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Characterization of Specific IgA Response to Antigenic Determinants of *Helicobacter pylori* Urease Encoded by *ureA* and *ureB* in Children

Min-Kyoung Shin[1,4], Jin-Su Jun[2], Soon-Wook Kwon[1], Dong-Hae Lee[1], Jong-Hun Ha[1], Jin-Sik Park[1], Dae Hyun Song[3], Myung Hwan Jung[1,4], Hyung-Lyun Kang[1,4], Seung-Chul Baik[1,4], Ji Sook Park[2], Hee-Shang Youn[2], Myung Je Cho[1,4], Ji-Hyun Seo[2*] and Woo-Kon Lee[1,4*]

Department of [1]Microbiology, [2]Pediatrics and [3]Pathology, College of Medicine, Gyeongsang National University, Jinju, Korea
[4]Research Institute of Life Science, Gyeongsang National University, Jinju, Korea

Corresponding
Woo-Kon Lee
Department of Microbiology, College of Medicine, Gyeongsang National University, 816-15, Jinju-daero, Jinju, Gyeong-Nam 52727, Korea
Phone : +82-55-772-8083
Fax : +82-55-772-8089
E-mail : wklee@gnu.kr

Ji-Hyun Seo
Department of Pediatrics, College of Medicine, Gyeongsang National University, 816-15, Jinju-daero, Jinju, Gyeong-Nam 52727, Korea
Phone : +82-55-772-8731
Fax : +82-55-772-9339
E-mail : seozee@gnu.kr

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Helicobacter pylori (*H. pylori*), a causative agent of chronic gastritis and gastric cancer, has several virulent factors for own survival and progression toward gastric diseases in human stomach. Of those, *H. pylori* produces mainly urease (10~15% total protein weight) that neutralize the gastric acid for survival. Here, we identified the antigenic epitope of urease and then developed an ELISA using the antigen including the epitope of urease. We identified the antigenic epitope of urease that induces IgA antibodies in human using truncated mutants. Eight kinds of serially-truncated mutant of UreA and UreB were prepared and subjected to immunoblot using pooled sera of patients with gastric disorders. UreBEnd protein containing UreB epitope was produced and investigated its diagnostic value via ELISA in children. As a result, mutants having last 24 amino acid residues of UreB carboxyl terminus deleted did not show IgA-reactive band. The clones that contained the downstream of 448th amino acid in UreB showed IgA-reactive band. The serodiagnostic value of the UreBEnd recombinant protein including identified epitope was confirmed via IgA ELISA and shown to have 97% sensitivity and 100% specificity. These results demonstrated that carboxyl terminal region of UreB carries an antigenic epitope for IgA response in human. It may be useful for detecting *H. pylori* infection with improved test accuracy and minimum use of endoscopy.

Key Words: *Helicobacter pylori*, Urease, Epitope, IgA, ELISA, Children

INTRODUCTION

Helicobacter pylori (*H. pylori*) has been known as a causative agent of chronic gastritis, peptic ulcer and gastric cancer (1~3). About half population of the world is estimated to be infected with *H. pylori*, but the infection trend has been declining because of improvement of socioeconomic conditions in the world (2, 3). However, *H. pylori* infection is still prevalent in developing countries with high infection rates (4). Not only in underdeveloped countries but also in developed countries, *H. pylori* infection mostly occurs in childhood, and the chances of a new infection are very rare in adulthood (5). In addition, since the probability that *H. pylori* infection in childhood is naturally healed is extremely low, most infected patients live their life together with the bacteria (6). Therefore, the diagnosis and treatment of *H. pylori*

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infection and various measures to prevent stomach diseases, including stomach cancer, should be focused on children.

