The Validation of the CagA Typing by Immunohistochemistry: Nationwide Applicability in Indonesia

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Submission date: 03-Jun-2019 12:29AM (UTC+0800)
Submission ID: 1139016553
File name: 2019.06.2_Validation_of_CagA.TKturnitin.accepted.docx (473.79K)
Word count: 5851
Character count: 33630

The Validation of the CagA Typing by Immunohistochemistry: Nationwide Application in Indonesia

Running head: CagA immunohistochemistry validation in Indonesia

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ABSTRACT

Aim: To validate 2 types of antibodies, anti-CagA antibody and anti-East Asian CagA specific antibody (α-EAS antibody) for the determination of CagA status by immunohistochemistry in Indonesia. Additionally, we also confirmed the performance of α-EAS antibody for the detection of East Asian-type CagA *H. pylori*.

Methods: Immunohistochemistry was performed using anti-CagA antibody and α-EAS antibody on total of 1038 gastric biopsy specimens from Indonesian subjects. Immunohistochemistry performance was evaluated with PCR-based sequencing as gold standard.

Results: Immunohistochemistry using anti-CagA antibody had high sensitivity,

specificity, and accuracy (85.7%, 100%, and 98.7%, respectively) for determining CagA status. The α-EAS antibody was not suitable for the purpose of CagA status determination, as it had a low sensitivity (23.5%). High specificity (97.7%) but low sensitivity (40%) and accuracy (65.3%) was observed in α-EAS antibody to detect East Asian-type CagA . Subjects with positive result of immunohistochemistry using anti-CagA antibody had significantly higher monocyte infiltration score in antrum (P <0.001) and corpus (P = 0.009)

Conclusion: The anti-CagA antibody is still suitable to be used in Indonesia for determining the *cagA* status, whilst the α-EAS antibody was not suitable to discriminate East Asian-type *cagA* and non-East Asian-type *cagA* in Indonesia.

Keywords: CagA typing, immunohistochemistry, East Asian-CagA antibody

INTRODUCTION

Helicobacter pylori infection is an important causative factor to the development of wide spectrum of gastrointestinal disease such as gastritis, gastric ulcer, duodenal ulcer and gastric cancer [1-3]. Severity of disease was reported to be associated with the *H. pylori* virulence factors, such as duodenal ulcer promoting factor (dupA), outer inflammatory protein (oipA), and cytotoxin-associated gene A (CagA) [4].

CagA, which encoded by the *cagA* gene, is believed to be one of the most essential *H. pylori* protein related to the gastric mucosal inflammation and therefore, as the most extensively studied *H. pylori* virulence factor. CagA protein is inserted into the host cell by a syringe-like structure called *cag* pathogenicity island (*cag* PAI) type IV secretion system [[5]6, 7]. CagA is structurally differentiated by the presence of repeated five-amino-acid sequence located in the C-terminus, consist of glutamic acid-proline-isoleucine-tyrosine-alanine (EPIYA) [8]. There were several types of EPIYA motifs according to the amino acid downstream to the repeat sequence; EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D. The alignment of these EPIYA motifs is divided into Western-type CagA and East Asian-type CagA and. Western-type CagA is characterized by the continuous order of EPIYA-A, EPIYA-B, and one or more repeats of EPIYA-C. On the other hand, East Asian-type CagA has EPIYA-A and EPIYA-B, followed by EPIYA-D. East Asian-type CagA is associated with more severe inflammation and morphological alterations in gastric mucosa compared to Western-type CagA [9, 10]. We previously reported a unique type of CagA type called ABB-type CagA, which was found in the *H. pylori* strains isolated mainly in Papua Island, Indonesia [11]. The ABB-type CagA is identified by the EPIYA-A and EPIYA-B, but followed by EPIYA-B. Recently, we also reported that there were unique *cagA* genotypes found in Papua Island, possessing AB-

and B- motifs [12]. However, due to the similarity of the B-segment of the AB-type and B-type CagA with the ABB-type CagA, these two genotypes were considered as ABB-type subtypes [12].

The determination of CagA status and genotypes is mainly by polymerase chain reaction and sequencing of the variable EPIYA region in the C-terminus of the *cagA* gene. However, this method is considerably expensive and inaccessible in several areas due to the lack of *H. pylori* culture and genome sequencing facilities. Therefore, immunohistochemistry method might become a valuable tool for determining the CagA status of *H. pylori* infected patients. Anti-East Asian CagA specific antibody (α-EAS) which specific for the East Asian-type CagA was previously developed [13]. This antibody is reported to be useful for the purpose of detecting East Asian-type CagA immunohistochemically in East Asian and South East Asian countries, such as Japan [14, 15], Thailand and Vietnam [16].

