Acta Histochemica 122 (2020) 151594

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

The validation of the *Helicobacter pylori* CagA typing by immunohistochemistry: nationwide application in Indonesia

Muhammad Miftahussurur[a,b,1,*], Dalla Doohan[b,c,1], Ari Fahrial Syam[d], Iswan Abbas Nusi[a], Langgeng Agung Waskito[b,c], Kartika Afrida Fauzia[b,c], Yudith Annisa Ayu Rezkitha[b,e], Astri Dewayani[f,g], Reny I'tishom[b], Hasan Maulahela[d], Tomohisa Uchida[h], Yoshio Yamaoka[a,c,i,j,**]

[a] Gastroentero-Hepatology Division, Department of Internal Medicine, Faculty of Medicine-Dr. Soetomo Teaching Hospital, Universitas Airlangga, Surabaya, 60131, Indonesia
[b] Institute of Tropical Disease, Universitas Airlangga, Surabaya, 60115, Indonesia
[c] Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Yufu, 879-5593, Japan
[d] Division of Gastroenterology, Department of Internal Medicine, Faculty of Medicine, University of Indonesia, Jakarta, 16424, Indonesia
[e] Faculty of Medicine, University of Muhammadiyah Surabaya, Surabaya, 60113, Indonesia
[f] Department of Anatomy and Histology, Faculty of Medicine, Universitas Airlangga, Surabaya, 60131, Indonesia
[g] Department of Infectious Disease, Oita University Faculty of Medicine, Yufu, 879-5593, Japan
[h] Department of Molecular Pathology, Oita University Faculty of Medicine, Yufu, 879-5593, Japan
[i] Department of Gastroenterology and Hepatology, Baylor College of Medicine and Michael E. DeBakey Veterans Affairs Medical Center, Houston, TX, 77030, USA
[j] Global Oita Medical Advanced Research Center for Health (GO-MARCH), Yufu, 870-1192, Japan

ARTICLE INFO

Keywords:
CagA typing
Immunohistochemistry
Infectious disease
anti-CagA antibody
Anti-East Asian CagA antibody

ABSTRACT

We aimed 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 in Indonesia. We also confirmed the performance of α-EAS antibody for the detection of East Asian-type CagA *H. pylori*. Immunohistochemistry was performed using anti-CagA antibody and α-EAS antibody on gastric biopsy specimens from a total of 967 Indonesian patients. Diagnostic values of immunohistochemistry were evaluated with PCR-based sequencing as gold standard. Anti-CagA antibody had high sensitivity, specificity, and accuracy (87.0 %, 100 %, and 98.8 %, 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 (23.9 %). High specificity (97.6 %) but low sensitivity (41.2 %) and accuracy (66.3 %) was observed in α-EAS antibody to detect East Asian-type CagA. Patients with positive result of immunohistochemistry using anti-CagA antibody had significantly higher monocyte infiltration score in antrum ($P < 0.001$) and corpus ($P = 0.009$). In 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 appropriate to discriminate between East Asian-type and non-East Asian-type CagA in Indonesia.

1. 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 (Amieva and Peek, 2016; Graham, 2014; Peek and Blaser, 2002). 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) (Yamaoka, 2010).

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

* Corresponding author at: Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu-City, Oita, 879-5593, Japan.
** Corresponding author at: Gastroentero-Hepatology Division, Department of Internal Medicine, Faculty of Medicine-Dr. Soetomo Teaching Hospital, Universitas Airlangga, Jalan Mayjend Prof. Dr. Moestopo No. 6-8, Surabaya, 60131, Indonesia.
E-mail addresses: muhammad-m@fk.unair.ac.id (M. Miftahussurur), yyamaoka@oita-u.ac.jp (Y. Yamaoka).
[1] These authors are equally contributed.

https://doi.org/10.1016/j.acthis.2020.151594
Received 16 April 2020; Received in revised form 25 June 2020; Accepted 3 July 2020

system (Backert et al., 2017, 2015). 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) (Higashi et al., 2002). Generally, the CagA type is divided into Western-type CagA and East Asian-type CagA. However, we previously reported a unique CagA type called ABB-type CagA, which was found in the *H. pylori* strains isolated mainly in Papua Island, Indonesia (Miftahussurur et al., 2015b). The ABB-type CagA is identified by the EPIYA-A and EPIYA-B, but followed by EPIYA-B. We also reported that there were unique *cagA* genotypes found in Papua Island, possessing AB- and B- motifs (Waskito et al., 2018). However, due to the similarity of the B-segment of the AB-type and B-type CagA with the ABB-type CagA, these two types were considered as ABB-type subtypes (Waskito et al., 2018). The predominant CagA type might different between populations and reported to be associated with geographical factor. For example, East Asian-type is reported to be the predominant type in Japan (Yamaoka et al., 1998), Thailand (Subsomwong et al., 2017), and Indonesia (Miftahussurur et al., 2015b), while Western-type CagA is the predominant type in Bangladesh (Aftab et al., 2017), Myanmar (Doohan et al., 2019), and Mongolia (Tserentogtokh et al., 2019).

