Abstract

Aim: Infection with Helicobacter pylori (H. pylori) possessing the cag pathogenicity island (PAI) has been associated with severe clinical outcome and CagA-antibody has been used to indicate cagPAI-positive infection. The aim of this study was to examine the accuracy of CagA seropositivity to indicate the virulence of the cagPAI in Japan.

METHODS: Sixty isolates of H. pylori cultured from gastric biopsies were examined by polymerase chain reaction assays for the presence of cagA, cagE and VirD4. Anti-CagA IgG antibody in matching sera was tested by both ELISA and immunoblot assay. Histological grade of gastritis was graded according to the updated Sydney System.

RESULTS: Amongst 53 patients infected with cagA+/cagE+/VirD4+ strain, 38 were CagA seropositive. There were four patients infected with strains possessing incomplete cagPAI. Two out of three patients with cagA+/cagE+/VirD4 infection were CagA seropositive, while a patient with cagA-/cagE+/VirD4+ infection was CagA seronegative. Accuracy of ELISA to predict bacterial possession of cagA was 61.7% whereas 58.3% for cagE and VirD4. The immunoblot assay showed relatively higher sensitivity and showed better accuracy. The lower grade of gastric mucosal inflammatory infiltration was seen in false CagA-seronegative patients.

CONCLUSION: Some serodiagnosis does not seem to have enough accuracy to indicate virulence of cagPAI, particularly in infection of strains with incomplete cagPAI. The degree of gastric mucosal inflammation may affect the results of CagA serodiagnosis.

Key words: CagA seropositivity; cagPAI; Gastric inflammation

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CagA serology and the structure of the cagPAI to examine the accuracy of CagA serology to indicate the virulence of cagA- or cagPAI-positive H pylori in Japan. Furthermore, we compared the histologic grade of gastritis between CagA seropositive and seronegative patients who were infected with cagA-positive H pylori strain.

**MATERIALS AND METHODS**

**Patients**

Patients scheduled for upper gastro-intestinal endoscopy for routine screening for gastric cancer at Hirosaki University Hospital were enrolled in the study. Patients were considered to be eligible for inclusion when their endoscopic diagnosis was normal or atrophic gastritis was present without any evidences of neoplasia. Patients were excluded if they had received at least one of acid suppression agents, anti-ulcer agents and antibiotics during the 2 mo before the examination or had previous histories of gastric tumors and gastric surgery. All subjects provided informed consent before their endoscopy and this study was approved by the ethics committee of Hirosaki University. Peripheral venous blood was obtained at the time of endoscopy and biopsy specimens were also taken from the antrum and the corpus of the stomach. Patients were eligible if H pylori was isolated from their biopsy specimens.

**H pylori culture**

Biopsy specimens were homogenized and cultured for 3-5 d on Skirrow blood agar at 37°C. Biopsy specimens were homogenized and cultured for 3-5 d

**DNA preparation and PCR assay**

One milliliter of aliquots of bacteria in PBS were centrifuged at 10 000 g for 5 min. After the supernatant was discarded, the bacterial pellets were digested with 0.3 units of proteinase K at 55°C for 2 h and DNA was extracted with phenol-chloroform-isooamylalcohol. The concentration and quality of DNA samples were estimated by measuring $A_{260\, \text{nm}}/A_{280\, \text{nm}}$. The presence of the cagA, cagE, and VirD4 homolog were determined by polymerase chain reaction (PCR) using the oligonucleotide primer pairs described previously $^{[13-15]}$. In brief, after 5 min of denaturing at 94°C, 35 cycles of PCR, including denaturing at 94°C for 40 s (cagA) or 30 s (cagE and VirD4 homolog), annealing at 50°C for 40 s (cagA) or 30 s (cagE and VirD4 homolog), and polymerization at 72°C for 1 min (cagA) or 2 min (cagE and VirD4 homolog) were performed. The final cycle included an extension step for 10 min at 72°C. Each PCR amplification was performed using 0.1 µg of extracted DNA, which was added to 50 µL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 200 µmol/L each deoxynucleotide (dNTPs), 100 pmol (cagA) or 200 pmol (cagE and VirD4 homolog), and 0.5 units of Taq polymerase (TaKaRa Taq, Takara Biochemicals, Tokyo, Japan). PCR amplification was performed in duplicate for each DNA sample. PCR products were electrophoresed in 3% (cagA) or 2% (cagE and VirD4 homolog) agarose gel. Each sample was also tested for the presence of the 23S rRNA gene by PCR to confirm the existence of H pylori DNA as described previously $^{[18]}$.

