sera were serially diluted over 100 times and antibody units for each dilution of standard

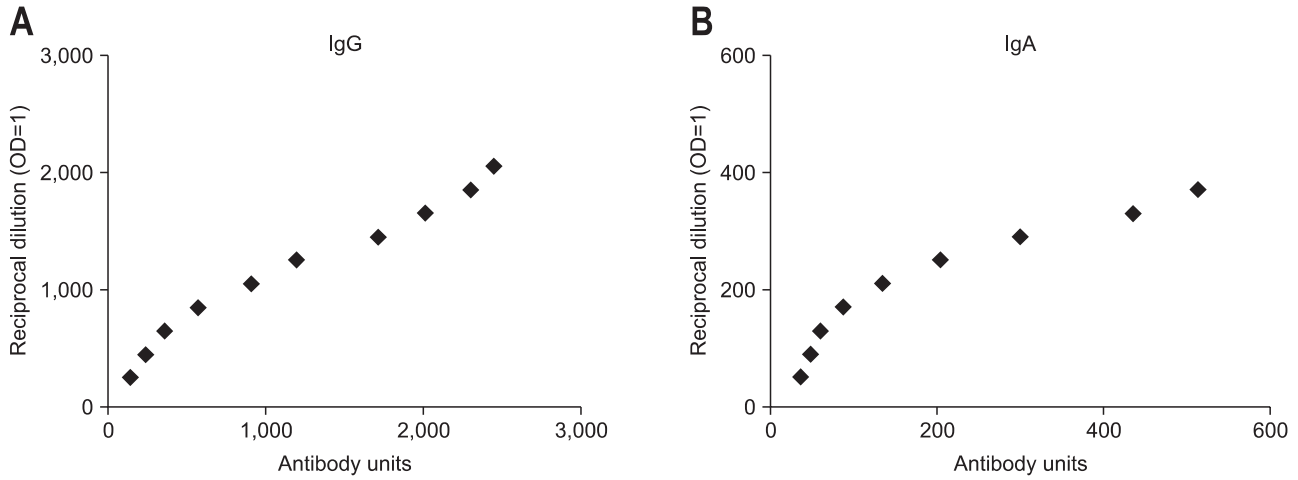

Fig. 1. Comparison of enzyme-linked immunosorbent assay antibody units derived from single versus multiple serial dilutions. (A) Immunoglobulin G (IgG). (B) IgA. Antibody units were calculated from the OD_{492} reading of a single dilution referred to a standard curve and plotted on the X-axis. The standard sera were serially diluted to generate a complete dilution curve. The reciprocal of the dilution giving an OD_{492} reading of 1 was calculated (on the Y-axis) from the dilution curve.
OD, optical density.

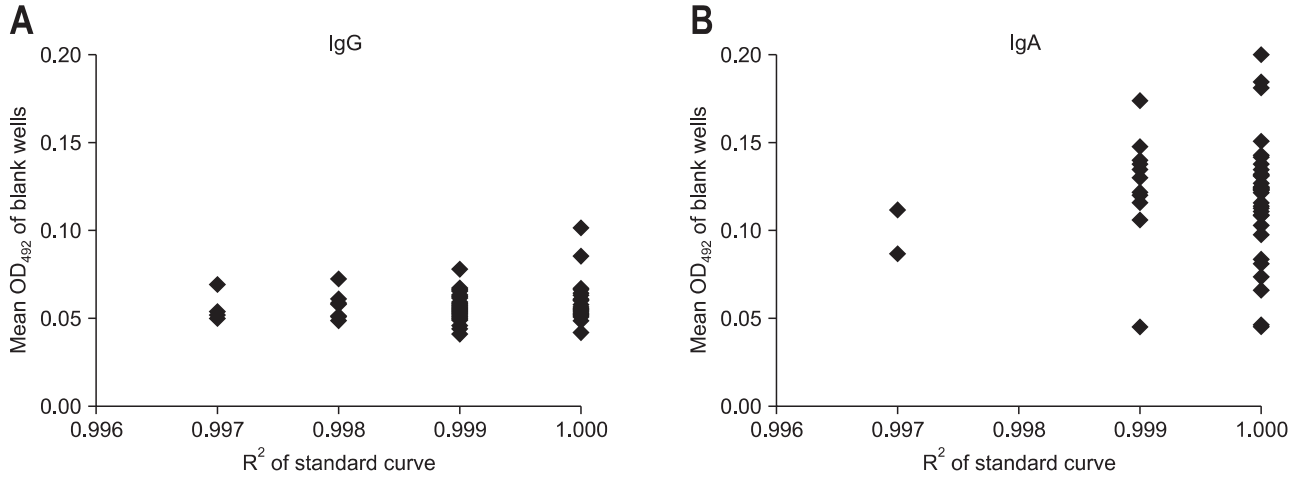

Fig. 2. Quality-control parameters for an enzyme-linked immunosorbent assay standard curve. The R^2 of a standard curve (fitted to a 4-parameter hyperbolic curve) is plotted on the X-axis, and the arithmetic mean value of OD_{492} of 4 blank wells on the same plate is plotted on the Y-axis. (A) Immunoglobulin G (IgG). (B) IgA.
OD, optical density.

