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ORIGINAL INVESTIGATION

Characterization of a novel *Helicobacter pylori* East Asian-type CagA ELISA for detecting patients infected with various *cagA* genotypes

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Abstract

Currently, Western-type CagA is used in most commercial *Helicobacter pylori* CagA ELISA kits for CagA detection rather than East Asian-type CagA. We evaluated the ability of the East Asian-type CagA ELISA developed by our group to detect anti-CagA antibody in patients infected with different *cagA* genotypes of *H. pylori* from four different countries in South Asia and Southeast Asia. The recombinant CagA protein was expressed and later purified using GST-tag affinity chromatography. The East Asian-type CagA-immobilized ELISA was used to measure the levels of anti-CagA antibody in 750 serum samples from Bhutan, Indonesia, Myanmar, and Bangladesh. The cutoff value of the serum antibody in each country was determined via Receiver-Operating Characteristic (ROC) analysis. The cutoff values were different among the four countries studied (Bhutan, 18.16 U/mL; Indonesia, 6.01 U/mL; Myanmar, 10.57 U/mL; and Bangladesh, 6.19 U/mL). Our ELISA had better sensitivity, specificity, and accuracy of anti-CagA antibody detection in subjects predominantly infected with East Asian-type CagA *H. pylori* (Bhutan and Indonesia) than in those infected with Western-type CagA *H. pylori* predominant (Myanmar and Bangladesh). We found positive correlations between the anti-CagA antibody and antral monocyte infiltration in subjects from all four countries. There was no significant association between bacterial density and the anti-CagA antibody in the antrum or the corpus. The East Asian-type CagA ELISA had improved detection of the anti-CagA antibody in subjects infected with East Asian-type CagA *H. pylori*. The East Asian-type CagA ELISA should, therefore, be used in populations predominantly infected with East Asian-type CagA.

Keywords East Asian-type CagA · ELISA · *Helicobacter pylori* · Anti-CagA antibody

Introduction

Since past 4 decades, *Helicobacter pylori* has become the most studied bacterial pathogen in the human stomach. *H. pylori* is a causative agent of several gastroduodenal diseases, such as chronic gastritis, peptic ulcers, and gastric cancer [1, 2]. The capability of *H. pylori* to induce the development of gastroduodenal diseases is strongly associated with cytotoxin-associated gene A (CagA) [3]. The translocated CagA is reported to be able to activate or inactivate multiple host signaling cascades either in a phosphorylation-dependent or phosphorylation-independent way [4–6].

Numerous repeat sequences exist within the 3′ regions of *cagA*, and the C-terminus of CagA possesses a variable number of tyrosine phosphorylation sites located within the Glu-Pro-Ile-Tyr-Ala (EPIYA) motif [3]. The presence of repeat sequences results in various sizes of the CagA protein in different strains [7] and classification of the protein is generally divided into Western-type and East Asian-type CagA [8]; they possess EPIYA-C and EPIYA-D segments, respectively. The binding capability of EPIYA-D to the proto-oncogenic SH2-domain-containing tyrosine phosphatase (SHP2) was reported to be stronger than that of EPIYA-C, leading

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to hyper-stimulation of Ras-Erk signaling [9–11]. Therefore, the East Asian-type CagA is associated with greater virulence than the Western-type CagA.

The anti-CagA antibody level may be dependent on whether CagA is East Asian-type or Western-type, which will dictate the type of serologic assay to be used [12]. Although the ELISA system using immobilized antigen derived from Western-type CagA is widely commercially available, there was no ELISA system that used antigen derived from East Asian-type CagA [12]. Our previous home-made East Asian-type CagA ELISA had comparable performance to commercial Western-type CagA-based ELISA in Vietnam [13]. However, considering the high variation of *cagA* genotypes, it is important to examine the capability of the East Asian-type CagA ELISA to detect antibody in respect to the genotypic variations of *cagA*. We evaluated the performance of our newly constructed East Asian-type CagA ELISA in detecting the anti-CagA antibody in subjects infected with *H. pylori* possessing various *cagA* genotypes from four different countries in South Asia and Southeast Asia. Furthermore, we analyzed the association between anti-CagA antibody value, *H. pylori* density, and the gastric histological scores.

Materials and methods

Study participants

We used samples from four different countries in South Asia and Southeast Asia as the representative of an East Asian-type CagA predominant countries (Bhutan and Indonesia) [14, 15] and a Western-type CagA predominant countries (Myanmar and Bangladesh) [16, 17]. We performed upper endoscopy on 150 subjects with dyspeptic symptoms in Mawlamyine and Mingaladon City, Myanmar during February 13–17, 2017. The sample population consisted of 98 males and 52 females with a mean age of 47.1 ± 13.0 years (range 17–87 years). In this study, we included samples and data from our previous studies including 372 samples from Bhutan [14, 18] and 133 from Bangladesh [16]. We also included samples and data of 1139 patients from our previous studies in Indonesia [19, 20], including our recent endoscopic surveys held in Palu and Ternate. We performed an upper endoscopy on 100 subjects with dyspeptic symptoms in Palu and Ternate, Indonesia on March 2017. The sample population consisted of 47 males and 53 females with a mean age of 44.5 ± 13.0 years (range 19–83 years) and fasting sera samples were collected immediately after endoscopy. During the endoscopies, we collected four biopsy specimens, including three samples from the lesser curvature of the antrum approximately 2 cm from the pyloric ring and one sample from the greater curvature of the corpus. Each sample from the antrum was used for rapid urease test, *H. pylori* culture, and histological examination. Corpus specimens were used for histological examination. Fasting sera samples were collected immediately after endoscopy session and then were stored at − 20 °C until used.