The determination of CagA status and genotypes is mainly by polymerase chain reaction (PCR) and sequencing of the EPIYA region in the 3' of the *cagA*. 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 (Uchida et al., 2007). This antibody is reported to be useful for the purpose of detecting East Asian-type CagA immunohistochemically in East Asian and Southeast Asian countries, such as Japan (Kanada et al., 2008; Yasuda et al., 2009), Thailand and Vietnam (Nguyen et al., 2009).

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 (Syam et al., 2015). 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 (Miftahussurur et al., 2018). An easier and quicker method following endoscopy to help ascertain CagA status is needed, such as immunohistochemistry. 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 analyzed the association between histological score and immunohistochemistry.

2. Material and methods

2.1. Study participants

A nation-wide, cross sectional and multicenter study was performed in 17 cities in Indonesia from August 2012–February 2016 (Fig. 1). Adult dyspeptic patients with minimum age 18 years old were recruited and were stated in our previous studies in Indonesia (Miftahussurur et al., 2015a, 2016; Miftahussurur et al., 2019; Syam et al., 2015; Waskito et al., 2018). 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 of 1,072 patients underwent upper endoscopy. In each upper endoscopic session, 3 biopsy specimens were taken, including 2 biopsies from the antrum and 1 biopsy from the corpus. Each sample from the antrum was used for histology analysis and *H. pylori* culture. Corpus biopsy specimen was used for the histology analysis. In this study, we excluded 88 samples with incomplete histological data in both antrum and corpus. We also excluded 17 samples with culture test yielded negative results, but histology or immunohistochemistry using anti-*H. pylori* antibody were positive.

Ethical approval was obtained from the Ethics Committee of Dr. Soetomo Teaching Hospital (Surabaya, Indonesia), Dr. Cipto Mangunkusumo Teaching Hospital (Jakarta, Indonesia), Dr. Wahidin Sudirohusodo Teaching Hospital (Makassar, Indonesia), and Oita University Faculty of Medicine (Yufu, Japan).

2.2. H. pylori culture and infection status

The details of culture method has been reported previously (Syam et al., 2015). Briefly, *H. pylori* selective media (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; Ref. Number 51035) was used to isolate bacteria from a homogenized biopsy specimen and then incubate up to 7 days in microaerophilic conditions (10 % O_2, 5% CO_2, and 85 % N_2) at 37 °C. The *H. pylori* colonies were then inoculated onto Brucella Agar medium (Becton Dickinson, Sparks, MD, USA; Ref. Number 211086) supplemented with 7% horse blood. *H. pylori* was identified on the basis of Gram-negative staining result, bacteria morphology, and positive result of oxidase, urease, and catalase. In this study, *H. pylori* infection positive status was determined via *H. pylori* culture from antral biopsy, while *H. pylori* uninfected status was determined via negative result of both culture and histology confirmed by the immunohistochemistry using the anti-*H. pylori* antibody (α-*H. pylori* Antibody, Dako, Glostrup, Denmark; Ref. Number B0471). The observation and the interpretation of the histology and immunohistochemistry were performed blindly by an experienced pathologist (TU).

2.3. α-EAS antibody

α-EAS antibody was generated as previously described (Uchida et al., 2007). Briefly, the East Asian CagA specific polypeptides, AINRKIDRINKIASAGKG was established in Uchida Laboratory (RRID: AB_2857922) and further produced by 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 antibodies were collected using the peptide-coupled HiTrap NHS-activated column (Cytiva Life Sciences, Cat. Number 17071601).