Endoscopic biopsy for rapid urease test, also known as the Campylobacter-like organism test (CLO test) and histopathologic examination, is considered as the most accurate methods in *H. pylori* infection diagnosis (6). However, the method is not recommended in children because they are invasive approaches, so they are not suitable for large scale screening of patients, especially, children and repeated manipulation. Therefore, serological diagnosis could be the most desirable test in children as it is non-invasive approach. Serological diagnosis methods have been mainly focused on IgG antibody reaction. However, different types of maternal immunoglobulins (Igs, IgG; IgA; IgM) are transferred from mother to fetus through the placenta with different efficacy (7). The transfer of IgG is steady and its process is the most intensive, whereas the transfer of IgA could be estimated in 75% of the cases, and of IgM in only 10% (7). In addition, the systemic potential of systemic IgA and IgG against *H. pylori* was already assessed via ELISA method with high sensitivity and specificity (8). Therefore, a new serodiagnosis method using IgA could be promising for *H. pylori* infection in children.

As a class I carcinogen by the World Health Organization, *H. pylori* has several critical virulent factors such as cytotoxin-associated gene pathogenicity island (*cagPAI*) and vacuolating cytotoxin gene (*vacA*), which were strongly considered to induce the gastric inflammation through cellular activities in host cells (9, 10). Together with the several virulent factors, *H. pylori* urease is an essential factor for colonization in gastric mucosa and suspected to be a cause of gastric damage (10, 11). In our previous study, a *H. pylori* antigen that specifically reacts to the IgA antibody was found and identified by proteome analysis (Under review). Various *H. pylori* proteins reactive with IgA were identified, thus by showing their serodiagnostic potential. Especially, urease was one of the most reactive proteins with IgA antibody. This enzyme is a high-molecular-mass multimeric enzyme composed of two distinct subunits, UreA (29.5 kDa) and UreB (66 kDa) (11). Of both subunits, UreB is known to have high immunogenicity and specificity to *H. pylori* from other organisms, which produce urease, including *Morganella morganii* and jack bean (11, 12).

In the present study, we produced the entire and truncated recombinant proteins of UreA and UreB, and then analyzed their epitopes using immunoblot assay with the patients' pooled sera infected with *H. pylori*. The determined epitopes were produced in *E. coli* expression system and the purified proteins were assessed their serodiagnostic value.

MATERIALS AND METHODS

Ethics statements

Sixty and ten sera from infant patients with and without gastric disorder at the Gyeongsang National University Hospital (GNUH, Jinju, Korea) were used in the present study, respectively. The serum samples of patients with gastric symptoms were divided into three groups based on the results of CLO test as follows: strong, weak, or negative. The gastric endoscopic biopsies of the patients were applied to the CLO test according to a procedure described previously (13). Briefly, a gastric antral biopsy sample was collected and then incubated in 0.3% urea broth (urea 20 g/l, phenol red 0.04 g/l, KH_2PO_4 0.2 g/l and NaCl 0.5 g/l, pH=6.8). The CLO tests were performed on three biopsy samples from same patient. Based on time of the color change of broth, urease activity was determined over 24 h following the grades: no color change (negative), weak (6~24 h), moderate (1~6 h) and strong (< 1 h). Sera were kept frozen at -70℃ until use. All sera were provided by GNUH, a member of the National Biobank of Korea, after the permission from the hospital ethics committee (GNUHIRB-2016-04-003-001).

PCR amplification and cloning of *H. pylori ureA* and *ureB* truncated genes

To analyze the antigenic epitope of *H. pylori* urease, a motif analysis was performed with the amino acid sequence of Strain 26695 using DNASTAR Lasergene program (Windows version; DNASTAR, Madison, WI, USA). Total genomic DNA was extracted from *H. pylori* #51 using a QiaAmp® DNA Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA).

