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Original Article

Comparison between Immunohistochemistry and Modified Giemsa Staining for Identification of *Helicobacter spp* in Stomach Biopsy

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Abstract

Background : *Helicobacter pylori* is classified as a grade 1 carcinogen by the International Agency for Research on Cancer. Identification of *Helicobacter pylori* infection is crucial for the prevention of malignancy. Immunohistochemistry is more specific and sensitive than modified Giemsa because it based on the antigen - antibody binding so it can exclude other organisms. However, it can be expressed in all species of *Helicobacter* genus. The objectives if this study was to compare the identification methods of *Helicobacter spp* in stomach biopsy between modified Giemsa and immunohistochemistry.
Methods : This research was a cross-sectional study. There were 64 biopsies taken by simple random sampling. They consisted of 32 positive and 32 negative *Helicobacter spp* based on the interpretation of modified Giemsa. Statistical analysis using x2 test.
Results : The number of atypical *Helicobacter spp* was 42.%. The number of samples that received Proton Pump Inhibitor (PPI) therapy was 93.75%. *Helicobacter spp* was positive in 31 samples and negative in 33 samples based on the interpretation of immunohistochemistry staining. There was no significant difference (p=0.617) between the identification results of *Helicobacter spp* using immunohistochemistry compared to the Modified Giemsa at both 400x and 1000x magnification.
Conclusion : Modified Giemsa is still reliable for identifying *Helicobacter spp*, especially in classical form, compared to immunohistochemistry. Due to the administration of PPI, there are a lot of cases with atypical form of *Helicobacter spp* which can be differentiated into coccoid form and intraepithelial located. Immunohistochemistry staining is useful in identify these cases.

Keywords : anti-*Helicobacter pylori* immunohistochemistry; *Helicobacter spp*; *Helicobacter pylori* identification; modified Giemsa; staining methods.

INTRODUCTION

Helicobacter pylori is classified as a grade 1 carcinogen by the International Agency for Research on Cancer.[1] Untreated *Helicobacter pylori* infection is a risk factor for gastric adenocarcinoma and primary B-cell lymphoma. The prevalence of *Helicobacter pylori* infection in 2015 was 22.1% in Indonesia.[2–6] *Helicobacter pylori* identification methods encompass non-invasive methods such as urea breath tests and invasive methods such as biopsies. A histopathological examination after biopsy has benefit of identifying *Helicobacter pylori* and simultaneous evaluation of any related mucosal lesions, such as inflammation, ulceration, atrophy, intestinal metaplasia, dysplasia, and/or malignancy.[7–11] The type of staining used includes routine histochemical staining of Hematoxylin Eosin (HE) followed by additional histochemical staining, immunohistochemistry, as well as FISH and PCR tests.[9,12] There has been controversies on these methods. Histochemical staining such as modified Giemsa is cheaper yet unspecific. Immunohistochemistry is specific to genus *Helicobacter* and not expressed in other organisms. However, there is still potential for cross-reactivity with *Helicobacter heilmannii*.[7] FISH and PCR are specific for *Helicobacter pylori* but are less applicable for daily practice. Boldt and Fan recommend routinely additional modified Giemsa histochemical staining,[13,14] while Glickman, Lash, Prabhu, and Loharamtaweethong recommend routine immunohistochemistry staining,[6,8,15,16] and others recommend HE staining for routine analyses; additional staining can be performed as needed.[2,10,11,17–19]

Modified Giemsa staining is routinely used to identify *Helicobacter spp* in Dr. Kariadi Central General Hospial. In this study we compare the identification methods of *Helicobacter spp* in stomach biopsy between modified Giemsa and immunohistochemistry. We aim for further understanding on the benefit of immunohistochemistry use for *Helicobacter spp* identification in daily practice.

METHODS

This research has been approved by the Health Research Ethics Committee of Dr. Kariadi Central General Hospital (1025/EC/KEPK-RSDK/2022) with research permission number DP.02.01/I.II/1485/2022. This analytical study used a cross-sectional design and was conducted from May to November 2022 in Anatomical Pathology Department of Integrated Laboratory Installation in Dr. Kariadi Central General Hospital. There were 320 gastric biopsies from patients undergoing endoscopic procedures with positive *Helicobacter spp* in 70 biopsies (21.9%) and negative in 250 biopsies (78.1%) using modified Giemsa. Simple random sampling was carried out to obtain 32 positive and 32 negative samples. The modified Giemsa slides used the Kwik-Diff (Shandon™) stain. Formalin- fixed, paraffin-embedded (FFPE) block were cut for immunohistochemistry slides with 4 microns in thickness. Antigen retrieval used citrate buffer pH 9.0 and heated in a decloaking chamber at 96°C for 40 minutes. Immunohistochemistry staining used primary monoclonal antibody anti-*Helicobacter pylori* (Biocare CM 383A, clone BC7, immunogen: whole lysate) with 1 hour incubation at 26.5°C. The positive control was attained from Awal Bros Hospital Batam.