2.4. Immunohistochemistry and histology examination

The immunohistochemistry was performed as previously reported (Kanada et al., 2008; Uchida et al., 2007). Briefly, after antigen retrieval, tissue sections were immersed in 10 mmol/L sodium citrate buffer pH 6.0, autoclaved at 120 °C for 10 min, and followed by cooling at room temperature. Tissue sections were treated with 3 % H_2O_2 for 10 min at room temperature to inactivate endogenous peroxidase activity before underwent blocking with 10 % goat serum for α-CagA (b-300) and α-*H. pylori* Antibody or rabbit serum for α-EAS Antibody (Histofine SAB-PO(M) Kit, Nichirei; Code. Number 424022) for 20 min at room temperature. Subsequently, they were incubated with α-*H. pylori* Antibody (Dako, Glostrup, Denmark; Ref. Number B0471) diluted 1:50 or α-EAS Antibody diluted 1:4000 or α-CagA (b-300) Antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; Cat. Number SC-25766) diluted 1:100 overnight at 4 °C. After being washed using PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Histofine SAB-PO (M) Kit, Nichirei; Code. Number 424022), followed by incubation with an avidin-conjugated horseradish peroxidase solution (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA, USA; Ref. Number PK-6100). Peroxidase activity was detected using an H_2O_2/diaminobenzidine substrate solution. For histopathology examination, the thin slices of paraffin-embedded biopsy with May-Grünwald-Giemsa and hematoxylin-eosin stains was prepared. The

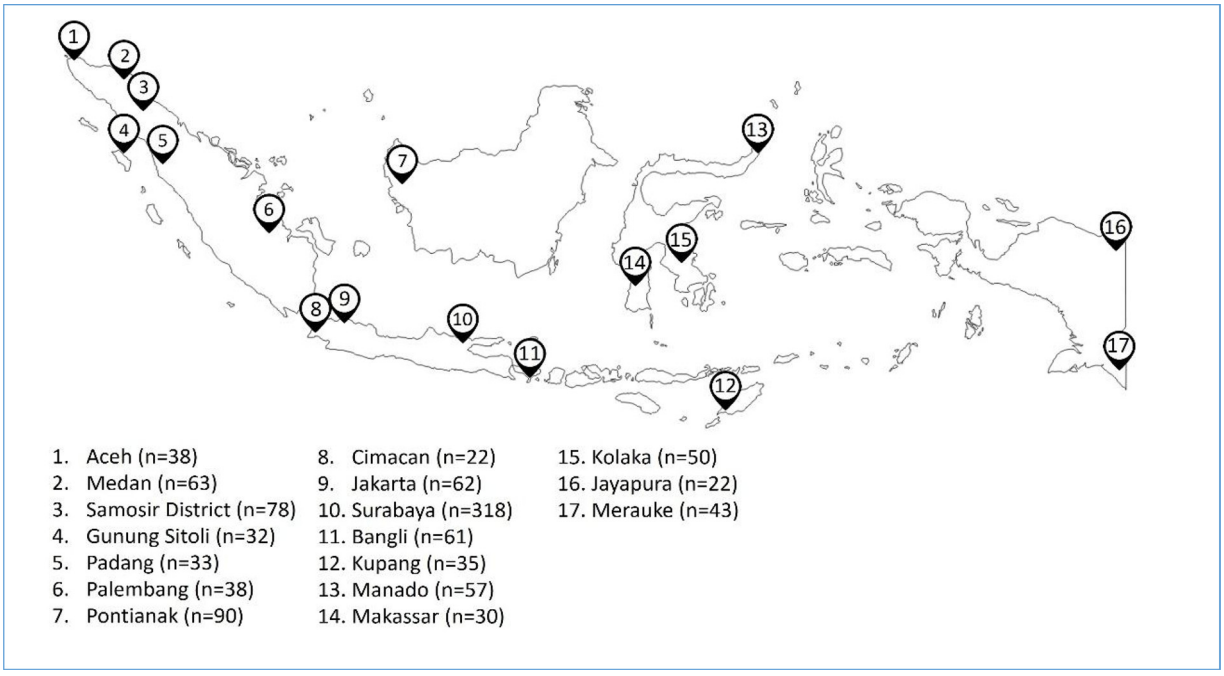

Fig. 1. Upper endoscopic survey was performed in 17 cities across Indonesia. The number presented in this figure are the number of patients recruited in this study.

Table 1
The distribution of *cagA* types.

