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Serum antibody against *Helicobacter pylori* assayed by a new capture ELISA

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Abstract: We developed a highly specific detection technique for serum antibody, using a monoclonal antibody to a specific antigen of *Helicobacter pylori*. A monoclonal antibody preparation that reacted with the 54-kDa molecule of *H. pylori* antigens was obtained. Using this preparation, an antigen-capture enzyme-linked immunosorbent assay (ELISA) was established by fixation of the monoclonal antibody, followed by reaction with sonicated whole cell antigens. The serum antibody titers of patients with gastritis and peptic ulcers were significantly higher than control titers, and the antibody titer correlated with the histological severity of gastritis. Patients positive for *H. pylori* by bacterial culture had higher titers than *H. pylori*-negative patients. Our new ELISA may be useful for the diagnosis of *H. pylori* infections and for evaluation of the severity of gastric inflammation.

Key words: *Helicobacter pylori*, enzyme-linked immunosorbent assay, monoclonal antibody

Introduction

Since the report of Warren and Marshall[1] in 1983, it has been generally agreed that *Helicobacter pylori* is closely associated with gastritis and peptic ulcers. In particular, the high detection rate of *H. pylori* in patients with duodenal ulcers and active chronic gastritis led to the belief that this bacterium was an etiological factor in these conditions. Methods used for the detection of *H. pylori* include microscopic, culture, and antibody detection methods. The microscopic method is simple, but has low specificity. The culture method is both specific and reliable and constitutes the basic method for the detection of this organism. However, *H. pylori* has a patchy distribution pattern in the stomach, and sampling errors may therefore lead to false negative results in cultures of gastric tissue. In that sense, a culture method using gastric juice is one of the most reliable and sensitive procedures.[2] Although antibody detection methods are non-invasive and useful, false-positive results have been obtained, depending on the antigen employed for the determination of antibody titers, due to cross-reactivity with other *Campylobacters*.[3] In this study, we attempted to develop a highly specific technique for the detection of serum antibody, using a monoclonal antibody to specific antigens of *H. pylori*.

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Materials and methods

Serum antibody against *H. pylori* was determined in 77 patients whose condition was diagnosed by gastroendoscopic examination. We observed 42 patients with gastritis, 19 patients with gastric ulcer, and 16 patients with duodenal ulcer. In 52 patients, the severity of histological inflammation of antral gastritis was also determined. As negative controls, we used the sera from 39 newborn infants.

Bacterial cultures

Gastric tissue samples were obtained from the mucosa of the greater curvature of the gastric corpus and from the pyloric antrum by endoscopic biopsy. The specimens were placed onto Brucella broth (B-broth), minced with the broth, and inoculated onto blood agar plates containing 10% lysed sheep blood. The cultures were incubated at 37°C for 3–5 days in a microaerophilic environment with 100% humidity.

Bacterial strains

All strains of *H. pylori* used were isolated at Kinan General Hospital, except for strains MR31 (M) and RD26 (R), which were kindly supplied by Dr. David Y. Graham and Dr. Patrice A. Michaletz (V.A. Medical Center, Houston, Tex.), and strain England (E), which was provided by Dr. Eiji Ishii (Osaka City Institute of Public Environmental Science, Osaka, Japan). Other *Campylobacter* species (*C.* sp.) used for comparative studies were *C. fetus*, kindly supplied by Dr. Toshiharu Hongo (Kurashiki Chuo Hospital, Okayama, Japan) and *C. coli* and *C. jejuni*, which were isolated at Kinan General Hospital, Wakayama, Japan. Human sera were obtained from patients on the day of endoscopic examination. Sera from 39 healthy newborn infants served as a negative control.

Monoclonal antibody production

The preparation and production of the monoclonal antibody followed our protocol, described previously.[4] Briefly, BALB/C mice were primed intraperitoneally with the sonicated E strain of *H. pylori* and Freund's complete adjuvant (FCA) and boosted two times at 2-week intervals. Four weeks after the last immunization, the animals received a final intraperitoneal injection of bacterial sonicates without adjuvant and, 3 days later, they were sacrified and their spleens were removed for fusion experiments. The methods used for the production and culture of hybridomas have been described elsewhere.[4]

Protein profiles of H. pylori

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) methods employed for the determination of the protein profiles of *H. pylori* have been described previously.[5] Briefly, whole organisms were placed in a disruption buffer containing 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 8% (v/v) glycerol, and 6.25 mM Tris-HCl. Samples were boiled at 100°C for 3 min and loaded onto a 12% polyacrylamide gel, following the method of Laemmli.[6] Electrophoresis was performed at a constant current (10 mM) until the indicator dye reached the bottom of the gel. Coomassie brilliant blue staining was performed for protein patterns.