Indonesia is a country consist of thousands of ethnics and generally had low *H. pylori* prevalence. However several ethnics had high *H. pylori* infection prevalence, such as Batak, Papuan, and Buginese [17]. Unfortunately, currently there are only 313 hospitals with endoscopy systems and there were only a very few centers with *H. pylori* culture and genome sequencing facilities, mainly located in the main island, Java [18]. An easier and quicker method following endoscopy to help ascertain CagA status is needed. Therefore, immunohistochemistry may become a valuable method for clinicians. Here we aimed to validate 2 types of antibodies, anti-CagA antibody and α-EAS antibody to determine CagA status by immunohistochemistry method in a nationwide scale in Indonesia. Additionally, we also validated α-EAS antibody for detecting East Asian-type CagA *H. pylori* in Indonesia.

MATERIALS AND METHODS

Study participants

A nation-wide, cross sectional and multicenter study was performed in 19 cities in Indonesia from August 2012–March 2017. Dyspeptic patients were consecutively enlisted and upper endoscopy examination was performed while excluding patients with the history of partial/total gastrectomy, *H. pylori* eradication, and contraindication for endoscopy examination. A total number of 1,236 dyspeptic patients were recruited; 849 of them were stated in our previous studies [11, 17, 19, 20], 387 of them were new recruited patients with dyspepsia from Gunungsitoli (n=32), Padang (n=33), Kolaka (n=50), Samosir District (n=47), Palembang (n=38), Palu (n=56), Cimacan (n=22), Surabaya (n=22), Merauke (n=43), and Ternate (n=44) (**Fig. 1**). However, 97 samples from Malang were excluded due to inadequate biopsy specimen to analyze. We also excluded patients without complete histological data and/or culture yielded negative results, but histology or immunohistochemistry were positive and samples with insufficient sequencing result. Finally, 1,038 samples were used for further analysis in this study. Antrum and corpus biopsy specimen were used for the histology analysis; antrum biopsy specimen were used for the *H. pylori* culture.

Ethical approval was obtained from the Ethics Committee of Dr. Soetomo Teaching Hospital (Surabaya, Indonesia), Dr. Cipto Mangunkusumo Teaching Hospital (Jakarta, Indonesia) and Oita University Faculty of Medicine (Yufu, Japan). A written informed consent was collected based on the guidelines of the Declaration of Helsinki.

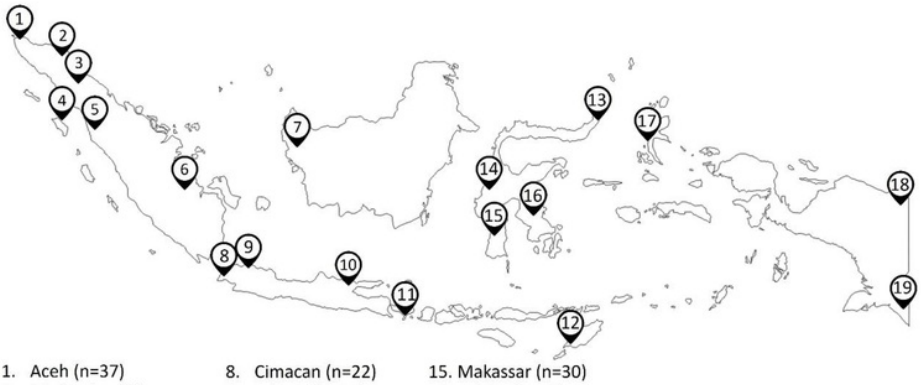

Fig. 1. Upper endoscopic survey was performed in 19 cities across Indonesia.

H. pylori culture and infection status determination

The details of culture method has been reported previously [17]. Briefly, *H. pylori* selective media (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) was used and up to 7 days of incubation in microaerophilic conditions (10% O_2, 5% CO_2, and 85% N_2) at 37°C is necessary to isolates bacteria from a homogenized biopsy specimen. The *H. pylori* colonies were The *H. pylori* colonies were inoculated onto Brucella Agar medium (Becton Dickinson, Sparks, MD, USA) supplemented with 7% horse blood (Nippon Bio-test, Tokyo, Japan) and stored at −80°C in Brucella Broth (Becton Dickinson, Sparks, MD, USA) with 10% glycerol and 10% horse serum. *H. pylori* infection positive status was determined via *H. pylori* culture from antral biopsy

East Asian CagA specific antibody

East Asian CagA antibody (α-EAS) was generated as previously described [13]. Briefly, the East Asian CagA specific polypeptides, AINRKIDRINKIASAGKG was synthetized (OPERON Biotechnologies, Tokyo, Japan). Subcutaneously injection of 1 mg of keyhole limpet hemocyanin (KLH)-conjugated synthetic peptide emulsified (1:1, v/v) with Freund's complete adjuvant was then performed to immunize New Zealand white rabbits. The antisera were collected using the peptide-coupled HiTrap NHS-activated column (Amersham Biosciences, UK).