**Serological assays**

Serum samples were assayed for the presence of antibodies to CagA. CagA antibodies were assayed by ELISA using a recombinant fragment of CagA as antigen $^{[17]}$. In brief, flat-bottom 96-well microplates were coated with 0.1 µg/well antigen in 0.1 mol/L bicarbonate buffer for 24 h at 4°C. Plates were washed with PBS containing 0.1% Tween-20 and blocked with 1% bovine serum albumin (BSA) in PBS-Tween for 1 h at 26°C. Serum samples diluted 1/75 in 1% BSA/PBS were incubated in duplicate for 90 min at 26°C. Following incubation with goat anti-human IgG alkaline phosphatase conjugate (Sigma, Poole, Dorset, UK) for 2 h at 26°C, bound antibodies were detected with p-nitrophenyl phosphate substrate (Sigma) at 1 mg/mL in diethanolamine-MgCl2 buffer. IgG antibodies to CagA were also measured using Chiron Diagnostics' Recombinant Immunoblot Assay (RIBA™). The relative intensity of antibodies to CagA was determined by comparing the intensity of CagA labeling to that of the human IgG internal control. The cut off for positivity was determined using sera of known H pylori status $^{[18]}$.

**Histological grading of gastritis**

The specimens were embedded in paraffin and stained with hematoxylin and eosin and also Warthin-Starry method. Mononuclear cell (MNC) infiltration, polymorphonuclear cell (PMN) infiltration, glandular atrophy, and the density of H pylori were graded into 0-3, according to the updated Sydney System by an experienced pathologist without the knowledge of bacterial genotype.

**Statistical analysis**

The differences of histological grade of gastritis were tested by Mann-Whitney U test. A P value of less than 0.05 was considered significant.

**RESULTS**

H pylori strains and serum samples were obtained from a total of 60 patients (34 males and 26 females, mean age 62.1±11.7 years). Fifty-six of the sixty H pylori isolates had cagA and three of them did not have both cagE and VirD4. Four strains did not possess cagA and three of them were also negative for cagE and VirD4. There were four strains, which had incomplete cagPAI; three strains were cagA+/cagE-/VirD4- and another strain was cagA-/cagE+/VirD4+. Antibodies to CagA was positive in 33 patients (55.0%) by ELISA and in 46 patients by immunoblot assay (76.7%).

Table 1 shows the results of CagA serology and diversity of cagPAI. Although the sensitivity was relatively low, ELISA did not detect anti-CagA antibody in cagA-negative strains and the accuracy of ELISA to predict bacterial possession of cagA was 61.7%. In six patients infected with cagE and VirD4-negative strain, two were CagA seropositive. Thus, the accuracy of CagA ELISA to define cagE and VirD4 positivity was only 58.3%. The immunoblot assay showed relatively
higher sensitivity and showed better accuracy (76.7%) than ELISA. However, the high sensitivity of immunoblot assay was associated with false positive results. There were seven patients infected with *H. pylori* without intact cagPAI, and their individual results of CagA serology and diversity of cagPAI were shown in Table 2. In two patients with cagA positive infection, both serological assays determined cagA-positivity correctly, while the strains did not possess both cagE and VirD4. In a patient with cagA-/cagE+/VirD4+ infection, immunoblot assay showed a positive result.

### DISCUSSION

Since early studies had demonstrated the association between gastric inflammation and the presence of CagA, a number of studies have implicated the virulence of this protein. CagA is now recognized as a protein encoded by the cagA gene, which is one of the genes inside the cagPAI. Recent studies have shown that CagA protein is delivered into gastric epithelial cells through the type IV secretion system. Phosphorylated CagA forms a physical complex with a tyrosine phosphatase SHP-2 and perturbs cellular functions by deregulating SHP-2 activity[8]. Other genes inside the cagPAI, which encode the type IV secretion system, play a role in gastric mucosal inflammation. VirD4 is recognized as a possible adaptor to guide CagA into the secretion system and necessary for the virulence of CagA[9]. On the other hand, there are other virulent functions, which are independent of CagA-VirD4. Inactivation of cagE but not cagA causes a marked reduction of the ability of *H. pylori* to stimulate gastric epithelial IL-8 production[7,20]. A recent study also showed the association between cagE status and NF-κB activation *in vitro*, although genes inside the cagPAI have different pathogenic mechanisms, presence of anti-CagA antibody has been only one serological test to indicate the virulence of cagPAI. Furthermore, CagA serology has been usually used without examining the structure of cagPAI. Although most Japanese *H. pylori* strains possess cagPAI, some strains lack cagPAI totally or partially[14,22]. Therefore, it was necessary to examine whether CagA serology can indicate the structure of cagPAI accurately in Japan. In the present study, only 3 out of 60 (5%) *H. pylori*

### Table 1 CagA serology and bacterial phenotypes

| cagA | cagE and VirD4 | Serology | ELISA       | Immunoblot |
|------|----------------|----------|-------------|------------|
| ELISA| Positive       | Negative | Positive    | Positive   |
| Positive | 33  | 23         | 31  | 23         |
| Negative | 0   | 4          | 2   | 4          |
| Accuracy (%) | 61.7 | 58.3          |
| Immunoblot| Positive | 44 | 12        | 43 | 11        |
| Negative | 2   | 2          | 3   | 3          |
| Accuracy (%) | 76.7 | 76.7          |

