Detecting the *Helicobacter pylori* 16S rRNA Gene in Dyspepsia Patients Using Real-Time PCR

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ABSTRAK

Latar belakang: deteksi dini *H. pylori* sangat diperlukan untuk mencegah berkembangnya infeksi menjadi keganasan lambung. Bentuk kokoid dari *H. pylori* sulit dideteksi dengan biakan dan histopatologi, namun dapat terdeteksi dengan metode molekuler misalnya real-time PCR. Penelitian ini diharapkan dapat memberikan informasi baru tentang pengembangan deteksi *H. pylori*. *Metode:* studi potong lintang dilakukan di Pusat Endoskopi Saluran Cerna, Rumah Sakit Cipto Mangunkusumo (RSCM) sejak Oktober 2016 hingga Agustus 2017. Metode pengambilan sampel yang digunakan yaitu consecutive sampling. Biopsi dari antrum dan korpus lambung dilakukan terhadap 64 pasien. Dua spesimen masing-masing dari antrum dan korpus dikumpulkan untuk pemeriksaan real-time PCR dan histopatologi. Mula-mula dilakukan optimasi kondisi real-time PCR, kemudian aplikasi pada sampel klinis. Analisis data menggunakan uji McNemar's χ2 dan Kappa. *Hasil:* proporsi positif hasil pemeriksaan real-time PCR sebesar 25%; sedangkan proporsi positif pemeriksaan histopatologi sebesar 14%. Real-time PCR memiliki sensitivitas dan spesifisitas sebesar 88,9% dan 85,5%. Hasil uji Mc Nemar χ2 menunjukkan terdapat perbedaan yang signifikan (p=0,039) antara kedua uji dengan nilai kappa (p=0,561). *Kesimpulan:* pemeriksaan real-time PCR lebih sensitif dibandingkan dengan histopatologi. Teknik ini mampu meningkatkan diagnosis sebesar 11% dibandingkan pemeriksaan histopatologi.

Kata kunci: H. pylori, histopatologi, real-time PCR.

ABSTRACT

Background: early detection of *H. pylori* is essential to prevent the development of infections into gastric malignancies. The coccoid form of *H. pylori* is difficult to detect either by culture or histopathology; however, it can be detected using molecular methods, such as real-time PCR. The study was expected to provide new information on the development of *H. pylori* detection. *Methods:* a cross-sectional study was conducted at the Gastrointestinal Endoscopy Center of Cipto Mangunkusumo Hospital between October 2016 and August 2017. The sampling method used was consecutive sampling. Biopsy from gastric antrum and corpus were performed in 64 patients. We collected 2 specimens from each site to be examined using real-time PCR and histopathology. Initially, we conducted real-time PCR optimization followed by application of clinical samples from gastric biopsy. Data analysis using McNemar's χ2 and Kappa tests. *Results:* the real-time PCR showed 25% positivity, while the positive proportion of histopathological examination was 14%. Real-time PCR has a sensitivity and specificity 88.9% dan 85.5%, respectively. The McNemar's χ2 test showed that there is significantly different (p=0.039)

between the two tests; kappa value (p=0.561). **Conclusion:** *the real-time PCR examination is more sensitive than histopathology. This technique can improve diagnosis by 11% compared to histopathological examination.*

Keywords: H. pylori, histopathology, real-time PCR.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, motile, spiral-shaped or curved rod bacteria that induces the development of chronic gastritis, gastric or duodenal ulcers, and gastric carcinoma.[1] It is estimated that *H. pylori* infection affects almost half of adult population worldwide and it accounts for 75% of all gastric cancer cases.[1,2] In 1994, the International Agency for Research on Cancer (IARC) classified *H. pylori* infection as a definitive type I carcinogen for humans.[2] The prevalence of *H. pylori* infection in developed countries is approximately 30 to 40%; while in developing countries, it may reach 80%. Indonesia as one of developing countries has a prevalence of 22.1 to 68% with the youngest patients of 6 years of age; moreover, the prevalence in Jakarta is 9.7 to 52.3%. It indicates that *H. pylori* infection occurs at an early age, especially in developing countries.[3-5] Therefore, laboratory procedures are essential for determining the diagnosis of *H. pylori* infection.