The sequences of *ureA* and *ureB* primers were designed based upon GenBank (Table 1). The *ureA* and *ureB* fragments were amplified using *Taq* polymerase (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions. 1 μl of genomic DNA template, 5 μl of 10X PCR buffer (20 mM Mg^{2+}), 5 μl of 10 mM dNTP mixture (2.5 mM of each: dATP, dCTP, dGTP, and dTTP), 1 μl of external forward primer (10 pmol μl^{-1}), 1 μl of external reverse primer (10 pmol μl^{-1}), 1 μl of *Taq* polymerase (5 units μl^{-1}), and 36 μl of triple-distilled H_2O in a total volume of 50 μl. The PCR products were purified with a QIAquick Gel extraction kit (Qiagen) and cloned into pET15b vector. The clones were confirmed through DNA sequencing with an ABI 377L automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The cloned *ureA* and *ureB* truncated genes were then transformed into competent *E. coli* BL21 cells for expression.

Production of *E. coli* lysate containing the truncated UreA and UreB proteins and the recombinant UreBEnd

The clones were cultured in LB media until the culture reached an optical density (OD600) of 0.6~0.8, and then isopropyl β-D-1-thiogalactopyranosidec (IPTG) was added at a final concentration of 1 mM and incubated for 4 h. The harvested cells were resuspended and lysed by sonication in lysis buffer (50 mM Tris-hydrogen chloride, 100 mM sodium chloride, pH 8.0). *E. coli* lysates containing UreA or UreB recombinant protein were obtained as a supernatant by centrifugation at 5,000 ×g for 30 min.

The *ureBEnd* fragment was amplified using the following primers: forward, 5'-GTGTTCGTGGATGGC-3'; reverse, 5'-CTAG-AAAATGCTAAA-3'. The fragment was cloned into pGEM-T vector. The *ureBEnd* fragment was produced by gel extraction using a QIAquick Gel extraction kit after treatment with restrict enzymes, *Ned*I and *BamH*I. The ureBEnd fragment was cloned into pEGexp vector and the recombinant plasmid was transformed into *E. coli* BL21 cells for expression. The recombinant UreBEnd was purified with glutathione-affinity chromatography using Glutathione Sepharose resin following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The purified recombinant proteins were stored at -20℃ until use.

Immunoblot analysis of recombinant UreA and UreB using sera from patients infected with *H. pylori*

E. coli lysate containing the truncated UreA and UreB proteins were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining and immunoblotted using patients' pooled sera verified previously (14). The transferred membrane was incubated with pooled sera from the patients infected with *H. pylori* at a 1:500 dilution and an alkaline phosphatase-conjugated anti-human IgG (H+L) or anti-human IgA at a 1:1,000 dilution. Blots were developed with an alkaline phosphatase kit (BIO-RAD, Hercules, CA, USA).

Development of UreBEnd-enzyme linked immunosorbent assays

UreBEnd (10 μg/ml) was coated in 96-well microplates at 4℃ overnight for enzyme linked immunosorbent assay (ELISA). The plates were blocked with 3% bovine serum albumin by incubation at 37℃ for 3 h. The plates were incubated with positive and negative control sera and the patients' sera (1:100) at 37℃ for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-human IgA (1:10,000; Bethyl Laboratories Inc., Montgomery, TX, USA) at 37℃ for 1 h. Between each step, the plates were washed with PBST. Color formation was done with *o*-phenylene diamine as substrate and then stopped by 2 N sulfuric acid. OD was measured at 492 nm using an Emax Precision microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Statistical analysis

The data were expressed as mean ± standard deviation (SD), and statistical significance was analyzed by student's *t*-test using Statistical Package for Social Sciences software (version 17.0, SPSS, Chicago, IL, USA). Differences were considered significant if a value of $p < 0.05$ was obtained.