Immunoblotting (Western blotting)

Western blotting with monoclonal antibodies was carried out according to the method of Towbin et al.,[7] with slight modifications, which have been described elsewhere.[8] Briefly, after SDS-PAGE, the proteins were transferred from the gel onto a nitrocellulose sheet (Nihon Millipore Kogyo, Tokyo, Japan). The nitrocellulose blots were soaked in blocking solution containing 5% (w/v) skim milk and 0.05% (v/v) Tween 20 in phosphate-buffered saline (PBS-T-SM), and were then incubated with monoclonal antibody or serum for 1 h at 37°C. After washings in PBS with 0.05% Tween 20 (PBS-T), they were incubated with peroxidase-conjugated anti-mouse immunoglobulins for 30 min at 37°C. After further washings in PBS-T, the coloring reaction was performed, with the substrate solution being 0.02% (w/v) 3-3′-diaminobenzidine and 0.05% (v/v) H_2O_2 in 0.05 M Tris-HCl.

Enzyme-Linked immunosorbent assay (ELISA)

Monoclonal antibody in 0.05 M carbonate-bicarbonate buffer (4 μg/ml), pH 9.6, was coated onto micro-ELISA plates overnight at 4°C. The excess protein-binding sites were blocked with PBS-T-SM for 1 h at room temperature. After washings with PBS-T, the sonicated whole cell suspension of E strain *H. pylori* was added and the plates were incubated for 2 h at 37°C. After washings, sera (diluted 1:100) were added and the plates were again incubated for 2 h at 37°C; this was done in duplicate. Further washings were carried out, and the plates were incubated with peroxidase-conjugated anti-human IgG (Cappel products, USA) for 2 h at 37°C. Conjugated peroxidase was detected with 2-2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and hydrogen peroxide. The absorbance was measured at 410 nm after 20 min.

Statistical analysis

The Kruskal-Wallis test and Wilcoxon's nonparametric test (two-tailed) were used, with $P < 0.05$ being taken as significant. Data are expressed as means ± SE.

Results

Monoclonal antibodies (Fig. 1)

Eight hybridoma clones were established from the mice immunized with the E strain of *H. pylori*. Two clones that reacted with major antigens of the E strain were selected. Their characteristics and immunological behavior have been described previously.[5] Briefly, the monoclonal antibody 102 (mAb 102, subclass IgG 2b) reacted with a polypeptide with a molecular weight of 54 kDa in the bacterial cells. This antibody cross-reacted with other *H. pylori* strains, including both the M and R strains; however, no reaction was observed with the other bacterial enteropathogens tested, i.e., *C. jejuni*, *C. fetus*, and *Escherichia coli*. These results

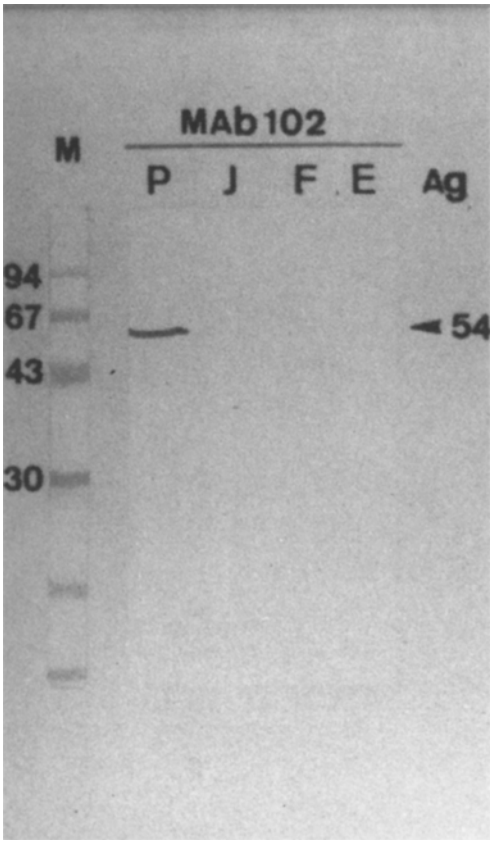

Fig. 1. Western blotting of polypeptides from various bacterial cells reacted with monoclonal antibody. Numbers on the left side (*M*) indicate the molecular weight (×1000) of the polypeptides. Antigens (*Ag*) used were *H. pylori* (*P*), *C. jcjuni* (*J*), *C. fetus* (*F*), and *E. coli* (*E*)