Immunohistochemistry and histology examination

The immunohistochemistry was done as previously reported [13]. Briefly, we fixed biopsy specimens in 10% (v/v) formaldehyde and followed by paraffin embedding. After cutting the block into approximately 5 µm-thickness tissue section, deparafinization and rehydration procedure was performed. Then, sections were autoclaved at 120°C for 10 minutes in the sodium citrate buffer (10 mmol/L, pH 6.0) (Nichirei, Tokyo, Japan) solution and subsequently cooled down at room temperature. The 3% H_2O_2 solution (v/v) was applied to the sections at room temperature for 10 minutes in order to inactivate the endogenous peroxidase activity, then blocked with 10% goat serum (Nichirei, Tokyo, Japan) for 20 minutes at room temperature. Incubation with 3 types of antibodies, including 1:50 dilution of anti-*H. pylori* Ab (Dako, Glostrup, Denmark) , 1:100 dilution of anti-CagA (b-300) Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 1:4000 dilution of α-EAS Ab was done at 4°C overnight. The next day, the section were washed with PBS, incubated with biotinylated goat anti-rabbit IgG (Nichirei, Tokyo, Japan) for 20 minutes, applied with second PBS washing, and incubated with avidin-conjugated

horseradish peroxidase solution (Vecstatin Elite ABC kit, Vector Laboratories, CA, USA) for 20 minutes . Diaminobenzidine substrate solution was applied for peroxidase activity determination, then counterstained with hematoxylin.

The thin slices of paraffin-embedded biopsy with May-Grünwald-Giemsa and hematoxylin-eosin stains was prepared for histopathology examination. The degree of inflammation, atrophy, and bacterial density based on Updated Sydney system to one of four grades: 0, normal; 1, mild; 2, moderate; and 3, marked [21] was determined by the experienced pathologist (TU).

cagA genotyping

H. pylori genomic DNA was extracted using a DNA extraction kit (QIAGEN, Santa Clarita, CA). The polymerase chain reaction (PCR) method was used to amplified conserved *cagA* gene as previously described [22] and followed by direct sequencing using AB 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Detection of *cagA* empty site by PCR confirmed the absence of *cagA* gene [23]. The *cagA* genotypes based on the sequences of EPIYA segment (East Asian-type, Western-type or ABB-type) was defined as previously described [4, 11, 12].

Statistical analysis

All the statistical test was executed in SPSS statistical software package version 23 (SPSS, Inc., USA). The chi-squared test or Fisher's exact was used to analyze categorical data with P less than 0.05 was accepted as statistically significant. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of immunohistochemistry were evaluated using PCR-based sequencing as gold standard.

The amino acid sequence similarity between the East Asian specific peptide sequence and the amino acid sequences of Indonesian East Asian-type *cagA* strains were analyzed using blast algorithm.

Nucleotide sequencing

Nucleotide sequence data reported are available under the DDBJ accession numbers LC471286-LC471291 (*cagA*).

Results

Study participants

We included a total of 1,038 subjects, including 101 *H. pylori*-positive and 937 *H. pylori*-negative subjects. The demographic data and clinical outcome of the subjects are shown in **Table 1**. Based on ethnicity, the patients were divided into 15 ethnic groups, with Javanese being the most prevalent ethnic (230/1,038, 22.2%) and Kaili being the lowest. Based on endoscopy, most patients had gastritis (961/1,038, 92.6%). There were 76 patients (7.3%) with peptic ulcer disease and only 1 patient with gastric cancer (0.1%).

Table 1. Association between demographic data and clinical outcome

Variable	n	Clinical outcome (%)		
		Gastritis	PUD	Gastric cancer
Total	1038	961 (92.6)	76 (7.3)	1 (0.1)
Sex				
Male	591	548 (92.7)	42 (7.1)	1 (0.2)
Female	447	413 (92.4)	34 (7.6)	0 (0.0)
Age				
<30	126	119 (94.4)	7 (5.6)	0 (0.0)
30-39	226	215 (95.1)	11 (16.5)	0 (0.0)
40-49	256	241 (94.1)	14 (5.5)	1 (0.4)
50-59	243	220 (90.5)	23 (9.5)	0 (0.0)
≥60	187	166 (90.5)	21 (13.7)	0 (0.0)
Ethnic				
Aceh	37	26 (70.3)	11 (29.7)	0 (0.0)
Balinese	61	60 (98.4)	1 (1.6)	0 (0.0)
Batak	96	80 (83.3)	16 (16.7)	0 (0.0)
Bugis	99	96 (97.0)	3 (3.0)	0 (0.0)