To date, accurate assessment of incidence and route of infection is still difficult to perform due to diagnostic inaccuracy. On the other hand, the effectiveness of treatment depends on the sensitivity and accuracy of the diagnostic approach. The importance of early detection of *H. pylori* infection and prompt treatment may prevent the development of infections into gastric malignancies. So far, there are two common diagnostic methods for *H. pylori* infection with different accuracy, i.e. non-invasive sampling method (without endoscopy) and invasive method (with endoscopy). The non-invasive examinations include serological tests, Urea Breath Test (UBT) and Stool Antigen Test. The invasive methods are histopathology, culture and Rapid Urease Test (RUT).[6] Histopathological examination of biopsy samples has an important role in the diagnosis of H.pylori for pathological changes.[7] Improper biopsies, observer-related

factors, topographical changes in the stomach, *H. pylori* density including its patchy distribution and types of staining techniques may cause false results.[8] Often, characteristic histological pattern of chronic active gastritis prompts a pathologist to scrutinize the biopsy specimen for typical helical morphologic profile of *H. pylori*.[8]

Under certain conditions, in which the patients must continue their treatment, a biopsy examination is still recommended for histopathology. However, classical histopathological staining methods cannot identify low numbers or coccoid forms of *H. pylori*. PCR method offers advantages over culture and histopathology as it can detect the coccoid form of the bacteria.[9] PCR is also a faster diagnostic techniques compared to culture since there is a slow proliferation of *H. pylori*, in which it needs 4 to 10 days to grow in cultures and it requires very specific (microaerophilic) condition.[10] Therefore, sensitive and specific tests, such as real-time PCR are essential. Over the last few years, real-Time PCR has developed rapidly. Real-time PCR has been known to be 10 times more sensitive than conventional PCR.[11,12] In Indonesia, the real-time PCR method has not been developed for the diagnosis of *H. pylori* infection. Our study was aimed to develop a real-time PCR test as a diagnostic approach for detecting *H. pylori* 16S rRNA gene using specific primer. The study was expected to provide new information on the development of *H. pylori* detection.

METHODS

This is a cross-sectional study conducted at the Gastrointestinal Endoscopy Center of Cipto Mangunkusumo Hospital between October 2016 and August 2017. Patients with a complaint of dyspepsia and undergone endoscopic examination as well as biopsy were recruited. Biopsy from gastric antrum and corpus were performed in 64 patients. We collected 2 specimens from each site and the specimens were examined using real-time PCR

and histopathology. Each of biopsy specimens was placed in a tube containing 0.9% NaCl for real-time PCR and 10% formalin for histopathological examination. We excluded patients who had taken any antibiotic treatment within the previous 2 weeks. Informed consent had been obtained from all participants. The study had been approved by the Ethics Committee of Faculty of Medicine Universitas Indonesia on October 17th, 2016, with a reference number of 888/UN2.F1/ETIK/2016.

Histopathology

Specimens were stained with hematoxylin and eosin (H&E) staining and Giemsa, which were evaluated by several pathologists blinded to the results of the real-time PCR tests. Histopathology was considered as the gold standard.

Real-Time PCR

The study used primers and probes based on Lazaro et al.[24] The DNA of bacterial control was obtained from a paraffin block gastric biopsy specimen that showed positive result of *Helicobacter pylori* by histopathological examination.

The isolation process was performed following the user guide of commercial DNA Extraction Kit (QIAGEN) protocol with a modification on overnight tissue incubation; while the optimization process included primer annealing temperature, primer concentration, probe concentration, DNA template volume testing as well as sensitivity and specificity of the technique.