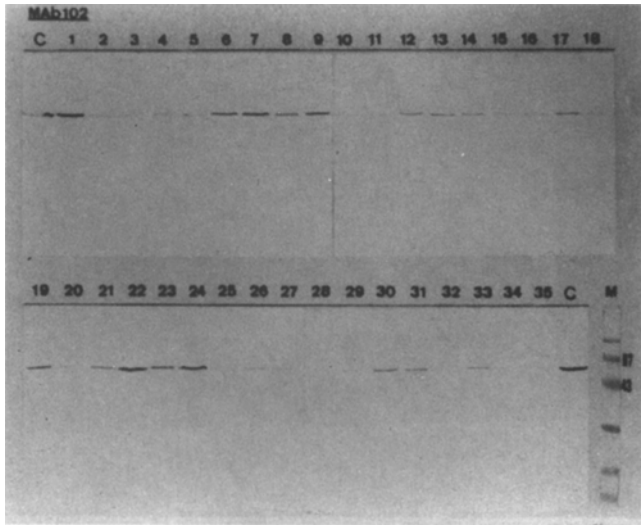

Fig. 2. Reactivity of monoclonal antibody (*mAb* 102) with various *H. pylori* isolates from human gastric materials on Western blotting. The E strain of *H. pylori* and 35 samples were analyzed

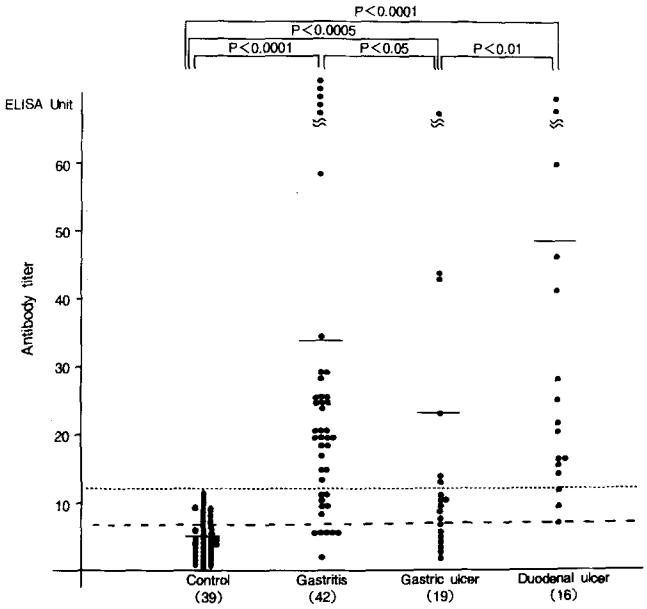

Fig. 3. Serum antibody titer in patients with gastroduodenal disease and control subjects. *Horizontal bar* indicates the mean value for each group. *Dotted line* indicates the upper limit of mean + 2 SD in control subjects. Broken line indicates 7 EU. *Numbers in parentheses* indicate numbers of subjects examined. *ELISA*, Enzyme-linked immunosorbent assay

suggest that the mAb 102 was specific for H. pylori antigens.

Reactivity with antigens of heterogenous H. pylori

We analyzed the polypeptide antigens of 78 *H. pylori* isolates by Western blotting. As shown in Fig. 2, mAb 102 reacted with the 54-kDa polypeptide of all *H. pylori* isolates investigated.

*Anti-*H. pylori *antibody in serum*

An ELISA was established for the detection of anti-*H. pylori* antibody in serum. For the standardization of antibody titers in serum, an ELISA unit (EU) was determined, using the serum of one patient, according to methods previously described by Sugiyama et al.[9] The cut-off value, determined from the mean (4.97 EU) and the standard deviation (3.19 EU) of the negative controls, was 12 EU. In the culture-negative patients, the serum antibody titer increased slightly with age; however, the differences between age groups were not significant.