Chinese	126	119 (94.4)	7 (5.6)	0 (0.0)
Dayak	47	44 (93.6)	3 (6.4)	0 (0.0)
Javanese	230	216 (93.9)	14 (6.1)	0 (0.0)
Kaili	11	11 (100.0)	0 (0.0)	0 (0.0)
Malay	37	34 (91.9)	3 (8.1)	0 (0.0)
Minahasa	53	49 (92.5)	4 (7.5)	0 (0.0)
Nias	69	62 (89.9)	7 (10.1)	0 (0.0)
Papuan	65	60 (92.3)	4 (6.2)	1 (1.5)
Ternate	46	44 (95.7)	2 (4.3)	0 (0.0)
Timor	38	37 (97.4)	1 (2.6)	0 (0.0)
Tolaki	23	23 (100.0)	0 (0.0)	0 (0.0)

PUD: peptic ulcer disease

cagA genotyping

In this study, we utilized the *cagA* sequence data from our previous studies in Indonesia [11, 12]. We also added data from 6 *cagA*-positive samples from Palu and Ternate survey. Among 101 isolated *H. pylori* strains, 98 (97.0%) were *cagA*-positive and were 3 *cagA*-negative strains. Of 98 *cagA*-positive strains, 55 (56.1%) possessed East Asian-type *cagA*, 25 (25.5%) were Western-type *cagA*, and 18 (18.4%) were ABB-type *cagA*. The distribution of *cagA* genotype within ethnics is shown in **Table 2**.

Table 2. The distribution of *cagA* genotype

Ethnic	*cagA*-positive	*cagA* genotypes		
		East Asian-type	Western-type	ABB-type
Total	98	55	25	18
Aceh	0	0 (0.0)	0 (0.0)	0 (0.0)
Balinese	7	4 (57.1)	3 (42.9)	0 (0.0)
Batak	22	22 (100.0)	0 (0.0)	0 (0.0)
Bugis	14	4 (28.6)	10 (71.4)	0 (0.0)
Chinese	7	6 (85.7)	1 (14.3)	0 (0.0)

Dayak	2	0 (0.0)	2 (100.0)	0 (0.0)
Javanese	1	0 (0.0)	0 (0.0)	1 (100.0)
Kaili	1	0 (0.0)	1 (100.0)	0 (0.0)
Malay	2	1 (50.0)	1 (50.0)	0 (0.0)
Minahasa	7	7 (100.0)	0 (0.0)	0 (0.0)
Nias	1	1 (100.0)	0 (0.0)	0 (0.0)
Papuan	18	1 (5.6)	0 (0.0)	17 (94.4)
Ternate	3	3 (100.0)	0 (0.0)	0 (0.0)
Timor	13	6 (46.2)	7 (53.8)	0 (0.0)
Tolaki	0	0 (0.0)	0 (0.0)	0 (0.0)

Immunohistochemistry and the detection of CagA

The gastric biopsies from 1,038 patients were analyzed for the immunoreactivity with 2 different types of CagA antibodies (i.e. anti-CagA antibody and α-EAS antibody) (**Table 3** and **Figure 1**). Forty-three patients (43/55, 78.2%) infected with East Asian-type *cagA*, 24 patients (24/25, 96.0%) infected with Western-type *cagA* strains, and 17 patients (17/18, 94.4%) infected with ABB-type *cagA* strains were immunoreactive to anti-CagA antibody. In contrast, all *H. pylori*-uninfected patients (937/937, 100%) and all patients infected with *cagA*-negative *H. pylori* (3/3, 100%) were non-immunoreactive to the anti-CagA antibody. By using PCR-based sequencing as gold standard, the performance of immunohistochemistry to determine *cagA* status was analyzed. The sensitivity, specificity, PPV, NPV, and accuracy of immunohistochemistry using anti-CagA antibody were 85.7%, 100%, 100%, 98.5%, and 98.7%, respectively.

On the other hand, immunohistochemistry using α-EAS antibody, we found that only 22 patients infected with East Asian-type *cagA* strains (22/55, 40.0%) were immunoreactive. All patients infected with Western-type *cagA* strains (25/25, 100.0%) and almost all of the patients infected by ABB-type *cagA* strains (17/18, 94.4%) were

non-immunoreactive towards α-EAS antibody. All the *H. pylori*-uninfected patients (937/937, 100%) and all patients infected with *cagA*-negative strains (3/3, 100%) were also showed to be non-immunoreactive towards α-EAS antibody. The sensitivity, specificity, PPV, NPV, and accuracy of immunohistochemistry using α-EAS antibody were 23.5%, 100%, 100%, 92.6%, and 92.7%, respectively.