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RESULTS

Analysis of antigenic epitope motif of *H. pylori* urease and preparation of truncated clone

Based on the motif analysis data, a primer location was determined to prepare a truncated clone. An amino acid location where the Antigenic index was near zero was selected, and primers were designed as shown in Fig. 1 and Table 1. Two and six mutants were prepared by truncating the 3'-terminal of UreA and UreB, respectively. The antigenicity of expressed proteins

Table 1. Oligonucleotide primers for PCR amplification of *ureA* and *ureB* fragments

Truncated clone		Primer sequences (5'-3')	Position (size, bps)
UreA85	Forward	ATGAAACTCACCCC	1~255 (255)
	Reverse	CGCTTCAATACCCAC	
UreA142	Forward	ATGAAACTCACCCC	1~426 (426)
	Reverse	ACCGATTTGAACCGG	
UreB134	Forward	ATGAAAAAGATTAGCAG	1~402 (402)
	Reverse	GTCAATACCACCAGC	
UreB244	Forward	ATGAAAAAGATTAGCAG	1~732 (732)
	Reverse	TTGCACATCGTATTT	
UreB412	Forward	ATGAAAAAGATTAGCAG	1~1236 (1236)
	Reverse	GTTAATGGTGTATTTAG	
UreB495	Forward	ATGAAAAAGATTAGCAG	1~1485 (1485)
	Reverse	AGACACAAAAGTGATG	
UreB546	Forward	ATGAAAAAGATTAGCAG	1~1635 (1635)
	Reverse	CACATGGTAAGTTTC	
UreB448	Forward	TTGGCGTGAAACCCCATATG	1325~1710 (385)
	Reverse	CTAGAAAATGCTAAA	

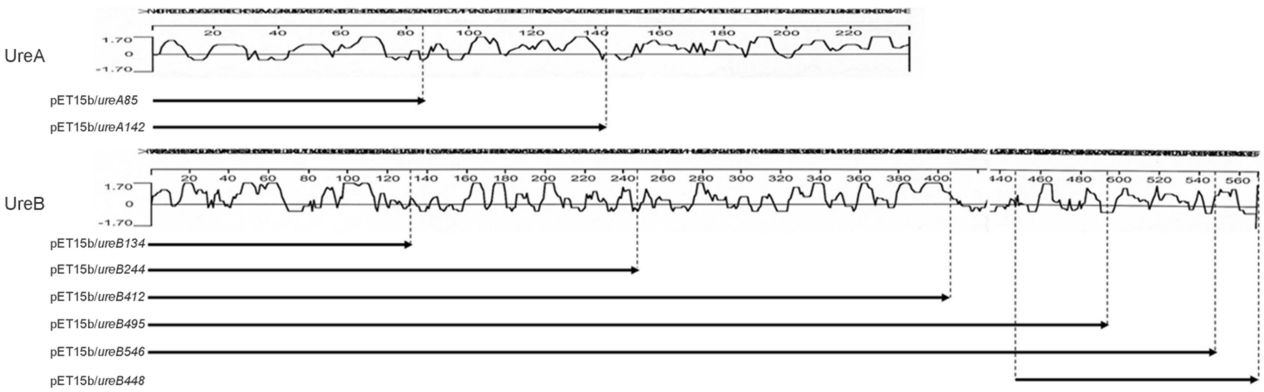

Figure 1. Schematic diagram of truncated mutant clones of UreA and UreB

was analyzed by an immunoblot assay using patient pooled sera. The result showed that IgG antibodies were bound to the entire UreA and the truncated UreA proteins, but UreA did not show IgA antibody reactivity (Fig. 2A). In addition, both IgG and IgA antibodies were bound to the entire UreB, but the mutant clones truncated at the 3'-terminal did not show antibody reactivity (Fig. 2B). However, the mutant clone of 3'-terminal sequence from UreB (448~569 amino acids) showed the IgA antibody reactivity clearly (Fig. 2B). This result indicated that the antigenic epitope is located between the 546th residue and the last 569th residue of UreB. To verify it, pET15b/UreB448 was prepared by inserting the UreB amino acid residues from the 448th residue to the last residue. The expressed pET15b/UreB448 showed antibody reactivity. This result suggested that the antigenic epitope of the urease B subunit to which the IgA and IgG antibodies are reactive is located between the 546th amino