Serum antibody titers in gastritis and peptic ulcers (Fig. 3). The antibody titers of the groups with gastritis (33.5 ± 7.87 EU, $P < 0.0001$), gastric ulcers (23.6 ±

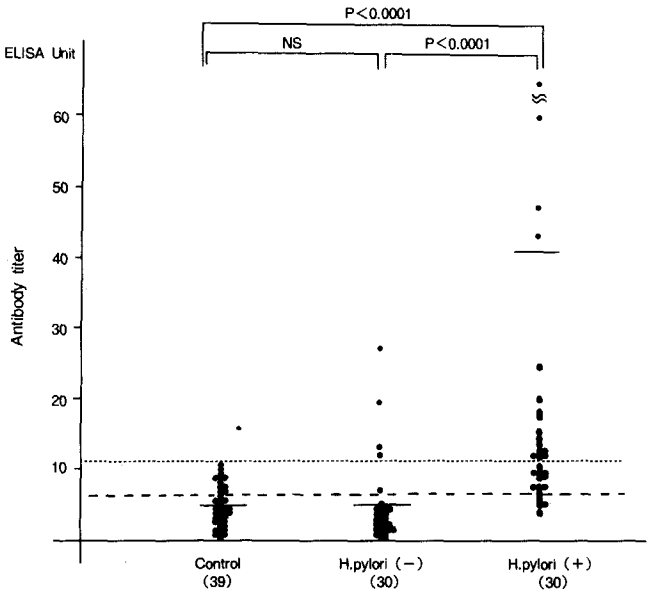

Fig. 4. Serum antibody titer in *H. pylori* culture-negative and -positive patients and in control subjects. *Horizontal bar* indicates the mean value of each group. *Dotted line* indicates the upper limit of mean + 2 SD in control subject. Broken line indicates 7 EU. *Numbers in parentheses* indicate numbers of subjects examined

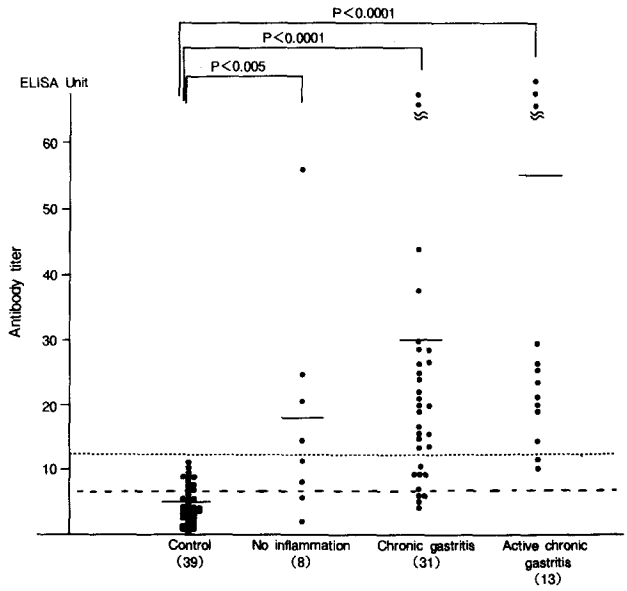

Fig. 5. Serum antibody titer in patients with antral gastritis as the grade of inflammation. *Horizontal bar* indicates the mean value for each group. *Dotted line* indicates the upper limit of mean + 2 SD in control subjects. Broken line indicates 7 EU. *Numbers in parentheses* indicate numbers of subjects examined

11.2 EU, $P < 0.0005$), and duodenal ulcers (47.8 ± 18.2 EU, $P < 0.0001$) were all significantly higher than the mean titer (4.97 ± 0.51 EU) of the negative control group.

Comparison with evaluation by culture (Fig. 4). The titer of the *H. pylori* culture-negative group was 5.02 ± 0.93 EU; that of the *H. pylori* culture-positive group was 30.3 ± 14.8 EU, this being significantly higher than the control titer of 4.97 EU ($P < 0.0001$). For the cut-off titer of 12 EU, this ELISA has a specificity of 87% and a sensitivity of 54%. At 7 EU, the specificity was 84% and the sensitivity was 87%.

Relationship between severity of histological inflammation and serum antibody titer in chronic gastritis (Fig. 5). Patients with gastritis were divided into groups according to the histological classification of Warren and Marshall:[1] no inflammation (group N), chronic gastritis (group C), and active chronic gastritis (group A). For this purpose, specimens were taken from the pyloric antrum. The antibody titers of the N, C, and A groups were 18.0 ± 6.03, 29.1 ± 9.35, and 54.1 ± 22.2 EU, respectively, indicating a relation between inflammatory grade and antibody titer. The titers of group N ($P < 0.01$), group C ($P < 0.0001$), and group A ($P < 0.0001$) were significantly higher than the control titer. Thus, the anti-*H. pylori* antibody titer of the active chronic gastritis group was markedly elevated, indicating a close association with *H. pylori* infection.