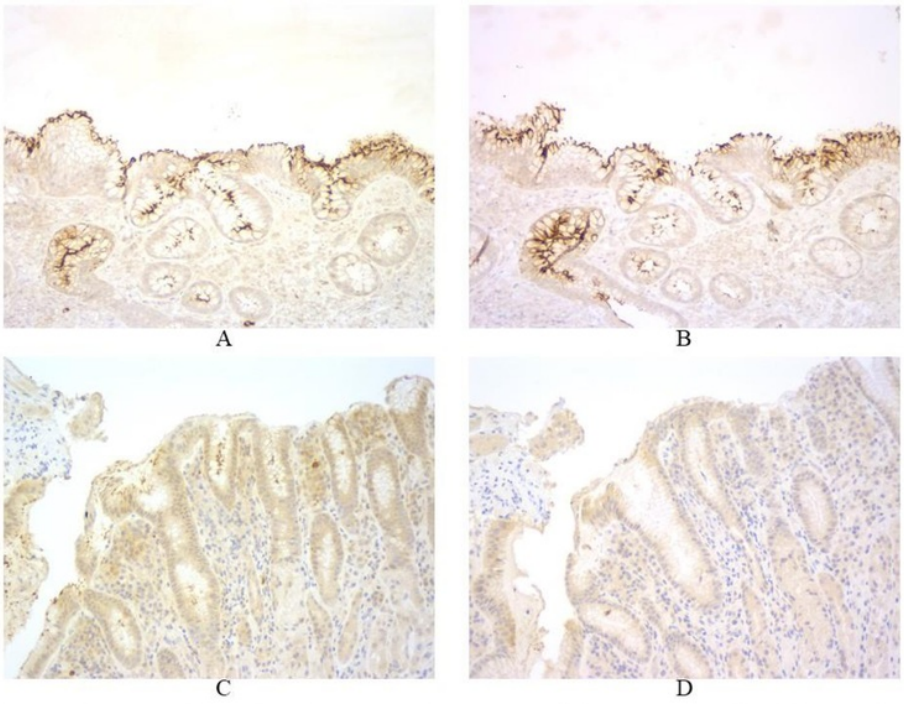

Figure 1. Immunohistochemistry by using 2 types of CagA antibodies. The specimen TER001 and KPG26 was used as example to show the typical immunohistochemistry staining result. TER001 specimen was positively immunostained with both anti-CagA antibody (A) and α-EAS antibody (B). KPG26 specimen was positively immunostained with anti-CagA antibody (C), but negatively immunostained with α-EAS antibody (D).

Table 3. The performance of immunohistochemistry using 2 types of CagA antibodies for the detection of CagA status

Immuno-reactivity	*H. pylori* (+) *cagA* (+)			*H. pylori* (-)(%)	*cagA* (-)(%)	Sens. (%)	Spec. (%)	PPV (%)	NPV (%)	Accuracy (%)
	EAT (%)	WT (%)	ABB (%)							
Total	55	25	18	937	3					
Anti-CagA						85.7	100	100	98.5	98.7
Positive	43 (78.2)	24 (96.0)	17 (94.4)	0 (0.0)	0 (0.0)					
Negative	12 (21.8)	1 (4.0)	1 (5.6)	937 (100)	3 (100)					
α-EAS						23.5	100	100	92.6	92.7
Positive	22 (40.0)	0 (0.0)	1 (5.6)	0 (0.0)	0					
Negative	33 (60.0)	25 (100)	17 (94.4)	937 (100)	3					

α-EAS antibody and the detection of East Asian-type *cagA H. pylori*

We analyzed the capability of α-EAS antibody for the detection of East Asian-type *cagA* strains **(Table 4)**. In this analysis, Western-type *cagA* and ABB-type *cagA* strains were regarded as non-East Asian-type. Therefore, patients infected with 55 East Asian-type *cagA* strains and those with 43 non-East Asian-type *cagA* strains were evaluated. We found that only 22 (22/55, 40.0%) patients infected with East Asian-type *cagA* strains were immunoreactive to the α-EAS antibody. Mostly the non-East Asian-type strains-infected patients (42/43, 97.7%) were not immunoreactive to the α-EAS antibody. The sensitivity, specificity, PPV, NPV, and accuracy of α-EAS antibody to detect East Asian-type *cagA* were 40.0%, 97.7%, 95.7%, 56.0%, and 65.3%, respectively. Therefore, the α-EAS antibody was not suitable to distinguish the East Asian-type *cagA* strains with non-East Asian-type *cagA* strains in Indonesia.

Table 4. α-EAS antibody immunoreactivity with East Asian-type *cagA*

Immunoreactivity with α-EAS antibody	*cagA* genotypes		Total
	East Asian-type (%)	Non-East Asian-type (%)*	
Total	55	43	98
Positive	22 (40.0)	1 (2.3)	23
Negative	33 (60.0)	42 (97.7)	75

Sensitivity: 40.0%; specificity: 97.7; PPV: 95.7%; NPV: 56.0%; accuracy: 65.3%
*Including Western-type CagA and ABB-type CagA subjects

False-negative samples analysis

We analyzed the false-negative samples in regards to the *cagA* genotypes (**Table 5**). In total, there were 14 patients failed to be detected by immunohistochemistry using anti-CagA antibody. Of these, 12 were infected with East Asian-type *cagA* strains, 1 was infected with Western-type *cagA* strains, and 1 was infected with ABB-type *cagA* strains. Most of the East Asian-type *cagA* that had false-negative results by anti-CagA antibody possessed a typical ABD-motif. There were 75 patients with negative result for immunohistochemistry using α-EAS antibody. Of 55 patients infected with East Asian-type *cagA* strains, 33 were non-immunoreactive towards α-EAS antibody.