Ethical approval was obtained from the Ethics Committee of Dr. Soetomo Teaching Hospital (Surabaya, Indonesia), Dr. Cipto Mangunkusumo Teaching Hospital (Jakarta, Indonesia), Bangladesh Medical Research Council (Dhaka, Bangladesh), Defense Services General Hospital (Myanmar), Thammasat University (Pathum Thani, Thailand), and Oita University Faculty of Medicine (Yufu, Japan). A written informed consent was collected before data collection based on the guidelines of the Declaration of Helsinki.

Histology, serology, and culture

Biopsy materials were fixed in 10% formaldehyde neutral buffer (Nacalai Tesque, Japan), followed by paraffin embedding. May–Grünwald–Giemsa and hematoxylin–eosin stains were applied to 5-μm slices of paraffin-embedded biopsy. On the basis of the updated Sydney system, an experienced pathologist (TU) assessed the degree of inflammation, atrophy, and bacterial density in each specimen and assigned each to one of four grades: 0, normal; 1, mild; 2, moderate; and 3, marked [21]. Serological tests were performed to determine the presence of *H. pylori* infection by measuring the anti-*H. pylori* antibody levels using an E-plate (Eiken Co., Ltd., Tokyo, Japan).

Helicobacter pylori culture was performed as previously described [22]. Briefly, biopsy specimens were homogenized in normal saline and streaked onto *H. pylori* selective media (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The plates were incubated for up to 10 days at 37 °C under microaerophilic conditions (10% O_2, 5% CO_2, and 85% N_2). The *H. pylori* colonies were sub-cultured onto Brucella Agar medium (Becton–Dickinson, Sparks, MD, USA) supplemented with 7% horse blood (Nippon Bio-test, Tokyo, Japan) without antibiotics. *H. pylori* was identified on the basis of bacteria morphology, Gram-negative staining result, and positive result of oxidase, urease, and catalase test. Isolated strains were stored at − 80 °C in Brucella Broth (Becton–Dickinson, Sparks, MD, USA) containing 10% glycerol and 10% horse serum.

The *H. pylori*-positive status was determined via *H. pylori* culture. However, *H. pylori*-negative status was defined as negative via *H. pylori* culture, rapid urease test, presence of *H. pylori* antibody in the serum, and histopathological examination.

cagA genotyping

Helicobacter pylori DNA was extracted using a commercially available kit (QIAGEN, Santa Clarita, CA). The conserved *cagA* gene was amplified by polymerase chain reaction (PCR) as previously described [23]. The absence of *cagA* was confirmed by the presence of a *cagA* empty site, as previously described [24]. The CagA type was confirmed by sequencing (e.g., East Asian-type, Western-type, or ABB-type). Direct DNA sequencing was performed using the AB 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The EPIYA segment types of CagA were defined as described previously [5, 8, 15, 25]. In this study, we also utilized the *cagA* sequences from our previous studies in Bhutan [14], Indonesia [15, 25, 26], and Bangladesh [16]. Additionally, we also analyzed the CagA N-terminus region [27, 28] to confirm the differences of N-terminus region of East Asian-type and Western-type CagA strains. N-terminus region was defined as amino acid from start codon up to just before EPIYA-A segment. We obtained the CagA N-terminus region sequences of several selected strains from four countries by performing BLAST search of the *cagA* against our next-generation sequencing database for the samples obtained from Myanmar and Bhutan, whereas the samples from Bangladesh and Indonesia were obtained from our previous studies [16, 25]. We constructed a phylogenetic tree based on N-terminus region of CagA protein sequence using poison model implemented in Mega 7 [29], with the addition of several reference strains obtained from GenBank.