Serum antibody profiles in the Western blotting method (Fig. 6)

Antibodies to the 96-, 62-, 54-, and 38-kDa antigens were frequently observed in the patients' sera. Antibodies to the 62-, 54-, and 38-kDa antigens were absent in the sera of newborns, although the antibody to the 96-kDa antigen was sometimes observed. The antibody to the 54-kDa antigen was the one most frequently observed in the patients.

Discussion

Initially, crude whole bacterial preparations of *Campylobacter* were used as ELISA antigens, as reported by Rathbone et al.[10] and Asaka et al.[11] However, in these studies, although *H. pylori*-positive patients had higher titers, there was considerable overlap between the *H. pylori*-positive and -negative groups. Rathbone et al.[12] reported that patients with high serum titers to *H. pylori* had consistently higher titers against other *Campylobacters*, such as *C. fecalis*, *C. fetus*, and *C. sputorum*, suggesting antigenic cross reactivity. It was confirmed that these antigens were of no diagnostic use, and, therefore, partly purified or highly purified antigens were sought for ELISA in later studies,[13–18] Rathbone[19] reported that if crude whole bacterial antigens were used for ELISA, the test resulted in many false positives, whereas partly purified antigens,

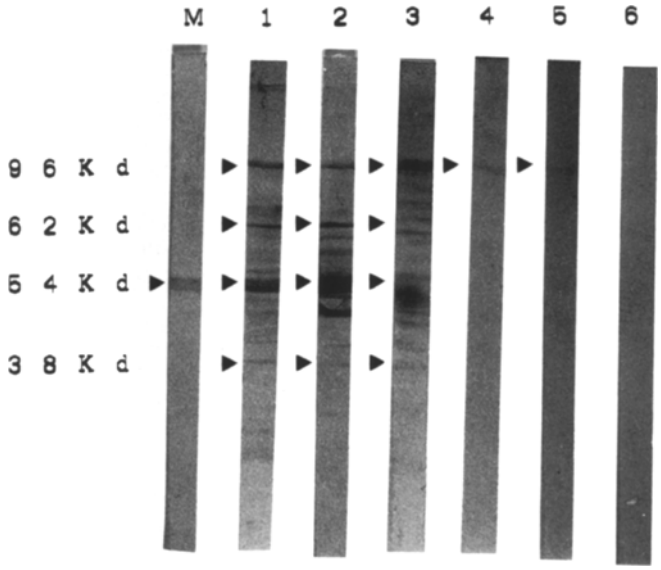

Fig. 6. Serum antibody profiles by the Western blotting method. Positive bands were observed for the monoclonal antibody (*lane M*), and for the sera of chronic gastritis patients (*lane 1*), gastric ulcer patients (*lane 2*), and duodenal ulcer patients (*lane 3*), compared with the sera of controls (*lanes 4, 5, and 6*)