Each slide of HE, modified Giemsa and immunohistochemistry was taken 1 field of 400x and 1000x magnification. Identification of *Helicobacter spp* was carried out by 2 observers blindly. Immunohistochemistry staining positivity defined by brown-stained bacterial structure. The bacterial structure is differentiated into 3 forms: classical form (spiral shaped), atypical coccoid and intraepithelial located.[20] Positivity with modified Giemsa staining defined by blue-stained bacterial structure.

Data analysis used SPSS version 22.0. Because there is no expected sample lower than 5 samples in each cell of the table, so it met the requirements for using the x^2 test. The x^2 was used to analyze the comparation in the identification of *Helicobacter spp* based on immunohistochemistry staining and modified Giemsa and the Kappa test to assess interobserver agreement.

RESULTS

The characteristics of the research sample based on the results of Observer I can be seen in Table 1. The patients' age ranges from 12 to 76 years old, with an average age of 51 years and a standard deviation of 14 years. The sample characteristics based on age group showed that *Helicobacter spp* infection was most found in adults (18–59 years). Based on gender, the results of positive identification of *Helicobacter spp* were found more frequently in men (Table 1).

The statistical analysis of the comparison between *Helicobacter spp* identification using immunohistochemistry and modified Giemsa (Table 2) was no significant difference ($p>0.05$), with Kappain moderate level of interobserver agreement (0.717 at 400x magnification and 0.748 at 1000x magnification). Representative images of *Helicobacter spp* cases based on immunohistochemistry and modified Giemsa can be seen in Figure 1.

The majority of the positive samples shows atypical *Helicobacter spp* (Figure 2). Sixty patients (93.75%) received intravenous or oral proton pump inhibitors such as omeprazole, lansoprazole and esomeprazole, whereas the other 4 patients (6.25%) had no information of proton pump inhibitor history. Those 4 patients had no history of antibiotic use and no previous endoscopic procedures and biopsies.

TABLE 1
Characteristics of the research samples

Variable	IHK (+) positive (n)	MG (+) positive (n)	IHK (-) negative (n)	MG (-) negative (n)	Total (n)	(%)
Age						
Younger than 18 years old	0	0	1	1	1	1.56
18–59 years old	21	23	25	23	46	71.88
60 years old or more	10	9	7	8	17	26.56
Gender						
Female	13	15	15	13	28	43.75
Male	18	17	18	19	36	56.25
Endoscopic finding						
Gastric within normal limits	2	2	3	3	5	7.81
Antral Gastritis	8	8	11	11	19	29.69
Erosive Gastritis	5	5	3	3	8	12.50
Chronic Gastritis	1	0	0	1	1	1.56
Superficial Gastritis	0	0	1	1	1	1.56
Gastroduodenitis	0	1	2	1	2	3.13
Duodenal ulcer	2	3	1	0	3	4.69
Pangastritis	7	8	6	5	13	20.31
Gastric ulcer	6	5	6	7	12	18.75

IHK: Immunohistochemistry; MG: Modified Giemsa

TABLE 2
Differences between the identification of *Helicobacter spp* using immunohistochemistry and modified Giemsa based on the evaluation of observer I

Helicobacter spp		Immunohistochemistry		P*
		Positive	Negative	
Modified Giemsa	Positive	17 (53.1%)	15 (46.9%)	0.617
	Negative	14 (43.8%)	18 (56.3%)	

*statistically significant if P value less than 0.05

DISCUSSION

There has been little data in recent studies about the prevalence of *Helicobacter pylori* in general because latest studies focus on the specific strain of *Helicobacter pylori* using molecular test or immunohistochemistry that specific to evaluate the antibiotic resistance types which is still not applicable to do widely in the daily practice.[21] Some recent articles in Indonesia still cite the large scale study about *Helicobacter pylori* prevalence in 2015.[4,22] The overall sample characteristic of this study consistent with previous study in prevalence, age and gender distribution. *Helicobacter pylori* infection is usually found in childhood and symptoms appear in adulthood.[2,10,17] The reduction in infections in the elderly group in this study was directly proportional to the number of patients in that age group. There was higher prevalence of infection in men. Some researchers have linked lifestyles such as smoking, alcohol consumption, and diet types with *Helicobacter pylori* infection.[23,24] *Helicobacter spp* can