Recombinant East Asian-type CagA preparation

The full-length *cagA* gene was amplified from the genomic DNA of clinical *H. pylori* isolated from a Japanese gastritis patient. The *cagA*-containing plasmid (pGEX-6P-1/*cagA)* was constructed using previously described methods [13]. The CagA-expressing plasmid pGEX-6P-1/*cagA* was transformed into the *E. coli* Rosetta Blue DE3 pLysS expression strain competent cells (Merck Millipore, Germany). The cells were cultured in Luria–Bertani liquid medium supplemented with carbenicillin (final concentration 100 µg/mL), chloramphenicol (final concentration 34 µg/mL), and glucose (final concentration 0.2%) at 30 °C until grown to an OD_{600} of 0.7. Isopropyl β-D-1-thiogalactopyranoside (final concentration 0.4 mM) was added to induce the expression of glutathione sulfate-transferase (GST) tag-fused recombinant CagA, and the culturing process was continued for 2 h at 30 °C. Recombinant CagA was purified using Glutathione Sepharose 4B (GE Healthcare) and was separated from the GST-tag using PreScission Protease enzyme (GE Healthcare) following the manufacturer's instruction. The newly purified protein was confirmed via SDS-PAGE and western blotting using anti-CagA rabbit polyclonal antibody (Austral Lineas Aereas), as well as the anti-CagA m24 peptide rabbit polyclonal antibody described in the East Asian-type CagA ELISA section.

East Asian-type CagA ELISA

ELISA was used to measure anti-CagA antibody according to our previous work [13] with modifications to the protocol. We developed an anti-CagA m24 peptide rabbit IgG antibody to be used as a standard curve. The m24 peptide (QKITDKVDNLNQAVSETKL) is located within the middle region of CagA protein and has been previously reported [30]. Using a 20mer-m24N peptide (NQKITDKVDNLNQAVSETKL), anti-CagA m24 peptide rabbit IgG antibody was generated, and then purified using m24N peptide affinity column chromatography (Sigma, St. Louis, USA) [31].

The purified recombinant East Asian-type CagA (0.1 µg/well) in 50 mM carbonate/bicarbonate buffer solution (pH 9.6) was immobilized overnight on a Maxisorp Loose ELISA plate (Thermo Fisher Scientific, Denmark). The East Asian-type CagA-immobilized plate was blocked with 2% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at 20–25 °C. Human serum samples (1:1000 dilution) were applied to the well and allowed to react with the immobilized recombinant CagA for 30 min at 20–25 °C. To generate the standard curve of antibody detection in the serum samples, serially diluted anti-CagA m24 peptide rabbit IgG was used as the primary antibody simultaneously in the recombinant East Asian-type CagA-immobilized 96-well plate. The plate was washed with wash buffer (PBS supplemented with 0.1% Tween-20 as final concentration). Anti-human IgG conjugated with horseradish peroxidase (HRP) (anti-human IgG-HRP; Jackson Immuno Research Labs) and anti-rabbit IgG conjugated with HRP (anti-rabbit IgG-HRP; Jackson Immuno Research Labs) were then applied to the sample and standard curve wells, followed by a 30-min incubation at 20–25 °C. After the plate was washed with wash buffer, ELISA peroxidase substrate (TMB; Nacalai Tesque, Japan) was added for the coupling reaction (10 min at 20–25 °C). Sulfuric acid was then added to stop the reaction and the absorbance was measured at 450 nm. The amount of anti-CagA antibody in human serum was calculated by applying the absorbance to the standard curve. The anti-CagA antibody calculated was then multiplied to the dilution factor to obtain the concentration of undiluted sample. In this study, we defined that 1 U/mL anti-CagA antibody from human was comparable to 1 µg/mL anti-CagA rabbit IgG.

Statistical analysis

Discrete variables were tested using the Chi square test. Continuous variables were tested using the Mann–Whitney

U. The cutoff value for the antibody levels measured via East Asian-type CagA ELISA was calculated using Receiver-Operating Characteristic (ROC) analysis. Spearman rank correlation model was used to determine the association between anti-CagA antibody, bacterial density, and histological score. Statistical significance was determined when the *P* value was less than 0.05. The statistical analysis was performed using the SPSS statistical software package version 23.0 (IBM Corp., Armonk, NY, USA).

Nucleotide sequencing

Nucleotide sequences data obtained in this study are available under the DNA Data Bank of Japan (DDBJ) accession numbers LC471224-LC471291, LC497423 (CagA EPIYA region in C-terminus), and LC497060-LC497072 (CagA N-terminus). The detailed information of the strains obtained in this study and the accession numbers are shown in the Supplementary Table 1.

Results

Samples characteristics

We isolated 65 strains from 150 gastric biopsies from a new survey at Mawlamyine and Mingaladon City, and 7 strains were isolated from 100 gastric biopsies from patients in Palu and Ternate. We used serum samples from culture-positive subjects, and excluded subjects who were culture negative, but histopathology or serology positive. We also excluded subjects with no serum and without complete histological data. Based on those criteria, 74 Bhutan samples, 13 Myanmar samples, and 12 Bangladesh samples were excluded. Given that Indonesia is a country with low prevalence of *H. pylori* infection [22], we utilized 56 *H. pylori* positive subjects from our previous studies [19], 7 *H. pylori*-positive from Palu and Ternate, and 131 *H. pylori* negative, which were randomly selected from among the Indonesian *H. pylori*-negative population. Finally, a total of 750 samples were further analyzed, and the demographic data and clinical outcomes of the subjects in each country are shown in Table 1.