Table 1. Antibody Titers in *Helicobacter pylori*-Positive and *H. pylori*-Negative Subjects

Group, yr	*H. pylori* status	No.	IgG antibody titer, mean, unit/mL	IgA antibody titer, mean, unit/mL	p-value
0-4	Positive	26	1,326.0	293.7*	<0.05
	Negative	29	593.7	205.3*	
5-9	Positive	33	1,740.9	327.0	<0.0001
	Negative	30	661.5	181.9	
10-15	Positive	29	1,925.4	350.0	<0.0001
	Negative	26	754.7	169.7	
20-29	Positive	27	2,036.0	430.9	<0.0001
	Negative	28	616.0	215.9	

IgG, immunoglobulin G; IgA, immunoglobulin A.
*p=0.021.

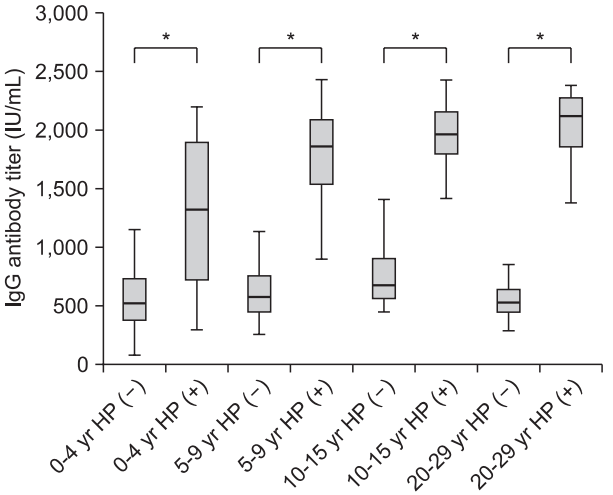

Fig. 3. Comparison of immunoglobulin G (IgG) antibody titers in *Helicobacter pylori* (HP)-positive and *H. pylori*-negative groups according to age. The median antibody titer is higher in the *H. pylori*-positive group than in the *H. pylori*-negative group.
*p<0.0001.

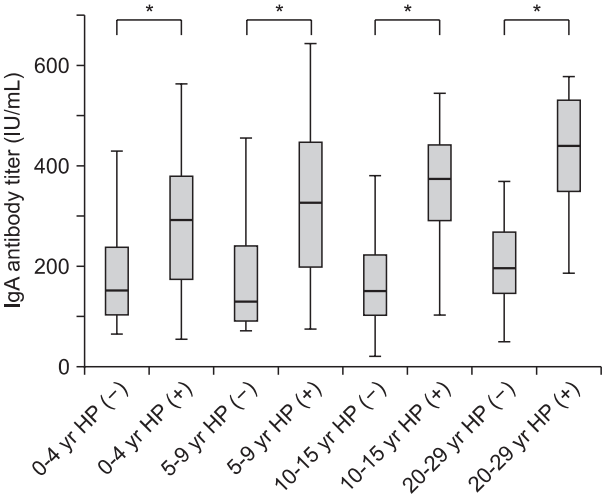

Fig. 4. Comparison of immunoglobulin A (IgA) antibody titers in the *Helicobacter pylori* (HP)-positive and *H. pylori*-negative groups according to age. The median antibody titer is higher in the *H. pylori*-positive group than in the *H. pylori*-negative group.
*p<0.05.

sera were calculated. The relative units were calculated by comparison with the antibody units of the sera that came from the dilution showing an OD_{492} closest to 1.0.

Based on the data above, we determined the criteria for acceptable quality for ELISA plates as the following:

1) R^2 of the standard curve should be equal to or greater than 0.997.

2) The OD_{492} of blank wells should be less than 0.04.

If the R^2 of standard curve and blank values did not match these criteria, all data were discarded.

3. Reproducibility of the ELISA data

Following the criteria listed above, antibody units of standard sera were analyzed by ELISA to evaluate the reproducibility of the data. Pooled standard sera were aliquoted and frozen at -80°C before use, and these sera were tested at an interval of several months. The concordance between the two sets of the data is highly significant judged by the linear regression analysis (coefficient was 1.01; 95% confidence interval [CI], 0.97 to 1.06; p<0.001). The reproducibility of data obtained with 224 antisera by two different operators using the same standard was also high (coefficient was 0.84; 95% CI, 0.97 to 1.06; p<0.001, data not shown).