Table 5. The false-negative results and the *cagA* genotypes

cagA genotypes	Antibody	
	Anti-CagA	α-EAS
Total true-positive	84	23
Total false-negative	14	75
Total	**98**	**98**

False-negative samples

East Asian-type (n=55)		
ABD	11/48 (22.9)	27/48 (56.3)
AABD	1/3 (33.3)	2/3 (66.7)
AAD	0/1 (0.0)	1/1 (100.0)
ABBD	0/3 (0.0)	3/3 (100.0)
Total	12/55 (21.8)	33/55 (60.0)
Western-type (n=25)		
ABC	1/20 (5.0)	20/20 (100.0)
ABCC	0/1 (0.0)	1/1 (100.0)
BC	0/4 (0.0)	4/4 (100.0)
Total	1/25 (4.0)	25/25 (100.0)
ABB-type (n=18)		
AB	1/3 (33.3)	3/3 (100.0)
ABB	0/8 (0.0)	8/8 (100.0)
B	0/7 (0.0)	6/7 (84.7)
Total	1/18 (5.6)	17/18 (94.4)
Total false negative	**14/98 (14.3)**	**75/98 (76.5)**

Immunohistochemistry diagnosis and the peptide sequence similarity

We performed protein sequence comparison analysis to analyze the similarity between the East Asian specific peptide sequence and the CagA amino acid sequences of Indonesian East Asian-type *cagA* strains (**Suppl. Table 1**). The East Asian specific peptide sequence comprised of 18 peptides (AINRKIDRINKIASAGKG). By using the blast algorithm, we found that 50 strains were detected to have similarity with the East Asian specific peptides and were able to calculate the similarity percentage. There were 5 East Asian-type *cagA* strains that were undetected by the blast algorithm, suggesting there were no similarities at all (n.a result). Ten strains were found to be identical (100%

similarity) to the East Asian specific peptides, while the other strains similarity varied between 35.3%–72.7%. Of 10 strains with identical sequence (100% similarity), 2 strains were not immunostained with the α-EAS antibody (MANADO29 and MANADO31). There was no specific pattern of similarity percentage to the immunohistochemistry result.

Suppl. Table 1. Similarity analysis between the East Asian specific peptide sequence and the CagA sequences of East Asian-type *cagA* strains.

Strain	EPIYA motif	*cagA* genotype	Similarity (%)	IHC result (α-EAS antibody)
IND68	ABD	East Asian	64.3	Negative
IND69	ABD	East Asian	100	Positive
IND71	ABD	East Asian	100	Positive
IND79	ABD	East Asian	64.3	Negative
JAY16	ABD	East Asian	64.3	Negative
JKT9	ABD	East Asian	64.3	Negative
MO17	ABD	East Asian	52.9	Negative
MN3	ABD	East Asian	64.3	Positive
MN10	ABD	East Asian	64.3	Negative
MN11	ABD	East Asian	64.3	Negative
SMS15	ABD	East Asian	55.6	Positive
SMS22	ABD	East Asian	64.3	Positive
SMS24	ABD	East Asian	64.3	Positive
SMS28	ABD	East Asian	64.3	Negative
SMS30	ABD	East Asian	71.4	Negative
MKS45	AABD	East Asian	64.3	Positive
MKS52	AABD	East Asian	64.3	Negative
PTK50	ABD	East Asian	52.9	Negative
MANADO5	ABD	East Asian	100	Positive
MANADO18	ABD	East Asian	57.1	Negative
MANADO20	ABD	East Asian	100	Positive
MANADO26	ABD	East Asian	100	Positive
MANADO28	ABD	East Asian	100	Positive