CagA types in each country

Table 2 shows the CagA types in each country. Among the 383 isolated strains, PCR analysis revealed that 365 (95.3%) contained *cagA*, including 199 (100%) from Bhutan, 61 (96.8%) from Indonesia, 64 (98.5%) from Myanmar, and 41 (73.2%) from Bangladesh. There were also 18 *cagA*-negative strains: 2 from Indonesia, 1 from Myanmar, and 15 from Bangladesh.

Sequence analysis showed that infected patients from Myanmar were predominant for the Western-type CagA, with 12 strains containing multiple EPIYA-C motifs (ABCC) and only 7.8% (5/64) were East Asian-type CagA. All Bangladesh samples were Western-type CagA. The East Asian-type CagA were high in Bhutan (92.0%) and moderate in Indonesia (54.1%). There were 8 Indonesia strains

Table 1 Demographic data and clinical outcome of subjects

Characteristic	Country				Total (%)
	Bhutan (%)	Indonesia (%)	Myanmar (%)	Bangladesh (%)	
Total samples					
n	298	194	137	121	750 (100)
H. pylori status[a]					
Positive	199	63[b]	65	56	383 (51.1)
Negative	99	131[b]	72	65	367 (48.9)
Sex					
Male	128 (43.0)	118 (60.8)	90 (65.7)	55 (45.5)	391 (52.1)
Female	170 (57.0)	76 (37.3)	47 (34.3)	66 (54.5)	359 (47.9)
Age					
Mean ± SD	39.1 ± 15.0	46.7 ± 13.6	47.3 ± 13.1	37.1 ± 12.5	42.2 ± 14.5
Disease					
Gastritis	249 (83.6)	166 (85.6)	99 (72.3)	114 (94.2)	628 (83.7)
Gastric ulcer	23 (7.7)	16 (8.2)	6 (4.4)	5 (4.1)	50 (6.7)
Duodenal ulcer	20 (6.7)	1 (0.5)	26 (19.0)	2 (1.7)	49 (6.5)
Gastric cancer	3 (1.0)	1 (0.5)	2 (1.5)	0 (0.0)	6 (0.8)
Reflux esophagitis	3 (1.0)	10 (5.2)	4 (2.9)	0 (0.0)	17 (2.3)

[a]Positive status was determined by culture method

[b]This number does not represent the actual prevalence of *H. pylori* infection in Indonesia

Table 2 CagA types in each country

CagA type	Country				Total
	Bhutan[a]	Indonesia[a]	Myanmar	Bangladesh[a]	
CagA status					
Positive	199	61	64	41	365
Negative	0	2	1	15	18
Total	199	63	65	56	383
CagA type (%)					
East Asian type	183 (92.0)	33 (54.1)	5 (7.8)	0	221
Western type	15 (7.5)	20 (32.8)	57 (89.1)	41 (100)	133
ABB type[b]	0	8 (13.1)	0	0	8
Undetermined	1 (0.5)	0	2 (3.1)	0	3
Total	199	61	64	41	365
East Asian type (%)					
ABD	81 (44.3)	29 (87.9)	4 (80.0)	0	114
ABBD	98 (53.5)	4 (12.1)	1 (20.0)	0	103
ABBBD	2 (1.2)	0	0	0	2
BD	2 (1.2)	0	0	0	2
Total	183	33	5	0	221
Western type (%)					
ABC	13 (86.7)	15 (75.0)	42 (73.7)	27 (65.9)	97
ABCC	0	0	12 (21.1)	8 (19.5)	20
ABCCC	0	0	0	2 (4.9)	2
ABBC	0	0	0	1 (2.4)	1
AB	1 (6.7)	1 (5.3)	1 (1.8)	3 (7.3)	6
BC	0	4 (21.0)	1 (1.8)	0	5
AC	1 (6.7)	0	1 (1.8)	0	2
Total	15	20	57	41	133

[a]These data were analyzed from our previous studies in Bhutan [14], Bangladesh [16], and Indonesia [15, 25, 26] with the addition of 7 *H. pylori* strains isolated from Palu and Ternate

[b]ABB-type is a unique CagA type found in Papua island [15, 25]

possessing ABB-type CagA. Two strains from Myanmar and one from Bhutan were PCR positive, but insufficient sequencing results prompted us to categorize these strains as undetermined-type CagA.

East Asian-type CagA ELISA performance

Table 3 shows the anti-CagA antibody value of the *cagA*-positive *H. pylori*-infected subjects. The median value of the anti-CagA antibody was higher in Bhutan (52.0 U/mL) than in the other three countries, while the median values in Indonesia, Myanmar, and Bangladesh were highly similar (18.8 U/mL, 18.7 U/mL, and 16.5 U/mL, respectively). The anti-CagA antibody levels in Bhutan were significantly higher than those in the other three countries ($P < 0.05$ for all). In Bhutan and Indonesia, the median value of the anti-CagA antibody of East Asian-type CagA *H. pylori*-infected subjects was higher than that of Western-type CagA *H. pylori*-infected subjects.