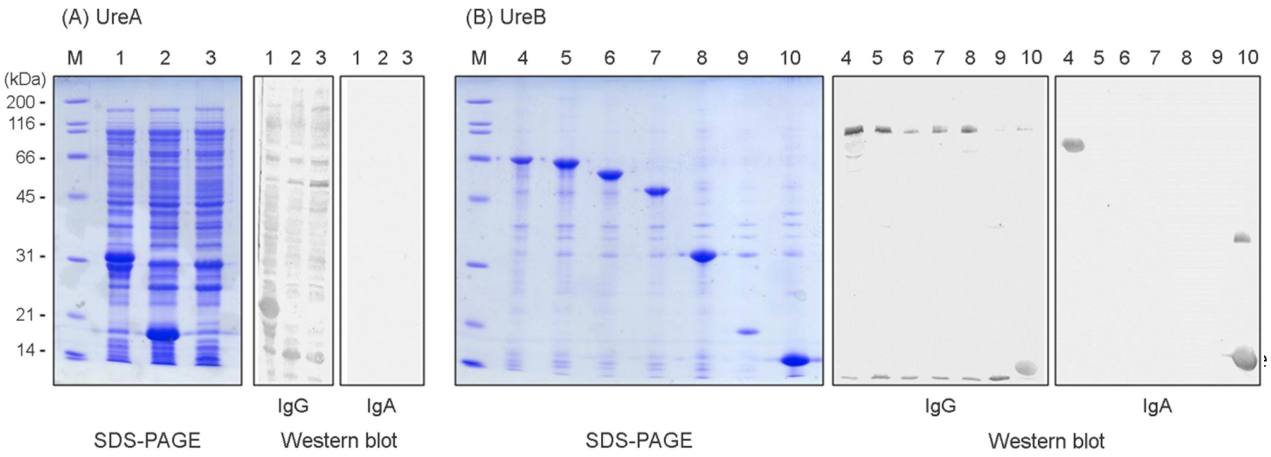

Figure 2. Reactivities of human antisera to truncated mutant proteins of UreA (A) and UreB (B). The truncated mutant proteins of UreA and UreB were expressed from pET15b clones. Reactivity of the proteins were analyzed by patient's sera. Lane M, molecular weight markers; 1, UreA; 2, UreA142; 3, UreA85; 4, UreB; 5, UreB546; 6, UreB495; 7, UreB412; 8, UreB244; 9, UreB134; 10, UreB448.

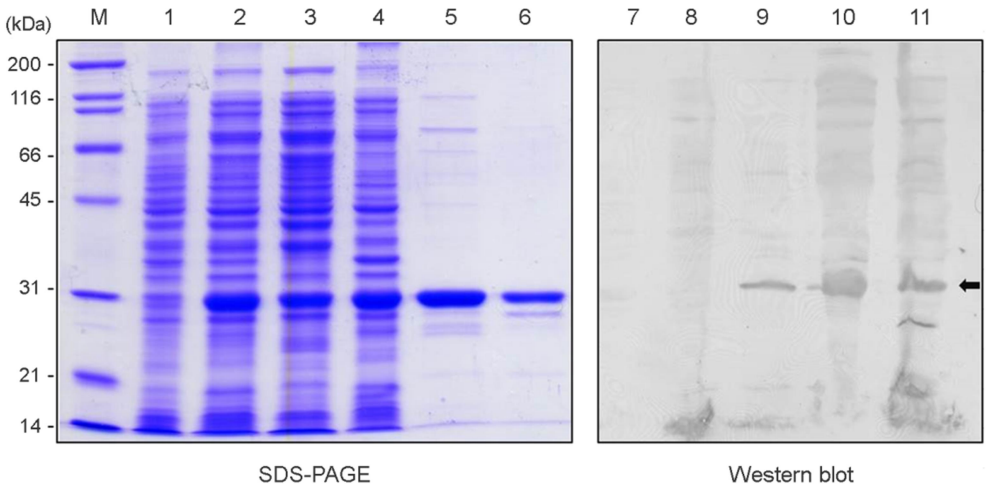

Figure 3. Production of recombinant UreBEnd protein. The recombinant UreBEnd protein was purified from pEGexs/UreBEnd clone by Glutathione column. Reactivity of the UreBEnd protein was analyzed with patients' pooled sera. Lane M, molecular weight markers; 1, Non-induction; 2, the whole cell lysate after IPTG induction; 3, the supernatant of whole cell lysate after IPTG induction; 4, the pellets of whole cell lysate after IPTG induction; 5, elution; 6, thrombin treated elution; 7, vector control; 8~11, elution 1~4.