Ethnic	*cagA*-positive	*cagA* types		
		East Asian-type (%)	Western-type (%)	ABB-type (%)
Balinese	6	3 (50.0)	3 (50.0)	0 (0.0)
Batak	22	22 (100)	0 (0.0)	0 (0.0)
Buginese	11	2 (18.2)	9 (81.8)	0 (0.0)
Chinese	7	6 (85.7)	1 (14.3)	0 (0.0)
Dayak	2	0 (0.0)	2 (100)	0 (0.0)
Javanese	1	0 (0.0)	0 (0.0)	1 (100)
Malay	2	1 (50.0)	1 (50.0)	0 (0.0)
Minahasa	7	7 (100)	0 (0.0)	0 (0.0)
Nias	1	1 (100)	0 (0.0)	0 (0.0)
Papuan	18	1 (5.6)	0 (0.0)	17 (94.4)
Ternate	2	2 (100)	0 (0.0)	0 (0.0)
Timor	13	6 (46.2)	7 (53.8)	0 (0.0)
Total	92	51	23	18

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 (Dixon et al., 1996) was determined by the experienced pathologist (TU).

2.5. CagA typing

In this study, we utilized the *cagA* sequences data from our previous studies in Indonesia (Miftahussurur et al., 2015b, 2016; Waskito et al., 2018). The CagA type (e.g. East Asian-type, Western-type, or ABB-type) was defined as previously described (Miftahussurur et al., 2015b; Waskito et al., 2018; Yamaoka, 2010).

2.6. Statistical analysis

The statistical analysis was performed using SPSS version 23.0 (IBM Corp., Armonk, NY, USA; RRID: SCR_002865). Discrete variables were analyze using the chi-squared test or Fisher's exact test, while continuous variables were analyzed using Mann-Whitney *U*. P value 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 BLASTN algorithm (RRID: SCR_001598) and converted the obtained sequences into amino acid.

3. Results

3.1. Study participants

Finally, 967 patients were included for further analysis in this study, consisted of 556 males and 411 females with mean age of 46.4 ± 13.8 years. The *H. pylori* infection status by histology showed 95 *H. pylori*-positive and 872 *H. pylori*-negative patients. Among 95 isolated *H. pylori* strains, 92 (96.8 %) were *cagA*-positive and 3 (3.2 %) were *cagA*-negative strains. Of 92 *cagA*-positive strains, 51 (55.4 %) possessed East Asian-type CagA, 23 (25.0 %) were Western-type CagA, and 18 (19.6 %) were ABB-type CagA. The distribution of CagA type within ethnics is shown in Table 1.

3.2. Immunohistochemistry and the detection of CagA

The gastric biopsies from 967 patients were analyzed for the immunoreactivity with 2 different types of CagA antibodies (i.e. anti-CagA antibody and α-EAS antibody) (Fig. 2 and Table 2). Forty patients (40/51, 78.4 %) infected with East Asian-type CagA, 23 patients (23/23, 100 %) 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 (872/872, 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 87.0 %, 100 %, 100 %, 98.6 %, and 98.8