Table 3 The anti-CagA antibody values of *H. pylori*-positive subjects infected with different CagA types in 4 countries

Anti-CagA antibody value (U/mL)	Country			
	Bhutan	Indonesia	Myanmar	Bangladesh
All types				
n	199	61	64	41
Median	52.0	18.8	18.7	16.5
Range	0.0–180.5	0.0–145.8	0.0–113.2	0.0–147.4
East Asian type				
n	183	33	5	0
Median	54.4	24.2	11.0	–
Range	0.0–180.5	0.0–145.8	1.6–28.3	–
Western type				
n	15	20	57	41
Median	48.9	13.2	19.1	16.5
Range	0.0–153.8	0.0–118.6	0.0–113.2	0.0–147.4

ROC analysis was performed on both the positive and negative groups (Table 4). The positive group comprised subjects infected with *cagA*-positive *H. pylori*, while the negative group comprised *H. pylori*-uninfected and *cagA*-negative *H. pylori*-infected subjects. The area under the curve (AUC) and cutoff values were different among countries. The cutoff value in Bhutan was 18.16 U/mL, with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy values of 89.4%, 90.9%, 95.2%, 81.1%, and 89.9%, respectively (AUC 0.940; 95% CI 0.910–0.970). The results in Bhutan highlight that the East Asian-type CagA ELISA showed better detection of anti-CagA antibody in East Asian-type CagA-infected subjects than in Western-type CagA-infected subjects, as demonstrated by better sensitivity (91.3% vs. 86.7%), specificity (90.9% vs. 81.8%), and accuracy (91.1% vs. 82.5%), respectively.

In Indonesia, with all CagA-type-infected subjects included in the positive group for the ROC analysis, we found that the cutoff value was 6.01 U/mL with the sensitivity, specificity, PPV, NPV, and accuracy values being 85.2%, 88.7%, 77.6%, 92.9%, and 87.6%, respectively (AUC 0.921; 95% CI 0.875–0.967). Similar to Bhutan, our result in Indonesia also showed that East Asian-type CagA ELISA had better detection of anti-CagA antibody in East Asian-type CagA-infected subjects than Western-type CagA-infected subjects with better sensitivity (90.9% vs. 75.0%), specificity (90.2% vs. 88.7%), and accuracy (90.4% vs. 86.9%), respectively.

Table 4 East Asian-type CagA ELISA performance in each country

CagA types	*n*	AUC[a]	95% CI	Cutoff (U/mL)	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)	Accuracy (%)
Bhutan									
All types	199	0.940	0.910–0.970	18.16	89.4	90.9	95.2	81.1	89.9
EAT	183	0.944	0.915–0.974	18.16	91.3	90.9	94.9	84.9	91.1
WT	15	0.884	0.773–0.995	12.30	86.7	81.8	41.9	97.6	82.5
Indonesia									
All types	61	0.921	0.875–0.967	6.01	85.2	88.7	77.6	92.9	87.6
EAT	33	0.947	0.898–0.995	7.04	90.9	90.2	69.8	97.6	90.4
WT	20	0.862	0.759–0.965	6.01	75.0	88.7	50.0	95.9	86.9
Myanmar									
All types	64	0.807	0.732–0.882	10.57	70.3	84.9	80.4	76.5	78.1
WT	57	0.818	0.742–0.894	10.57	71.9	84.9	78.8	79.5	79.2
Bangladesh									
All types (WT)	41	0.851	0.780–0.922	6.19	82.9	76.3	64.2	89.7	78.5

EAT East Asian type, *WT* Western type

[a]AUC was calculated from the negative group (*H. pylori*-uninfected subjects and *cagA*-negative *H. pylori*-infected subjects) in each respective country: 99 Bhutan samples, 133 Indonesian samples, 73 Myanmar samples, and 80 Bangladesh samples

The cutoff value in Myanmar subjects was lower than that in Bhutan subjects (10.57 U/mL vs. 18.16 U/mL) but higher than that in Indonesia subjects (10.57 U/mL vs. 6.01 U/mL). The sensitivity, specificity, PPV, NPV, and accuracy of the ELISA in Myanmar subjects were 70.3%, 84.9%, 80.4%, 76.5%, and 78.1%, respectively (AUC 0.807; 95% CI 0.732–0.882). Bangladesh subjects had a cutoff value of 6.19 U/mL with sensitivity, specificity, PPV, NPV, and accuracy of 82.9%, 76.3%, 64.2%, 89.7%, and 78.5%, respectively (AUC 0.851; 95% CI 0.780–0.922).

False-negative and false-positive analyses

To confirm the capabilities of the ELISA developed by our group, we also analyzed the false-negative subjects. There were 56 false-negative subjects, including 21 (10.6%) from Bhutan, 9 (14.8%) from Indonesia, 19 (29.7%) from Myanmar, and 7 (17.1%) from Bangladesh (Table 5). The number of false negatives may be attributed to the difference in the CagA types tested by the East Asian-type CagA ELISA and, therefore, we analyzed these false-negatives based on the CagA typing.