Detection sensitivity assay were done to identify the lower detection limit of the real-time PCR analyses. The analytical sensitivity of the real-time PCR assay was assessed using 10-fold serial dilutions of DNA (10-fold to 10^{-11}). Total DNA dissolved in DNase free water at a concentration of 3.8 x 10^{-11} ng/µl (Nanodrop). Using Avogadro's number to determine the amount of copy, this amount is equivalent to 1 copy/µl. This calculation is based on the assumption that the average weight of a base pair (bp) is 650 Daltons. This means that one mole of a bp weighs 650 g and that the molecular weight of any double stranded DNA

template can be estimated by taking the product of its length (in bp) and 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro's number, 6.022×10^{23} molecules/mole, the number of molecules of the template per gram can be calculated.

The specificity of the test was validated by examining other microorganisms, such as *Enterococcus faecalis*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Pseudomonas aeruginosa*, *Candida albicans*, *Haemophilus influenzae*, *Campylobacter jejuni*, *Herpes Simpex virus*, *Varicella Zooster*, *Cytomegalovirus*, and *Epstein Bar viruses*.

A 20µl PCR reaction mixture was prepared with 10 µl of Kappa probes fast qPCR kit, 0.8 µM primer concentrations, 0.6 µM probe concentrations and distilled water. Amplification 16S rRNA gene was accomplished by real-time monitoring of fluorescence intensity during PCR using TaqMan probes. The real-time PCR conditions were: 95°C for 3 min to activate the enzyme, 45 cycles of 15 sec at 95°C (denaturation) and 1 minute at 64°C (annealing and elongation). The real-time PCR was considered positive based on positive results of specimens obtained from gastric antrum and/or corpus.

Statistical Analysis

The data was analyzed using SPSS software program version 22.0 (Chicago, USA). The data of positive and negative real-time PCR and histopathology was presented in a 2x2 table. The results were subsequently represented as sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). Receiver-operating characteristics (ROC) curve was prepared (plot of sensitivity vs. 1-specificity) and the areas under the curves (AUC) estimated. AUC=1 indicates a perfect test, AUC>0.9 indicates high accuracy and AUC between 0.7 and 0.9 indicates moderate accuracy. The test results were compared using the McNemar's χ2 and kappa test.

Table 1. Primer and probe sequence for *H. pylori* detection

Gene	Forward Sequence	Reverse Sequence	Probe Sequence	Amplicon Size (bp)
16S rRNA	CTCATTGCGAAGGCGACCT	TCTAATCCTGTTTGCTCCCCA	FAM-ATT ACT GAC GCT GAT TGC GCG AAA GC-TAMRA	75

Table 2. Comparison of real-time PCR results between specimens obtained from gastric antrum and corpus biopsy

	Gastric biopsy		
	Antrum	**Corpus**	**Antrum and Corpus**
Real-time PCR Test			
Positive	13/64 (20%)	10/64 (16%)	7/64 (11%)
Negative	51/64 (80%)	54/64(84%)	57/64 (89%)
Total	64 (100%)	64 (100%)	64 (100%)

Table 3. Comparison of real-time PCR positive results between specimens obtained from gastric antrum and corpus in patients receiving PPI medication

	Gastric biopsy		
	Antrum	**Corpus**	**Antrum and Corpus**
PPI medication within the last 2 weeks			
Yes	3/17 (18%)	3/17 (18%)	1/17 (6%)
No	14/17 (82%)	14/17 (82%)	16/17 (94%)
Total	17 (100%)	17 (100%)	17 (100%)

RESULTS

In this study, the maximum used of tissue was 200 mg (according to the provisions of the extraction kit from Qiagen). One microlitter is equivalent to 1 mg, then 0.469 copy/μl is converted to 93.8 copies/200 mg. One amplification was found that there were 2 copies of 16S rRNA, so the DNA detection threshold was obtained by real-time PCR examination was 46 bacteria.

Real-time PCR and Histopathology examination on Clinical Samples

A total of 64 study subjects consisted of 43 (67%) women and 21 (33%) men were recruited in this study. Of the 17 positive (real-time PCR or histopathology) subjects, 12 (71%) were female and 5 (29%) were male subjects, between 22-80 years old and median 55 years. Thirty five out of 64 subjects without PPI (Proton Pump Inhibitor) therapy for 2 weeks. The antrum specimen showed 20% positivity, while the positive result of corpus specimen was 16 %. Comparison of Real-Time PCR results on antrum and corpus

gastric biopsy these results can be seen in **Table 2**.