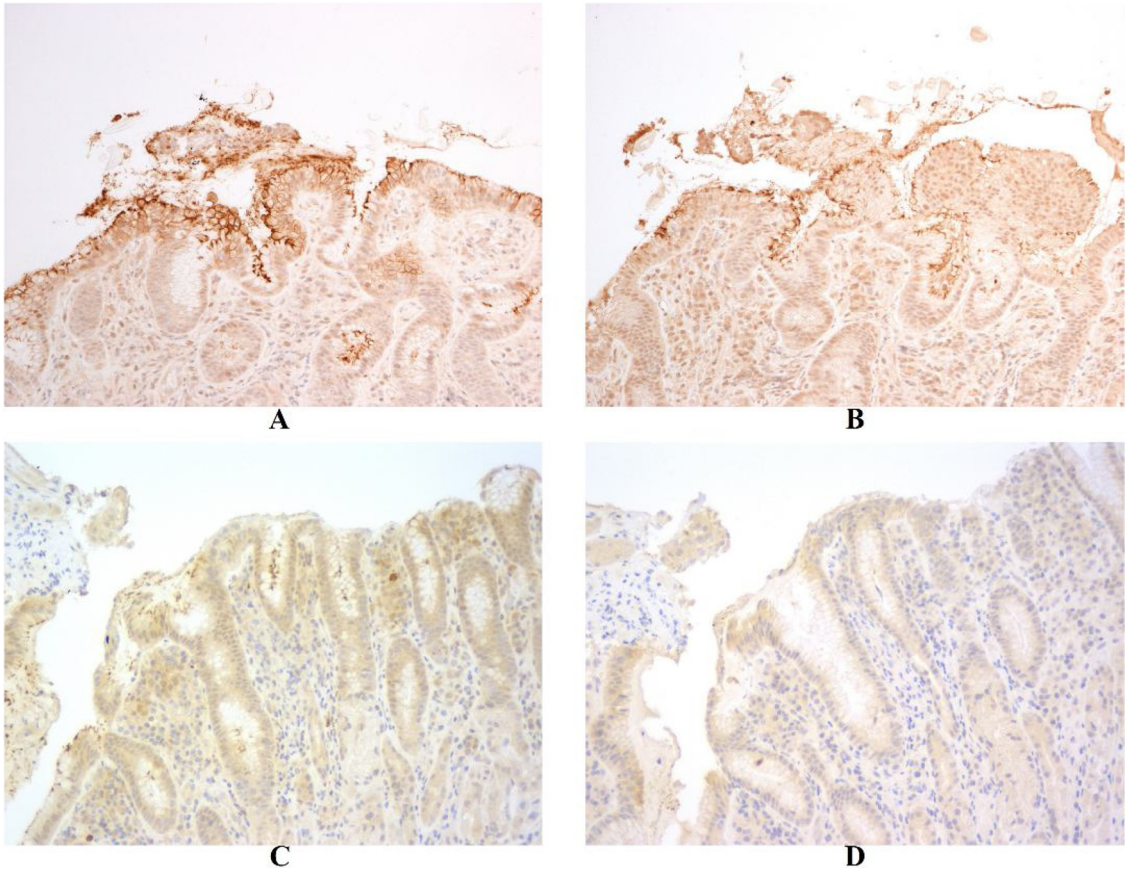

Fig. 2. Immunohistochemistry by using 2 types of CagA antibodies. The examples of the typical immunohistochemistry staining result. Positive immunostaining result is showed by brown color reaction. The specimen which was positively immunostained with both anti-CagA antibody (A) and α-EAS antibody (B). The specimen which was positively immunostained with anti-CagA antibody (C), but negatively immunostained with α-EAS antibody (D).

%, respectively.

On the other hand, immunohistochemistry using α-EAS antibody, we found that only 21 patients infected with East Asian-type CagA strains (21/51, 41.2 %) were immunoreactive. All patients infected with Western-type CagA strains (23/23, 100 %) 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 (872/872, 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 23.9 %, 100 %, 100 %, 92.6 %, and 92.8 %, respectively.

Table 3
α-EAS antibody immunoreactivity with East Asian-type *cagA*.

Immunoreactivity with α-EAS antibody	*cagA* types		Total
	East Asian-type (%)	Non-East Asian-type (%)*	
Total	51	41	92
Positive	21 (41.2)	1 (2.4)	22
Negative	30 (58.8)	40 (97.6)	70

Sensitivity: 41.2 %; specificity: 97.6; PPV: 95.4 %; NPV: 57.1 %; accuracy:66.3 %.

* Including Western-type CagA and ABB-type CagA subjects.

Table 2
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	51	23	18	872	3					
Anti-CagA						87.0	100	100	98.6	98.8
Positive	40 (78.4)	23 (100)	17 (94.4)	0 (0.0)	0 (0.0)					
Negative	11 (21.6)	0 (0.0)	1 (5.6)	872 (100)	3 (100)					
α-EAS						23.9	100	100	92.6	92.8
Positive	21 (41.2)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)					
Negative	30 (58.8)	23 (100)	17 (94.4)	872 (100)	3 (100)					

Table 4
The false-negative results and the *cagA* genotypes.

cagA genotypes	Antibody	
	Anti-CagA (%)	α-EAS (%)
Total true-positive	80	22
Total false-negative	12	70
Total	**92**	**92**
False-negative samples		
East Asian-type (n = 51)		
ABD	10/45 (22.2)	25/45 (55.6)
ABBD	1/6 (16.7)	5/6 (83.3)
Total	11/51 (21.6)	30/51 (58.8)
Western-type (n = 23)		
ABC	0/17 (0.0)	17/17 (100)
ABCC	0/1 (0.0)	1/1 (100)
AB*	0/1 (0.0)	0/1 (0.0)
BC	0/4 (0.0)	4/4 (100)
Total	0/23 (0.0)	23/23 (100)
ABB-type (n = 18)		
ABB	0/8 (0.0)	8/8 (100)
AB	1/3 (33.3)	3/3 (100)
B	0/7 (0.0)	6/7 (85.7)
Total	1/18 (5.6)	17/18 (94.4)
Total false negative	**12/92 (13.0)**	**70/92 (76.1)**

*The AB genotype was included into Western-type CagA due to the similarity with the B segment of Western-type CagA.
**The AB genotype was included into Western-type CagA due to the similarity with the ABB-type CagA, as previously reported (Waskito et al., 2018).