such as acid glycine extracts, gave results with very satisfactory sensitivities and specificities. Highly purified antigens, such as neuramin lactose-binding hemagglutinin (NLBH) and purified urease, tended to give a very specific but less sensitive ELISA system. Goodwin et al.[13] reported that when the antigen was an acid-glycine extract of *H. pylori*, antibody titers ranged from 80 to 22 000 ELISA units (EU), i.e., for a titer of 300 EU, the ELISA test had a specificity of 97% and sensitivity of 81%. At 150 EU, the specificity was 78% and the sensitivity 99%. Czinn et al.[14] showed that the outer membrane of *H. pylori* contained lipopolysaccharide (LPS) and proteins that consisted of 61-, 54-, and 31-kDa antigens. With this antigen, the sensitivity of the ELISA was 80% and the specificity was 100%. Sugiyama et al.[15] reported that a serum antibody directed against the 25-kDa molecule on *H. pylori* was both sensitive and specific for the diagnosis of *H. pylori* infection. In addition, the titer was correlated with the histologic severity of antral gastritis. Evans et al.[16] have developed a specific and sensitive ELISA that detects serum immunoglobulin G antibodies directed against a high molecular weight cell-associated protein (HM-CAP) of *H. pylori*. The specificity and positive predictive value of the HM-CAP ELISA (anti-urease) were both 100%; the sensitivity of the assay was 98.7%, and the negative predictive value was 98.6%. The HM-CAP ELISA and the urea breath test have both proven valuable for detecting *H. pylori* infection, the urea breath test being a more direct method, whereas the ELISA is less expensive and easier to perform. Evans et al.[17] reported another ELISA, in which NLBH purified from a single isolate was used as the antigen. A positive result was obtained with serum from 81.5% of individuals with ulcers, from 67.7% of *H. pylori*-infected volunteers, and from 1.7% of *H. pylori*-negative volunteers. Evans et al.[17] favored the hypothesis that there are probably several antigenically distinct *H. pylori* NLBH, i.e., NLBH which would stimulate antibodies that were undetectable with the antigen used in the ELISA. Stacey et al.,[18] using an enzyme immunoassay, investigated the antigenicity of *H. pylori* protein fractions separated by fast protein liquid chromatography size exclusion. They found that the antigenic material of *H. pylori* was confined to fractions 8 and 14–21. Urease-containing fractions (14/15) and flagella-containing fractions (17/18) were identified. With the urease-containing fraction, the sensitivity of the ELISA was 91% and the specificity was 91%; with the flagella-containing fraction, the sensitivity was 78% and the specificity 100%.

Our mAb 102 reacted with the 54-kDa antigen, which is one of the major constituent proteins of *H. pylori*; this antigen did not cross react with other *Campylobacters* tested, such as *C. jejuni*, *C. fetus*, or even *E. coli*. Thus, mAb 102 was proven to be highly specific to *H. pylori*. Using this antibody, we established an antigen-capture ELISA for determination of the antibody titer in patients with gastrointestinal disease. With this system, the antibody titers of gastritis and peptic ulcer patients were significantly higher than the control antibody titer of healthy newborns. Thus, it was possible to differentiate between healthy and gastric disease groups, and this ELISA with mAb 102 seemed to be a useful diagnostic tool for determining the presence of *H. pylori* infections.

On comparing results with this ELISA system with the results obtained by the culture method, we found that the *H. pylori* culture-positive group had a higher antibody titer than the *H. pylori* culture-negative group. However, there were some *H. pylori*-negative sera that had high anti-*H. pylori* antibody titers. These types of culture-negative, antibody-positive cases have been explained, by Sugiyama and Yabana,[20] in terms of the following possibilities: (1) tissue specimens for the culture method are taken from only one or two sites of the gastric mucosa, and infected areas may therefore have been missed; (2) the number of bacteria is under the detection limit, or the bacteria are located so deeply in the tissue that they escaped detection, or (3) the production of serum antibody persists even after the bacteria have disappeared from the tissue. These possibilities may also apply to our cases.

There was a correlation between the severity of histological inflammation and the antibody titer, i.e., the serum antibody titer increased as the severity, according to the classification of Warren and Marshall,[1] increased. A similar relationship was reported by Sugiyama et al.,[15] who used a 25-kDa antigen in their ELISA.

Pearson et al.[21] examined antibodies that reacted with many cell components of *H. pylori*, i.e., antibodies to the 62-, 53-, 50-, 39-, 25-, 21-, and 14-kDa antigens of the whole cell sonicate of *H. pylori*. Antibodies to the 74-, 64-, 58-, 43-, 21-, and 14-kDa antigens of *H. pylori* were reported by Megraud et al.[22] Drouet et al.[23] found that patients' sera reacted with polypeptides at 120, 88, 61, 54, 30, 25, and 19 kDa. In our analysis, the sera of gastritis patients revealed antibodies to the 96-, 62-, 54-, and 38-kDa antigens. Antibodies to the 62-, 54-, and 38-kDa antigens were not observed in the sera of newborns. The frequency of antibodies to the 54-kDa antigen in the sera of patients was high, indicating the usefulness of this antigen for diagnosis. However, antibody to the 96-kDa antigen was also observed in the sera of newborns. These results indicate that cross reactivity against other bacteria or viruses may be detected with the 96-kDa antigen.

Antibodies to *H. pylori* are multiple and their significance varies depending on the choice of antigen. Future analysis of the antigens that correspond to various antibodies is expected to shed more light on the pathology of gastric mucosal disease, the pathogenicity of *H. pylori*, and the progression of these disease conditions.

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