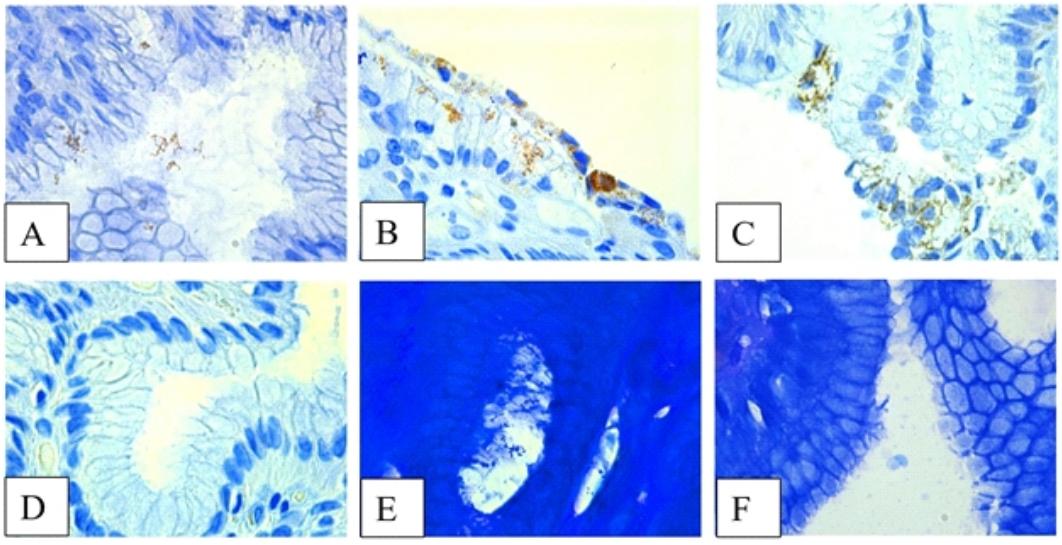

Figure 1. Microscopic figure of immunohistochemistry and modified Giemsa staining in 1000x magnification. A, *Helicobacter spp* in classical spiral form (immunohistochemistry). B, *Helicobacter spp* in atypical coccoid form (immunohistochemistry). C, *Helicobacter spp* are located intraepithelial (immunohistochemistry). D, *Helicobacter spp* negative (immunohistochemistry). E, *Helicobacter spp* positive (modified Giemsa staining). F, *Helicobacter spp* negative (modified Giemsa staining).

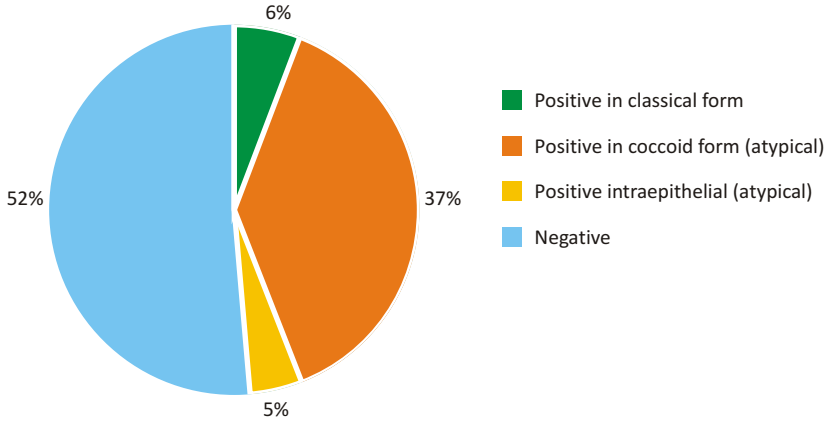

Figure 2. *Helicobacter spp* identification based on the morphology by immunohistochemistry. The positive results were classified as classical form, atypical in coccoid form and atypical in location (intraepithelial).

be found in the normal endoscopic findings, this was in accordance with previous studies which stated that *Helicobacter spp* infection can also be found in endoscopically normal mucosa, redness, and mild gastritis without signs of erosion.[25,26]

The results of the statistical analysis showed no significant difference in the identification of *Helicobacter spp* by modified Giemsa compared to immunohistochemistry. The result of this study is in line with other studies which stated that there was no difference in the identification of *Helicobacter pylori* using immunohistochemistry compared to modified Giemsa,[27,28] but differed from several previous studies which stated that there were significant differences.[8,11] Therefore, routine use of modified Giemsa can still be recommended.