3.3. 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 3). In this analysis, Western-type CagA and ABB-type CagA strains were regarded as non-East Asian-type. Therefore, patients infected with 51 East Asian-type CagA strains and those with 41 non-East Asian-type CagA strains were evaluated. We found that only 21 (21/51, 41.2 %) patients infected with East Asian-type CagA strains were immunoreactive to the α-EAS antibody. Mostly the non-East Asian-type strains-infected patients (40/41, 97.6 %) 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 41.2 %, 97.6 %, 95.4 %, 57.1 %, and 66.3 %, respectively.

3.4. False-negative samples analysis

We analyzed the false-negative samples regarding the CagA types (Table 4). In total, there were 12 patients (12/92, 13.0 %) failed to be detected by immunohistochemistry using anti-CagA antibody. Of these, 11 were infected with East Asian-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 70 patients (70/92, 76.1 %) with negative result for immunohistochemistry using α-EAS antibody. Of 51 patients infected with East Asian-type CagA strains, 30 were non-immunoreactive towards α-EAS antibody.

3.5. 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 (Supplementary Table 1). The East Asian specific peptide sequence comprised of 18 peptides. 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 was 1 East Asian-type CagA strain that was undetected by the blast algorithm, therefore no similarities were found (n.a result). Ten strains were found to be identical 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).

3.6. CagA immunohistochemistry and ethnics

We analyzed the result of immunohistochemistry using 2 types of CagA antibodies based on the ethnics in Indonesia (Supplementary Table 2). The immunohistochemistry using α-EAS antibody was not able to detect all *cagA*-positive strains of Balinese, Dayak, Javanese, and Nias. 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 Malay ethnics; however, it might be caused by the small number of samples in those ethnics. There was no significant association between the using of anti-CagA antibody and ethnicity ($P = 0.722$). There was a significant association between α-EAS antibody and the ethnicity ($P = 0.030$).

3.7. Association between histological score and immunohistochemistry

We analyzed the association between histological scores and the immunohistochemistry result of culture-positive patients. 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 $P = 0.226$, 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 ($P = 0.113$ and $P = 0.519$, respectively). Similar results were also found between the atrophic scores and immunoreactivity for α-EAS antibody both in the antrum and corpus ($P = 0.381$ and $P = 0.328$, respectively).

4. 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 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 appropriate 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* (Uchida et al., 2007). 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 (Nguyen et al., 2009).

The α-EAS antibody was reported to show high accuracy in several East Asian and Southeast Asian countries, such as Japan (Kanada et al., 2008), Thailand and Vietnam (Nguyen et al., 2009). However, we found that the accuracy of α-EAS antibody in Indonesia was considerably low compared to other countries (Indonesia: 66.3 % 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 (Matsunari et al., 2016). 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 (Matsunari et al., 2016).

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 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. It might be important to develop an antibody specific to the Indonesian East Asian-type CagA strains, thus 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 could be used to determine *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. Previous studies also reported the importance of CagA to the development of chronic gastritis in *H. pylori*-infected gastric mucosa (Correa and Houghton, 2007; Peek and Blaser, 2002; Suzuki et al., 2015).

There was 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.

5. Conclusions

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

Author contributions

Conceptualization, Writing – review & editing, Writing – original draft: MM, DD, and YY. Methodology, Data curation: TU, LAW, and DD. Formal analysis, Visualization, Validation: MM, DD, LAW, KAF, and TU. Resources: AFS, IAN, KAF, YAA, AD, RI, HM, TU, and YY. Funding acquisition: MM, YY. Supervision: YY. All authors read and approved the final version of the manuscript.

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. Asia-Africa Science Platform (YY). LAW, DD, and KAF are PhD students supported by the Japanese Government (MEXT) scholarship program for 2015, 2016, and 2017, respectively.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.acthis.2020.151594.

References

Aftab, H., Miftahussurur, M., Subsomwong, P., Ahmed, F., Khan, A.K.A., Matsumoto, T., Suzuki, R., Yamaoka, Y., 2017. Two populations of less-virulent Helicobacter pylori genotypes in Bangladesh. PLoS One 12 (8), e0182947.

Amieva, M., Peek Jr., R.M., 2016. Pathobiology of Helicobacter pylori-induced gastric cancer. Gastroenterology 150 (1), 64–78.