In Bhutan, the East Asian-type CagA ELISA had a lower rate of false negatives when detecting antibody from subjects infected with East Asian-type CagA *H. pylori* (16/183, 8.7%) than from subjects infected with Western-type CagA (5/15, 33.3%). Interestingly, in the East Asian-type CagA, subjects infected with ABBD-type CagA had a lower false-negative rate (5/98, 5.1%) than the typical ABD-type CagA (11/81, 13.5). In Indonesia, three false-negative subjects were infected with East Asian-type CagA, five were infected with Western-type CagA, and one was infected with ABB-type CagA. A similar pattern to that of Bhutan was observed in Indonesia, in which the East Asian-type CagA ELISA had a higher rate of false negatives in subjects infected with Western-type CagA (5/20, 25.0%) than East Asian-type CagA (3/33, 9.1%). However, the opposite result was observed in Myanmar (East Asian-type: 2/5, 40.0% vs. Western-type: 16/57, 28.1%).

Overall, we identified 56 (15.3%) false-negative samples in four countries. Specifically, 21 (9.5%) of the subjects with false-negatives were infected with the East Asian-type CagA, 33 subjects (24.8%) infected with Western-type CagA, one subject (12.5%) was infected with ABB-type CagA, and one subject (33.3%) was infected with an undetermined-type CagA. Our results showed that false negatives were more frequent in Western-type CagA-infected subjects than in East Asian-type CagA-infected subjects. In addition, there were 54 false positives, including 15 from Indonesia, 11 from Myanmar, 19 from Bangladesh, and 9 from Bhutan.

Anti-CagA antibody and *H. pylori* density

We analyzed the correlation between the anti-CagA antibody and *H. pylori* density (Table 6). There was no significant association between anti-CagA antibody and the antral *H. pylori* density in Bhutan, Indonesia, Myanmar, and Bangladesh ($P = 0.342$, $P = 0.456$, $P = 0.711$, and $P = 0.428$, respectively). Similar results were also found in the corpus ($P > 0.05$ for all).

Furthermore, we analyzed the correlation with respect to the CagA type. We divided the subjects into two groups depending on whether they were infected with Western-type CagA and East Asian-type and excluded subjects infected

Table 5 CagA types in samples with false-negative result

CagA types	Country				Total
	Bhutan	Indonesia	Myanmar	Bangladesh[a]	
True positive	178	52	45	34	309
False negative	21	9	19	7	56
Total	199	61	64	41	365
False-negative samples					
East Asian type (%)					
ABD	11/81 (13.5)	3/29 (10.3)	2/4 (50.0)	–	16/114 (14.0)
ABBD	5/98 (5.1)	0/4 (0.0)	0/1 (0.0)	–	5/103 (4.8)
Others	0/4 (0.0)	–	–	–	0/4 (0.0)
Total	16/183 (8.7)	3/33 (9.1)	2/5 (40.0)	–	21/221 (9.5)
Western type (%)					
ABC	4/13 (30.7)	2/15 (13.3)	12/42 (28.6)	5/27 (18.5)	23/97 (23.7)
ABCC	–	–	4/12 (33.3)	2/8 (25.0)	6/20 (30.0)
BC	–	3/4 (75.0)	0/1 (0.0)	–	3/5 (60.0)
AC	1/1 (100)	–	0/1 (0.0)	–	1/2 (50.0)
Others	0/1 (0.0)	0/1 (0.0)	0/1 (0.0)	0/6 (0.0)	0/9 (0.0)
Total	5/15 (33.3)	5/20 (25.0)	16/57 (28.1)	7/41 (17.1)	33/133 (24.8)
ABB type (%)	–	1/8 (12.5)	–	–	1/8 (12.5)
Undetermined (%)	0/1 (0.0)	–	1/2 (50.0)	–	1/3 (33.3)
Total false negative	21/199 (10.6)	9/61 (14.8)	19/64 (29.7)	7/41 (17.1)	56/365 (15.3)

[a]No East Asian-type CagA was found in Bangladesh

Table 6 Association between anti-CagA antibody level, *H. pylori* density, and histological score

Country	*n*	*Helicobacter pylori* density						Monocyte infiltration					
		Antrum			Corpus			Antrum			Corpus		
		Median	*P* value	*r*	Median	*P* value	*r*	Median	*P* value	*r*	Median	*P* value	*r*
All types													
Bhutan	199	2	0.342	− 0.068	2	0.513	− 0.047	2	0.084	0.123	1	0.109	0.114
Indonesia	61	1	0.456	0.097	1	0.553	− 0.077	2	0.002*	0.388	1	0.231	− 0.156
Myanmar	64	1	0.711	− 0.047	1	0.179	0.170	1	0.407	0.105	1	0.044*	0.253
Bangladesh	41	2	0.428	0.127	1	0.676	− 0.067	2	0.030*	0.340	1	0.260	0.180
EAT[a]													
Bhutan	183	2	0.075	− 0.132	2	0.394	− 0.063	2	0.318	0.074	1	0.266	0.083
Indonesia	33	1	0.972	0.006	1	0.749	− 0.058	2	0.016*	0.417	1	0.337	0.172
WT													
Bhutan	15	2	0.031*	0.558	2	0.559	0.148	2	0.008*	0.654	1	0.215	0.340
Indonesia	20	1	0.117	0.362	1	0.816	− 0.056	2	0.047*	0.449	1	0.046*	− 0.451
Myanmar	57	1	0.625	− 0.066	1	0.271	0.148	1	0.299	0.140	1	0.025*	0.298
Bangladesh	41	2	0.428	0.127	1	0.676	− 0.067	2	0.030*	0.340	1	0.260	0.180