Modified Giemsa staining is reliable to identify classical *Helicobacter spp*. In the normal condition, *Helicobacter pylori* has a classical a spiral or comma shape.[2,29] The classical form can turn to be atypical because of several factors. The atypical form consists of coccoid form and intraepithelial located.[20] When starved of nutrients, *Helicobacter pylori* converts to the coccoid form[30] which is metabolically active, but cannot be cultured. This change can be induced by administration of antibiotics and Proton Pump Inhibitors (PPI). Besides, PPI can affect the histopathological finding including minimal inflammation, atrophic mucosal changes,

changes in the density of bacteria, reduced bacterial colonization of the antrum, and a concomitant increase in bacterial colonization of the corpus, a phenomenon called 'proximal shift'. It can also affect the location of the bacteria that were originally in the mucous layer to become intraepithelial. The number of patients with minimal infection response and atypically shaped bacteria is increasing so that additional staining methods are needed to increase the accuracy of the diagnosis.[8,10–12,30,31]

This study showed that the number of samples of atypical bacteria was higher than in previous studies.[6,8,10,32] This is possibly due to the high number of PPI use prior to endoscopy. A previous study by Nurdin *et al* showed coccoid-shaped bacteria at 26.67% based on immunohistochemistry, although it did not consider the atypical category based on other criterias.[10] Glickman's study showed an atypical number of 10.7% of all samples with positive *Helicobacter pylori* with various methods. The difference may be caused by different criteria for atypical category including rare number of bacteria, minimal inflammation, and location of bacteria.[6]

Modified Giemsa cannot distinguish between *Helicobacter spp* in coccoid forms and cocci bacteria.[7,28,32] False positive results on modified Giemsa may be debris, thick straight rod-shaped bacteria, contaminants from consumed food, or contaminants from water baths that are also stained blue on modified Giemsa. Some studies note that false negatives can occur with modified Giemsa, especially in cases with low bacterial counts or patients undergoing incomplete therapy There is a possibility that the distribution of *Helicobacter pylori* is unequal and focal in the gastric mucosa, especially in cases with low bacterial density. Some small clusters or individual scatters of bacteria may not be sectioned in some slides.[7,11,12] Immunohistochemistry can identify bacteria with an intraepithelial location and coccoid forms because the positive stain colour contrasts with the background and it is specific for *Helicobacter spp* because of the principle of antibody and antigen binding.[7] Immunohistochemistry staining can be helpful in cases of negative bacteria on Hematoxylin Eosin (HE) and modified Giemsa with inflammation, post-therapy, atypical structures, intestinal metaplasia, gland atrophy for efficiency.[7,13,17,33,34]

If only histochemical stains such as Modified Giemsa are available for the identification of *Helicobacter spp* from gastric biopsy, it is necessary to ensure that the bacteria can be detected based on classic morphological features. In daily practice, it is often difficult to diagnose *Helicobacter spp* infection due to the finding of structures resembling atypical bacteria, although statistically there is no significant difference in this study. In such cases, an endoscopy should be performed after discontinuation of the consumption of PPI and antibiotics[33] at least for 2 weeks as in the Urea Breath Test. There should be a consensus with Gastroenterologists and Pathologist regarding patient preparation, criteria for collection, management, delivery of adequate samples for histopathological examination, and optimal Anatomic Pathology diagnosis results for patient management. Although the use of PCR may still be difficult to apply in daily practice, PCR analysis is considered as the gold standard compared to the Urea Breath Test, HE staining, modified Giemsa or immunohistochemistry. Previous studies have shown that immunohistochemistry has the same level of reliability as FISH and real-time PCR.[7,35]

This limitation of this study is on *Helicobacter spp* sampling from the FFPE. The modified Giemsa slides used for this study were readily available slides while the immunohistochemistry slides were newly cut from FFPE borrowed from the archives. The effect of cutting FFPE at different times can cause cutting levels that are too far apart so that there is a large depth level distance and cause differences in results due to sample heterogeneity. Thus, the results of the study may not reflect the actual conditions. This is also found in daily practice and is a common limitation in research.

CONCLUSION

There is no significant difference in identifying *Helicobacter spp* using immunohistochemistry compared with modified Giemsa. This study showed that modified Giemsa is still reliable for identifying *Helicobacter spp*, especially in classical form, compared to immunohistochemistry staining. Due to the administration of PPI, there are a lot of cases with atypical form of *Helicobacter spp* which can be differentiated into coccoid form and intraepithelial located. Immunohistochemistry staining is useful in identify these cases. Further studies are needed to define better comparison between immunohistochemistry and modified Giemsa staining with typical cases of *Helicobacter spp* which has not been treated by PPI. In addition, it is necessary to do research on the use of immunohistochemistry and modification of Giemsa when compared to molecular assays such as PCR as a gold standard.

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