4. Comparison of antibody titers in *H. pylori* positive and *H. pylori* negative groups

One hundred fourteen *H. pylori* positive patients and 114 *H. pylori* negative patients were tested. Of them, 55, 63, 55, and 55 patients were 0 to 4, 5 to 9, 10 to 15, and 20 to 29 years old, respectively (Table 1). The mean IgG antibody titer was 1,766.4 IU/mL in *H. pylori* positive patients and 654.3 IU/mL in *H. pylori* negative patients (p<0.0001). The mean IgG antibody titers of the four age-groups were higher in *H. pylori* positive patients than in *H. pylori* negative patients (p<0.0001) (Fig. 3). The mean

IgA antibody titer was 350.1 IU/mL in *H. pylori* positive patients and 193.5 IU/mL in *H. pylori* negative patients ($p<0.0001$). The mean IgA antibody titers of the four age-groups were also higher in *H. pylori* positive patients than in *H. pylori* negative patients ($p<0.05$) (Fig. 4). In the subgroup 0 to 4 years, the mean IgA antibody titer in *H. pylori* positive patients was higher than in *H. pylori* negative patients ($p=0.021$). The AUCs of IgG and IgA were 0.928 (95% CI, 0.89 to 0.97; $p<0.0001$) and 0.795 (95% CI, 0.74 to 0.85; $p<0.0001$), respectively. Altogether, these findings suggest adequate discrimination of *H. pylori* positive and *H. pylori* negative patients using this method.

DISCUSSION

The reliability of ELISA test is required with the standard curve for distinguishing positive and negative sera in infectious diseases, and for reproducibility. In this study, the pooled standard sera were used for making a standard curve. There are some advantages to using the pooled standard sera to make a standard curve in analyzing the quantity of antibody in *H. pylori* infection. For instance, it allows reliable assessment of the quantities of antibodies with fewer different diluted samples compared with endpoint titration, and also comparison of data obtained from different ELISA plates on different days. Nevertheless, although the present ELISA method produced reliable antibody levels with fewer dilution steps, it can also be assumed that all sera have the same shapes of dilution curves as the standard serum. The difference of antibody units between two dilutions was less than 30% if data were obtained at OD_{492} readings from 0.25 to 1.6.[11] In this study, two different operators using the standard sera pool produced the same shapes of dilution curves although tests were conducted at different time points. This suggests reliability and reproducibility of the current ELISA method. The results of this study showed that standard ELISA using whole cell lysate of *H. pylori* 51 strain and pooled standard sera gave reliable anti-*H. pylori* IgG and IgA antibody titer regardless of age of patients.

In a previous study, we showed that the Genedia IgG ELISA kit, which used *H. pylori* antigen obtained from Korean *H. pylori* strain, displayed the highest seropositivity rate than other ELISA kits (e.g., GAP IgG, HM-CAP, and Pyloriset EIA-G obtained from USA and Finland).[12] In this study, we found that more than 80% of seropositive ELISA results were CagA positive (the other 20% of seropositive result in ELISA might be attributed to its reaction to another *H. pylori* antigen). Therefore, the use of whole cell lysate of *H. pylori* strain 51 in ELISA might increase the yield in detecting anti-*H. pylori* antibody.

To evaluate the validity of our ELISA, we used it to compare antibody titers between *H. pylori* positive and negative patients. Interestingly, we found remarkable differences in IgG and IgA antibody titers between *H. pylori* positive and *H. pylori* negative patients suggesting its potential usefulness to diagnose *H. pylori* infection. Furthermore, the accuracy of commercial assays is greatly reduced when serum is obtained from younger patients, especially those under 5 years of age.[12] In the present study, we found that the discrepancy of antibody titers between the *H. pylori* positive and *H. pylori* negative patients increased as a function of age. However, the serum anti-*H. pylori* IgG and IgA levels in *H. pylori* positive patients were higher than those in *H. pylori* negative patients even those under 5 years old. The range of the AUCs using *H. pylori* positive and negative patients was 0.7 to 1.0 suggesting moderate to high accuracy of our ELISA. Additionally, using the same method, the antirecombinant purified proteins (CagA, UreB, and etc.) of *H. pylori* antibodies could be measured and this may prove useful in evaluating the relationship between *H. pylori* associated diseases and anti-*H. pylori* antibodies.

There are some limitations to the present study including the lack of comparison with other commercially available-ELISAs, the absence of negative standard sera, and a small study population for each age group to evaluate its validity.

In conclusion, we have developed a reliable ELISA method to detect anti-*H. pylori* IgG or IgA antibody in Korean patients, particularly in children under 5 years old. Our ELISA method can be a very useful tool to evaluate the IgG and IgA antibody titers in *H. pylori* infection and the relationship between *H. pylori*-associated disease and anti-*H. pylori* antibody titer.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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