MANADO29	ABD	East Asian	100	Negative
MANADO31	ABD	East Asian	100	Negative
KPG2	ABD	East Asian	64.3	Positive
KPG5	ABD	East Asian	62.5	Positive
KPG11	ABD	East Asian	64.3	Positive
KPG15	ABD	East Asian	100	Positive
KPG29	ABD	East Asian	64.3	Negative
KPG35	ABD	East Asian	64.3	Negative
KPG42	AABD	East Asian	71.4	Negative
KPG64	AAD	East Asian	35.3	Negative
KPG83	ABD	East Asian	71.4	Negative
MEDAN31	ABD	East Asian	64.3	Negative
MEDAN32	ABD	East Asian	64.3	Negative
NIAS9	ABD	East Asian	57.1	Negative
NIAS36	ABD	East Asian	55.6	Negative
NIAS37	ABD	East Asian	64.3	Positive
NIAS40	ABD	East Asian	64.3	Positive
NIAS49	ABD	East Asian	64.3	Negative
NIAS50	ABD	East Asian	64.3	Positive
NIAS56	ABBD	East Asian	64.3	Negative
NIAS67	ABD	East Asian	64.3	Negative
NIAS68	ABD	East Asian	64.3	Negative
NIAS73	ABBD	East Asian	64.3	Negative
NIAS75	ABBD	East Asian	64.3	Negative
PDG42	ABD	East Asian	57.1	Positive
SBY106	ABD	East Asian	100	Positive
SBY137	ABD	East Asian	64.3	Negative
SBY304	ABD	East Asian	n.a	Positive
TER001	ABD	East Asian	n.a	Positive
TER009	ABD	East Asian	n.a	Negative
TER018	ABD	East Asian	n.a	Negative
TER081	ABD	East Asian	n.a	Negative

275

276 **_cagA_ immunohistochemistry and ethnics**

277 We analyzed the result of immunohistochemistry using 2 types of CagA antibodies based

on the ethnics in Indonesia (**Supp. Table 2**). The immunohistochemistry using α-EAS antibody was not able to detect all *cagA*-positive strains of Balinese, Dayak, Javanese, Nias, and Kaili (100% of negative result). In Papuan ethnic which was constitutes of ABB-type *cagA*, almost all patients infected with *cagA*-positive strains (17/18, 94.4%) had negative result of the immunohistochemistry using anti-EAS antibody. In contrast, the immunohistochemistry using anti-CagA antibody was still able to detect patients infected with *cagA*-positive strains regardless the ethnicity, except for Kaili and Malay ethnics; however, it might be caused by the small number of samples in those ethnics. There were no significant association between the using of anti-CagA antibody and α-EAS antibody and the ethnicity (P = 0.414 and P = 0.110, respectively).

Supp. Table 2. CagA immunohistochemistry and ethnicity

Ethnic	Anti-CagA antibody		α-EAS antibody		Total
	Positive	Negative	Positive	Negative	
Balinese	6 (85.7)	1 (14.3)	0 (0.0)	7 (100)	7
Batak	18 (81.8)	4 (18.2)	7 (31.8)	15 (68.2)	22
Bugis	13 (92.9)	1 (7.1)	2 (14.3)	12 (85.7)	14
Chinese	5 (71.4)	2 (28.6)	3 (42.9)	4 (57.1)	7
Dayak	2 (100)	0 (0.0)	0 (0.0)	2 (100)	2
Javanese	1 (100)	0 (0.0)	0 (0.0)	1 (100)	1
Kaili	0 (0.0)	1 (100)	0 (0.0)	1 (100)	1
Malay	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	2
Minahasa	6 (85.7)	1 (14.3)	4 (57.1)	3 (42.9)	7
Nias	1 (100)	0 (0.0)	0 (0.0)	1 (100)	1
Papuan	17 (94.4)	1 (5.6)	1 (5.6)	17 (94.4)	18
Ternate	3 (100)	0 (0.0)	2 (66.7)	1 (33.3)	3
Timor	11 (84.6)	2 (15.4)	3 (23.1)	10 (76.9)	13
Total	84 (85.7)	14 (14.3)	23 (23.5)	75 (76.5)	98

Association between histological score and immunohistochemistry

We analyzed the association between histological scores and the immunohistochemistry result. We found that the patients with positive result of immunohistochemistry using anti-CagA antibody had significantly higher monocyte infiltration score in both the antrum and corpus compared to those with negative result ($P < 0.001$ and $P = 0.009$, respectively). Patients with positive result of immunohistochemistry using anti-CagA antibody also had significantly higher atrophic score in the antrum, but not in the corpus, compared to negative one ($P < 0.001$ and $P = 0.310$, respectively. However, no statistically significant results were found between the monocyte infiltration scores of patients with positive result of immunohistochemistry using α-EAS antibody compared to those with negative result both in the antrum and the corpus ($P = 0.111$ and $P = 0.467$, respectively). Similar results were also found between the atrophic scores and immunoreactivity for α-EAS antibody both in the antrum and corpus ($P = 0.354$ and $P = 0.314$, respectively).