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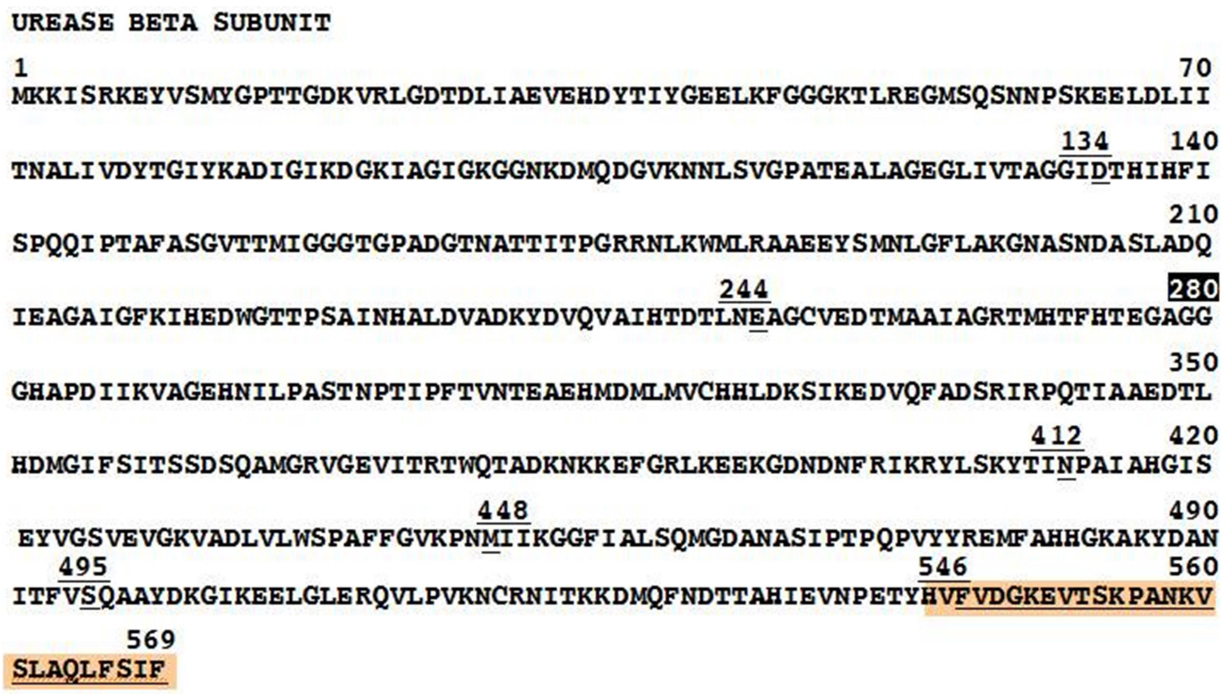

Figure 4. Location and sequence of antigenic determinant of UreB. Colored underlined sequence denotes the epitope sequence. Underlined numbers indicate 3'-termini's amino acid site of truncated mutant proteins.

acid residue and the last 569th residue. In other words, the antigenic epitope that specifically reacted to the IgA and IgG antibodies was the last 3'-terminal domain of UreB. The antigenic epitope was predicted to be VDGKEVTSKPANKVSLAQLFSIF based on the data, and then the predicted antigenic epitope was inserted to the pGEX4T1 vector to produce UreB antigenic epitope fusion glutathione-S-transferase (GST), which is a antigenic epitope recombinant protein using GST (Fig. 3). This recombinant protein (UreBEnd) was analyzed for its antigenicity using patients' pooled sera. The amino acid sequence of the motif acting as an antigenic epitope of UreB protein in human were shown in Fig. 4.