EAT East Asian type, *WT* Western type

*$P < 0.05$

[a]No East Asian-type CagA was found in Bangladesh and there were only 5 subjects infected with East Asian-type CagA *H. pylori* in Myanmar

with ABB-type CagA and undetermined-type CagA. There was a significant association between the anti-CagA antibody in subjects infected with Western-type CagA and the antral *H. pylori* density in Bhutan ($P = 0.031$, $r = 0.558$). However, there was no significant association between the anti-CagA antibody in subjects infected with Western-type CagA and the antral *H. pylori* density in Indonesia, Myanmar, and Bangladesh (P = 0.117, $P = 0.625$, $P = 0.428$,

respectively). There was no significant association between the anti-CagA antibody in subjects infected with the Western-type CagA and the corporal *H. pylori* density in four countries ($P > 0.05$). In subjects infected with East Asian-type CagA, there was no significant association between anti-CagA antibody and the antral and corporal *H. pylori* density in Indonesia and Bhutan ($P > 0.05$).

Anti-CagA antibody and histological score

The correlation between anti-CagA antibody and the histological score was analyzed (Table 6). We observed that there was a significant positive correlation between anti-CagA antibody and monocyte infiltration in the antrum in Indonesia ($P = 0.002$, $r = 0.388$) and in Bangladesh ($P = 0.030$, $r = 0.340$), and in the corpus in Myanmar ($P = 0.044$, $r = 0.253$).

When we divided the subjects infected with East Asian-type CagA and Western-type CagA, we found a significant correlation between the antral monocyte infiltration and the anti-CagA antibody in subjects infected with East Asian-type CagA in Indonesia ($P = 0.016$, $r = 0.417$). There was no significant correlation between anti-CagA antibody and monocyte infiltration in the antrum or the corpus of subjects infected with East Asian-type CagA in Bhutan ($P > 0.05$).

We found a significant correlation between antral monocyte infiltration and the anti-CagA antibody in subjects infected with Western-type CagA in Bhutan ($P = 0.008$, $r = 0.654$), in Indonesia ($P = 0.047$, $r = 0.449$), and in Bangladesh ($P = 0.030$, $r = 0.340$). We also found a significant correlation between corporal monocyte infiltration and the anti-CagA antibody in subjects infected with Western-type CagA in Indonesia ($P = 0.046$, $r = -0.451$) and in Myanmar ($P = 0.025$, $r = 0.298$).

Discussion

In this study, we evaluated the performance of our newly developed East Asian-type CagA ELISA to detect anti-CagA antibody in subjects infected with different CagA types *H. pylori*. We revealed that the East Asian-type CagA ELISA had improved detection of the anti-CagA antibody in subjects infected with East Asian-type CagA than in those infected with Western-type CagA. Thus, the East Asian-type CagA ELISA is better suited for use in the countries in which the population was predominantly infected with East Asian-type CagA *H. pylori*, such as Indonesia and Bhutan. The findings presented here are in agreement with our previous study in Vietnam [13]. Our results highlight the importance of an ELISA assay using East Asian-type CagA antigen to be used in populations that are predominantly infected with East Asian-type CagA *H. pylori*, such as Japan [32, 33], China [34], Indonesia [15], and Thailand [35].

The false-negative sample analysis also supports the findings presented here. The East Asian-type CagA ELISA had a higher rate of false-negatives when detecting the Western-type CagA *H. pylori*-infected subjects. Interestingly, we found that our East Asian-type CagA ELISA also had the ability to detect antibody in subjects infected with not only the typical ABD-type, but also the non-typical East Asian-type (such as ABBD-, ABBBD-, and BD-type CagA), as highlighted by the low rate of false-negatives. This result indicates that the East Asian-type CagA ELISA was still able to perform well on East Asian-type CagA *H. pylori*-infected subjects, regardless of the variation in CagA motifs. This is an important feature of a diagnostic test to ensure that the test is applicable for a wide variety of samples.

The cutoff value of East Asian-type CagA ELISA was different in four countries, and the cutoff value in Bhutan was higher than in Indonesia, Myanmar, and Bhutan. The high anti-CagA antibody values in Bhutan may be attributed to the strong host recognition toward CagA, resulting in a higher immunological response. A previous study also mentioned that host recognition could be associated with the difference of serum anti-CagA antibody titer [12]. In addition, it was suggested that the high antibody titer against CagA might be caused by differences in the transcription level of the *cagA* gene in the *H. pylori* strain [36]. The differences in the anti-CagA antibody level is not only affected by factors involving the host and pathogenic agent, but can also be related to the environmental factors. For example, the low pH and high salt level had been reported to increase CagA expression [37–39], and subsequently might affect anti-CagA antibody production. Despite the high cutoff value in Bhutan, the sensitivity, specificity, and accuracy levels were high, indicating that the East Asian-type CagA ELISA is suitable to be used in Bhutan.