Backert, S., Tegtmeyer, N., Fischer, W., 2015. Composition, structure and function of the Helicobacter pylori cag pathogenicity island encoded type IV secretion system. Future Microbiol. 10 (6), 955–965.

Backert, S., Haas, R., Gerhard, M., Naumann, M., 2017. The Helicobacter pylori type IV secretion system encoded by the cag pathogenicity island: architecture, function, and signaling. Curr. Top. Microbiol. Immunol. 413, 187–220.

Correa, P., Houghton, J., 2007. Carcinogenesis of Helicobacter pylori. Gastroenterology 133 (2), 659–672.

Dixon, M., Genta, R., Yardley, J., Correa, P., 1996. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am. J. Surg. Pathol. 20 (10), 1161–1181.

Doohan, D., Miftahussurur, M., Matsuo, Y., Kido, Y., Akada, J., Matsuhisa, T., Yee, T.T., Htet, K., Aftab, H., Vilaichone, R.K., Mahachai, V., Ratanachu-Ek, T., Tshering, L., Waskito, L.A., Fauzia, K.A., Uchida, T., Syam, A.F., Rezkitha, Y.A.A., Yamaoka, Y., 2019. Characterization of a novel Helicobacter pylori East Asian-type CagA ELISA for detecting patients infected with various cagA genotypes. Med. Microbiol. Immunol.

Graham, D.Y., 2014. History of Helicobacter pylori, duodenal ulcer, gastric ulcer and gastric cancer. World J. Gastroenterol. 20 (18), 5191–5204.

Higashi, H., Tsutsumi, R., Fujita, A., Yamazaki, S., Asaka, M., Azuma, T., Hatakeyama, M., 2002. Biological activity of the Helicobacter pylori virulence factor CagA is determined by variation in the tyrosine phosphorylation sites. Proc. Natl. Acad. Sci. U. S. A. 99 (22), 14428–14433.

Kanada, R., Uchida, T., Tsukamoto, Y., Nguyen, L.T., Hijiya, N., Matsuura, K., Kodama, M., Okimoto, T., Murakami, K., Fujioka, T., Yanagisawa, S., Moriyama, M., 2008. Genotyping of the cagA gene of Helicobacter pylori on immunohistochemistry with East Asian CagA-specific antibody. Pathol. Int. 58 (4), 218–225.

Matsunari, O., Miftahussurur, M., Shiota, S., Suzuki, R., Vilaichone, R.K., Uchida, T., Ratanachu-ek, T., Tshering, L., Mahachai, V., Yamaoka, Y., 2016. Rare Helicobacter pylori virulence genotypes in Bhutan. Sci. Rep. 6, 22584.

Miftahussurur, M., Shiota, S., Suzuki, R., Matsuda, M., Uchida, T., Kido, Y., Kawamoto, F., Maimunah, U., Adi, P., Rezkitha, Y., Nasronudin, Nusi, I., Yamaoka, Y., 2015a. Identification of Helicobacter pylori infection in symptomatic patients in Surabaya, Indonesia, using five diagnostic tests. Epidemiol. Infect. 143 (5), 986–996.

Miftahussurur, M., Syam, A.F., Makmun, D., Nusi, I.A., Zein, L.H., Zulkhairi, Akil, F., Uswan, W.B., Simanjuntak, D., Uchida, T., Adi, P., Utari, A.P., Rezkitha, Y.A., Subsomwong, P., Nasronudin, Yamaoka, Y., 2015b. Helicobacter pylori virulence genes in the five largest islands of Indonesia. Gut Pathog. 7, 26.

Miftahussurur, M., Syam, A.F., Nusi, I.A., Makmun, D., Waskito, L.A., Zein, L.H., Akil, F., Uwan, W.B., Simanjuntak, D., Wibawa, I.D., Waleleng, J.B., Saudale, A.M., Yusuf, F., Mustika, S., Adi, P., Maimunah, U., Maulahela, H., Rezkitha, Y.A., Subsomwong, P., Nasronudin, Rahardjo, D., Suzuki, R., Akada, J., Yamaoka, Y., 2016. Surveillance of Helicobacter pylori antibiotic susceptibility in Indonesia: different resistance types among regions and with novel genetic mutations. PLoS One 11 (12), e0166199.

Miftahussurur, M., Doohan, D., Nusi, I.A., Adi, P., Rezkitha, Y.A.A., Waskito, L.A., Fauzia, K.A., Bramantoro, T., Maimunah, U., Thamrin, H., Masithah, S.I., Sukadiono, S., Uchida, T., Lusida, M.I., Yamaoka, Y., 2018. Gastroesophageal reflux disease in an area with low Helicobacter pylori infection prevalence. PLoS One 13 (11), e0205644.