Measurement of IgA antibody titer of patient serum using antigenic epitope fusion protein

The UreB antigenic epitope fusion GST was purified and then used to measure the IgA antibody titer of the patient serum by ELISA. ELISA was performed with 60 patients whom *H. pylori* infection was confirmed and 10 subjects whom *H. pylori* infection was found to be negative. The average absorbance of anti-UreBEnd ELISA was 0.96±0.29 and 0.95±0.36 in the case of patients who showed strong and weak reactivity in CLO test, respectively, and that of the negative group was 0.35±0.19. Cut-off value (0.63) was determined by ROC analysis of 97% sensitivity and 100% specificity, as shown in Fig. 5, using the 10 negative and 60 positive sera noted above. According to the cut-off value, all the negative patients were found to be negative, and two, and nine of the strong and weak CLO test-reactive patients were found to be negative in UreBEnd-ELISA.

DISCUSSION

A number of *H. pylori* antigens have been identified to be applicable for the serological diagnosis of infection and have even been suggested as candidates for vaccination. *H. pylori* is the only organism that inhabit human stomach, but the environment

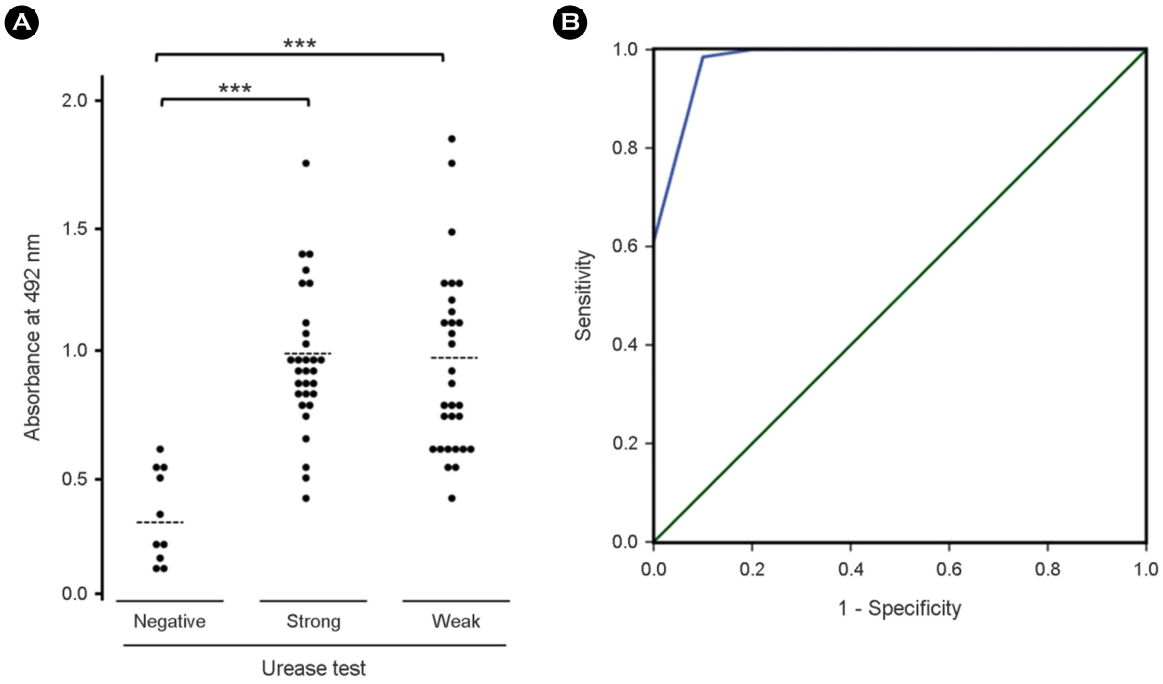

Figure 5. IgA distribution of sera from the patients with/without gastric symptoms measured by ELISA using purified recombinant UreBEnd. (A) Dashed line indicates the average of absorbance value: negative, 0.35; strong, 0.96; weak reactivity to CLO test, 0.95. (B) Receiver operating characteristic (ROC) curves of UreBEnd ELISA for the diagnosis of *H. pylori* infection. The area under the ROC of UreBEnd ELISA was 0.997 (95% confidence interval; $p < 0.001$).