An accurate standard curve is an important part of the ELISA not only for quantifying the amount of serum antibody, but also for the reproducibility of the test. Our current study used the newly anti-CagA m24 peptide rabbit IgG antibody as the standard curve. This antibody was prepared based on the m24 peptide, which is identical to the m24 middle region of the recombinant East Asian-type CagA. Previous studies reported that the CagA antibodies from children were reactive with the epitopes corresponding to m24 peptide region, which is containing sequences specific only to East Asian-type CagA [30]. The anti-m24 peptide antibody is a complement of and has a strong reaction to our recombinant East Asian-type CagA, thus making it possible to be used as a standard curve in this study. This standard curve system was based on IgG system, and generally it may give different affinity from antibodies reaction in human IgG system. However, this rabbit IgG-based standard curve was

useful to be used as detection control to adjust the variation between each ELISA plate in this study. In the future study, the calibration of human IgG level would be important to ensure the accuracy of the antibody level quantification.

Our data showed that the anti-CagA antibody displayed a trend toward positive correlation with the antral monocyte infiltration, and the same pattern was consistently found in all four countries, regardless the difference of CagA type. This result confirmed our previous studies in Japan using Western-type CagA ELISA and in Vietnam using East Asian-type CagA ELISA [12, 13]. There was no significant association between bacterial density and the anti-CagA antibody in the antrum or the corpus regardless the CagA type, suggesting that bacterial density alone cannot correlate with anti-CagA antibody level. These findings are the current general conclusion with two previous studies in Japan and Vietnam [12, 13]. However, the correlation between the anti-CagA antibody with *H. pylori* density and monocyte infiltration could also be dependent on the compatibility of the used ELISA test for a given infecting *H. pylori* strain.

More than 90% of *H. pylori* strains reported in several countries were *cagA* positive, including in Japan [40–42], South Korea [43, 44], China [45, 46], Indonesia [15], and Bhutan [14]. Additionally, most countries in East Asia and Southeast Asia were infected with East Asian-type CagA *H. pylori*, which supports the use of the East Asian-type CagA ELISA to detect *H. pylori* infection with high sensitivity, specificity, and accuracy. An alternative approach to rapid diagnostic methods based on recombinant CagA such as immunochromatography also warrant further investigation [47]. The high immunogenicity of the CagA antigen [48, 49] also highlights the potential use of this antigen in the development of future *H. pylori* vaccines [50, 51].

There were several limitations in this study. The recombinant protein used as an antigen was a crude mixture of products from the protein purification procedure, which contained not only the full-sized protein, but also protein with certain degree of denaturation. As a result, the protein with denaturation in C-terminus region might be putatively containing no EPIYA motifs, subsequently reduce the amount of antibody measurement for the C-terminus. In addition, although we determined the CagA type by examining the EPIYA repeat region located in the C-terminus, the constructed East Asian-type CagA protein was based on whole CagA residues. Therefore, the N-terminus region of the CagA might also have an important role to the performance of this ELISA system. To understand the importance of the CagA N-terminus (approximately 100 kDa), the phylogenetic tree analysis was performed using only the N-terminus region of several selected *H. pylori* strains isolated from the patients in this study (Supplementary Fig. 1). Interestingly, the phylogenetic tree was separated into two main branches, one (upper branches) consisted of Western-type CagA strains and the other (bottom branches) consisted of East Asian-type CagA strains, including KYJP001 strain that the *cagA* gene was cloned to develop the recombinant protein used in this study. The phylogenetic tree separation was in concordance with the genotyping based on the C-terminus EPIYA repeat region. This result suggests that the performance of the ELISA might also be affected by N-terminus region differences. Further study is needed to confirm the importance of N-terminus region for the ELISA. Moreover, additional efforts to develop full-sized CagA protein and to use it as antigen might be important to increase the performance of ELISA system in the future.

Conclusion

The East Asian-type CagA ELISA display greater capabilities of detecting the anti-CagA antibody in subjects infected with East Asian-type CagA *H. pylori*. These results highlight the importance of using an ELISA assay based on the East Asian-type CagA in a population that is predominantly infected with East Asian-type CagA *H. pylori*.

Author contributions Conceived and designed the experiments: DD and YY. Performed the experiments: DD and YM. Analyzed the data: DD, MM, JA, and YY. Contributed reagents/material/analysis tools: YK, YM, TM, TTY, KH, HA, RV, VM, TR, LT, LAW, KAF, TU, AFS, and YAA. Wrote the paper: DD, MM, JA, and YY.

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Compliance with ethical standards

Conflict of interest No competing interests declared.

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