Miftahussurur, M., Waskito, L.A., Syam, A.F., Nusi, I.A., Siregar, G., Richardo, M., Bakry, A.F., Rezkitha, Y.A.A., Wibawa, I.D.N., Yamaoka, Y., 2019. Alternative eradication regimens for Helicobacter pylori infection in Indonesian regions with high metronidazole and levofloxacin resistance. Infect. Drug Resist. 12, 345–358.

Nguyen, L.T., Uchida, T., Kuroda, A., Tsukamoto, Y., Trinh, T.D., Ta, L., Mai, H.B., Ho, D.Q., Hoang, H.H., Vilaichone, R.K., Mahachai, V., Matsuhisa, T., Kudo, Y., Okimoto, T., Kodama, M., Murakami, K., Fujioka, T., Yamaoka, Y., Moriyama, M., 2009.

Evaluation of the anti-East Asian CagA-specific antibody for CagA phenotyping. Clin. Vaccine Immunol. 16 (11), 1687–1692.
Peek Jr., R.M., Blaser, M.J., 2002. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nature reviews. Cancer 2 (1), 28–37.
Subsomwong, P., Miftahussurur, M., Vilaichone, R.K., Ratanachu-Ek, T., Suzuki, R., Akada, J., Uchida, T., Mahachai, V., Yamaoka, Y., 2017. Helicobacter pylori virulence genes of minor ethnic groups in North Thailand. Gut Pathog. 9, 56.
Suzuki, N., Murata-Kamiya, N., Yanagiya, K., Suda, W., Hattori, M., Kanda, H., Bingo, A., Fujii, Y., Maeda, S., Koike, K., Hatakeyama, M., 2015. Mutual reinforcement of inflammation and carcinogenesis by the Helicobacter pylori CagA oncoprotein. Sci. Rep. 5, 10024.
Syam, A.F., Miftahussurur, M., Makmun, D., Nusi, I.A., Zain, L.H., Zulkhairi, Akil, F., Uswan, W.B., Simanjuntak, D., Uchida, T., Adi, P., Utari, A.P., Rezkitha, Y.A., Subsomwong, P., Nasronudin, Suzuki, R., Yamaoka, Y., 2015. Risk factors and prevalence of Helicobacter pylori in five largest Islands of Indonesia: a preliminary study. PLoS One 10 (11), e0140186.
Tserentogtokh, T., Gantuya, B., Subsomwong, P., Oyuntsetseg, K., Bolor, D., Erdene-Ochir, Y., Azzaya, D., Davaadorj, D., Uchida, T., Matsuhisa, T., Yamaoka, Y., 2019. Western-type Helicobacter pylori CagA are the most frequent type in mongolian patients. Cancers 11 (5).
Uchida, T., Kanada, R., Tsukamoto, Y., Hijiya, N., Matsuura, K., Yano, S., Yokoyama, S., Kishida, T., Kodama, M., Murakami, K., Fujioka, T., Moriyama, M., 2007. Immunohistochemical diagnosis of the cagA-gene genotype of Helicobacter pylori with anti-East Asian CagA-specific antibody. Cancer Sci. 98 (4), 521–528.
Waskito, L.A., Miftahussurur, M., Lusida, M.I., Syam, A.F., Suzuki, R., Subsomwong, P., Uchida, T., Hamdan, M., Nasronudin, Yamaoka Y., 2018. Distribution and clinical associations of integrating conjugative elements and cag pathogenicity islands of Helicobacter pylori in Indonesia. Sci. Rep. 8 (1), 6073.
Yamaoka, Y., 2010. Mechanisms of disease: helicobacter pylori virulence factors. Nat. Rev. Gastroenterol. Hepatol. 7 (11), 629–641.
Yamaoka, Y., Kodama, T., Kashima, K., Graham, D.Y., Sepulveda, A.R., 1998. Variants of the 3' region of the cagA gene in Helicobacter pylori isolates from patients with different H. pylori-associated diseases. J. Clin. Microbiol. 36 (8), 2258–2263.
Yasuda, A., Uchida, T., Nguyen, L.T., Kawazato, H., Tanigawa, M., Murakami, K., Kishida, T., Fujioka, T., Moriyama, M., 2009. A novel diagnostic monoclonal antibody specific for Helicobacter pylori CagA of East Asian type. APMIS 117 (12), 893–899.