of the stomach is too harsh to live for the bacteria (15, 16). Urease is the most expressed *H. pylori* cell component (10~15% total protein weight) that determines the representative biochemical feature of the strain (12). Urease, which hydrolyzes urea into NH_3 and CO_2, plays important role in *H. pylori* colonization in the acidic condition of stomach (pH 3) (10). According to the report by Ghalehnoei *et al.*, some variants of UreB could be shown depending on the strains (17). Thirty percent of patients with intestinal metaplasia showed a single amino acid sequence change in UreB, but this change did not affect the urease activity at all (17). As mentioned above, urease is a relatively conserved protein for *H. pylori* survival in stomach, and although it may have some changes depending on the strains, it does not seem to affect the function and activity of urease. Furthermore, UreB subunit was found highly reactive to IgA antibody while all other antigenic spots were highly reactive to IgG antibody in the analysis of immunogenic proteome components of our previous study (data not shown, under review). Here, we identified the antigenic epitope of urease that induces IgA antibodies in human using truncated mutants. Based on the identified epitopes of UreB, the UreB protein was purified and characterized for its serodiagnostic potential.

Since the protein size of UreB is large (61.5 kDa), the antigenic epitope reacting with the IgG antibody may be different from the antigenic epitope reacting with the IgA antibody. Thus, an amino acid sequence deleted mutant was produced to prepare an antigenic epitope map. First, five mutant genes prepared by deleting the C-terminal in steps were cloned to the pET15b vector to produce deletion mutant proteins and analyzed their antigenicity. The result showed that the antigenic epitope to which the IgG antibody was reactive was the same as the one to which the IgA antibody was reactive. The antigenic epitope was located between the 546th amino acid residue and the last 569th residue of UreB, and the sequence of the antigenic epitope was found to be VDGKEVTSKPANKVSLAQLFSIF. This motif was reported as the sequence that connects the 12 monomers constituting the urease of *H. pylori*.

Although UreB is expressed as a dissolved form when the entire ORF is expressed in *E. coli*, all the deletion mutants were

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expressed in an undissolved form. To solve this problem, the antigenic epitope was expressed by binding it to the GST. The resulting UreB antigenic epitope fusion GST protein was expressed in a dissolved form. The fusion protein was purified, and the IgA antibody titer of the patient serum was measured by ELISA to evaluate the diagnostic value. In the present experiment, the antigen concentration for attachment and the dilution factor of the patient serum were determined as 10 μg/ml and 1:100, respectively. The average absorbance of the serum of the infected patients was 0.95, and that of the negative group was 0.35. In addition, among the infected patients, the average absorbance was 0.96 and 0.95 in the group with strong and weak reactivity to CLO test, respectively. Although there was no difference in the UreBEnd-ELISA results according to the degree of reactivity of CLO test, 97% sensitivity and 100% specificity were shown in serum samples from the patients with gastrointestinal disturbances and positive for CLO test.

As urease is a protein that is expressed at a high level, when the gastric mucosa is infected by *H. pylori*, urease will be exposed to the immune system of the host more than any other components, resulting in an increased antibody titer of urease. Hence, the antibody titer of IgA to the UreB antigenic epitope will also be kept at high level in adult patients. The results from the present study showed that the UreB antigenic epitope strongly reacts with not only the IgG antibody but also the IgA antibody, indicating that the UreB antigenic epitope may be used as a diagnostic material for the specific diagnosis of *H. pylori* infection in both adults and children.

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