### Table 2 CagA serology in seven patients with *H. pylori* without intact cagPAI

| (Age,sex) | cagPAI | cagE and VirD4 | Serology | ELISA | Immunoblot |
|-----------|--------|----------------|----------|-------|------------|
| Patient A (73, F) | Positive | Negative       | Positive | Positive | Positive |
| Patient B (83, M) | Negative | Positive       | Negative | Negative | Negative |
| Patient C (74, F) | Positive | Negative       | Negative | Negative | Negative |
| Patient D (64, F) | Negative | Positive       | Negative | Positive | Positive |
| Patient E (62, F) | Negative | Negative       | Negative | Negative | Negative |
| Patient F (51, F) | Negative | Negative       | Negative | Negative | Negative |
| Patient G (69, M) | Negative | Negative       | Negative | Negative | Negative |

### Table 3 Grade of gastritis in CagA seropositive and negative patients

| Grade | ELISA | Western blot |
|-------|-------|--------------|
| Atrophy | 0   | 0       |
| PMN    | 0   | 0       |
| H pylori | 0   | 0       |
| Atrophy | 3   | 3       |
| PMN    | 3   | 3       |
| H pylori | 3   | 3       |

MNC, mononuclear cells; PMN, polymorphonuclear cells. Histological grades are 0: none, 1: mild, 2: moderate, and 3: marked. *P<0.05, **P<0.01* (when seropositivity was defined by Western blot).
isolates lack all of tested genes inside the cagPAI. The proportion of cagPAI possession was consistent with previous studies in Japan[^23^]. CagA ELISA was negative in these three patients, while immunoblot assay showed a false positive result in one patient. Immunoblot assay has been shown to have a better sensitivity than ELISA to detect antibody to CagA[^24^] and better sensitivity was also observed in the present results. However, high sensitivity sometimes cause false positive results. In another patient, who was infected with H pylori of cagA+ / cagE+ /virD4+ genotype, anti-CagA antibody was detected by only immunoblot assay. However, it is possible that PCR might demonstrate the presence of cagA in this patient, if cagA was examined by different primers. In three patients infected with H pylori of cagA+ /cagE-/ virD4- genotype, both ELISA and immunoblot assay were positive in two patients. Thus, both serological methods may be inadequate to indicate the virulence of the genes inside the cagPAI other than cagA.

In 53 patients infected with H pylori, which possess all the three genes tested, CagA seropositivity was significantly lower by ELISA (58.5%) than immunoblot assay (79.2%; P<0.05). The low sensitivity is a major cause of the low accuracy of CagA ELISA in this study. The recombinant CagA antigen used in ELISA may affect sensitivity. Basso et al[^23^], compared four different ELISA methods to detect serum anti-CagA antibody and showed different sensitivity, which varied from 79% to 100%. The difference of CagA antigen has caused controversial results of the association between CagA serology and gastric cancer in Japanese patients infected with H pylori. Three studies have examined the association by ELISA using different CagA antigen and two of them found significant association while another did not[^24-26,27^]. Therefore, before examining anti-CagA antibody by ELISA, sensitivity and specificity should be carefully investigated. Furthermore, when infected H pylori is cagA positive but lacks cagPAI partially, it is more questionable to indicate the virulence of the cagPAI by ELISA.

We also studied the association between histological gastritis and CagA seropositivity in patients infected with cagA-positive H pylori. Significantly lower degree of MNC infiltration was observed in CagA seronegative patients, whereas both bacterial density and glandular atrophy were not different between groups. Thus, recognition of CagA by host, but not CagA expression by bacteria, could be a relevant factor for CagA seropositivity. Prevalence of anti-H pylori antibody was similar in the two groups (anti-H pylori antibody was negative in only one CagA seropositive patient). It follows that host recognition of H pylori did not affect gastric mucosal inflammatory infiltration in patients infected with cagA-positive H pylori. The results were in consistent with general understanding that CagA seropositivity is associated with enhanced gastritis.

In conclusion, immunoblot assay showed a better sensitivity to indicate bacterial possession of cagA than ELISA in this series of patients. However, in this study, both ELISA and immunoblot assay for anti-CagA antibody were unreliable to indicate the virulence of cagPAI, particularly in strains, which lack genes inside the cagPAI other than cagA. Although, in Asia, some serological tests have shown that the CagA seropositivity in patients with H pylori infection up to 90%, serodiagnosis of CagA requires careful evaluation of their accuracy before use. Analysis of bacterial genotype via isolation of H pylori from endoscopic gastric biopsy specimens is recommended to determine the presence of genes inside the cagPAI and its virulence.

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