Discussion

In this present study, we aimed to validate 2 different types of antibody for CagA status determination and the use of specific East Asian-type CagA antibody to specifically distinguish the East Asian-type CagA and other CagA types. We found that the anti-CagA antibody is still suitable to be used in Indonesia for the purpose of the CagA status determination, showed by considerably high sensitivity, specificity, and accuracy. On the contrary, the α-EAS antibody was found to be not suitable for the purpose of CagA status determination, as it had a considerably low sensitivity. The low sensitivity of α-EAS antibody is not surprising, as it developed not for CagA status determination purpose, but to differentiate the East Asian-type CagA *H. pylori* and non-East Asian-type CagA *H. pylori* [13]. Both anti-CagA antibody and α-EAS antibody were showing negative result for all patients with *H. pylori*-uninfected patients, confirming the antibodies were not cross-reactive with uninfected gastric mucosa, in concordance with our previous study using Thai and Vietnamese population [16].

The α-EAS antibody was reported to show high accuracy in several East Asian countries, such as Japan [14], Thailand and Vietnam [16]. However, we found that the accuracy of α-EAS antibody in Indonesia was considerably low compared to other countries (Indonesia: 63.5% vs. Japan: 91.6% vs. Thailand and Vietnam: 97.1%). This

antibody sensitivity was very low to differentiate the East Asian-type *cagA* and non-East Asian-type *cagA*, suggesting that the α-EAS antibody was not suitable to be used in Indonesia. Similar to this result, previous study in Bhutan reported that the α-EAS antibody had low sensitivity (36.2%) and low accuracy (41.0%) to detect samples infected with East Asian-type CagA strains [24]. They also showed that the increasing number of amino acid differences from the designed α-EAS antibody sequences might be responsible to the decrease of positivity result [24].

To analyze the possible reason of low sensitivity of the α-EAS antibody in Indonesian *H. pylori* isolates, we performed the blast algorithm to compare the protein sequence of the East Asian-type CagA specific antigen used to generate α-EAS antibody (AINRKIDRINKIASAGKG) with the protein sequences of Indonesian East Asian-type *cagA H. pylori* isolates. We found that most of the strains was detected to have similarity with the East Asian-type CagA specific peptide. However, they mostly in range between 60%–75% similarity. This analysis showed that the α-EAS antibody were not suitable enough to be used as *cagA* genotyping method in Indonesia. It might be important to develop an antibody specific to the Indonesian East Asian-type CagA strains, as it may increase diagnostic accuracy.

Based on ethnicity, our result showed that the immunohistochemistry using α-

EAS antibody was not suitable to determine the *cagA* positivity status, as it showed high false negative result in all Indonesian ethnics. On the other hand, the immunohistochemistry using anti-CagA antibody was able to determine the *cagA* positivity status with low false negative in almost all ethnics in Indonesia. Our result suggesting that the anti-CagA antibody is still suitable be used in the immunohistochemistry for the purpose of determining *cagA* status in Indonesia, regardless the ethnic differences.

We found that the patients with positive result of immunohistochemistry using the anti-CagA antibody had significantly higher monocyte infiltration score in both the antrum and corpus compared to those with negative result of immunohistochemistry using anti-CagA antibody. Supporting our result, previous studies also reported the importance of CagA to the development of chronic gastritis in *H. pylori*-infected gastric mucosa [3, 25, 26].

There were limitation in this study. Given that Indonesia is a country with low *H. pylori* prevalence, the number of *H. pylori*-positive samples were considerably smaller than the *H. pylori*-negative samples. Therefore, when we divided the samples based on ethnicity, it yielded very low sample number in several ethnic groups. Further study with bigger sample size in each ethnic may be necessary. However, given that the samples

were collected from different locations throughout Indonesia, this study may give a better understanding of the applicability of CagA immunohistochemistry in general.

Conclusion

The anti-CagA antibody is suitable to be used in Indonesia for the purpose of the *cagA* status determination. The α-EAS antibody were not suitable to distinguish East Asian-type *cagA* and non-East Asian-type *cagA* in Indonesia.

Potential competing interests

The authors declare that they have no competing interests.

Financial Support

This report is based on work supported in part by grants from the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan (15H02657, 16H05191, 16H06279, 18KK0266, and 19H03473) (YY) and the National Institutes of Health (DK62813) (YY). This work was also supported by the Japan Society for the Promotion of Science (JSPS) Institutional Program for Core-to-Core Program; B. Africa-Asia Science Platform (YY) and The

Ministries of Research, Technology and Higher Education of Indonesia for World Class Professor Program (no. 123.4/D2.3/KP/2018) to MIL and MM. LAW, DD, and KAF are PhD students supported by the Japanese Government (MEXT) scholarship program for 2015, 2016, and 2017, respectively.

Author contributions

Conceived and design the experiments: MM, DD, and YY. Performed the experiments: TU, LAW, and DD. Analyzed the data: MM, DD, LAW, and YY. Contributed reagents/material/data acquisition: AFS, KAF, YAA, TU, and YY. Wrote the paper: DD and MM. YY revised the manuscript and added important content. All authors read and approved the final version of the manuscript.

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