The positive results from antrum or corpus on subjects who consumed PPI in the last 2 weeks were 3 subjects (18%). In addition, only one positive subject from antrum and corpus were known to has consumed PPI in the last 2 weeks.

The real-time PCR showed 25% positivity, while the positive result of histopathology examination was 14%. There were 8 subjects (13%) positive based on real-time PCR but negative histopathology. While 1 subject (2%) was negative based on real-time PCR but positive histopathology. There were 8 (13%) subjects showing positive results from both real-time PCR and histopathology. (**Table 4**)

McNemar's χ2 showed ample evidence that the positive proportion of the real-time PCR compared to the reference tests was significantly different p=0.039 (p≤0.05). The agreement when assessed with kappa statistic showed a good to moderate agreement of real-time PCR with the reference test.

**Table 4.** Comparison of real-time PCR and histopathology test

	Histopathology		Total
	Positive	Negative	
Real-time PCR			
Positive	8/64 (13%)	8/64 (13%)	16/64 (25%)
Negative	1/64 (2%)	47/64 (73%)	48/64 (75%)
Total	9/64 (14%)	55/64 (85%)	64/64 (100%)

Table 5. Result of the study

	Histophatology					
	Se % CI (95%)	Sp % CI (95%)	PPV % CI (95%)	NPV % CI (95%)	LR+ CI (95%)	LR- CI (95%)
Real-time PCR	88.9 (68.3-100)	85.5 (76.1-94.8)	50 (25.5-74.5)	97.9 (93.9-100)	6.11 (3.1-12.1)	0.13 (0.02-0.83)

Definition of abbreviations: Se=sensitivity; Sp=specificity; PPV=positive predictive value; NPV=negative predictive value; LR+=likelihood ratio+; LR-=likelihood ratio

The ROC curve was drawn and the AUC was calculated by considering the histopathologic report as the standard test. We found the AUC to be 0.872 (CI 95%: 0.74 – 1.00) indicating a moderate accuracy for real-time PCR (**Figure 1**).
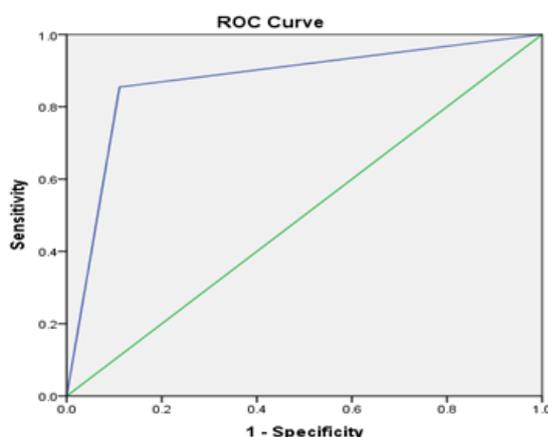
Figure 1. ROC curve

DISCUSSION

Before optimizing the real-time conditions, we selected the target genes used for real-time PCR. The 16S rRNA gene has been known to show better sensitivity than other target genes for *H. pylori* detection. A study conducted by Lazaro et al[24] evaluated the sensitivity of several genes for *H. pylori* detection including the 16S rRNA, 23S rRNA and UreA. The sensitivity of the three genes was 55%, 41% and 43% for 16S rRNA, 23S rRNA and UreA, respectively. Another study performed by Sugimoto et al[13] has compared five primers representing the 16S rRNA gene, HP0075-0076, ureA, VacA, glmM. The sensitivity of the five genes for *H. pylori* detection in gastric biopsy samples were as follows: 16S rRNA (94%), HP0075-0076 (84%), UreA (88%), VacA (88%) and glmM (90%). Another study by Peek et al,[14] which utilized the 16S rRNA, ureA, and cagA genes for detecting of *H. pylori*, has demonstrated the sensitivity of 24/25 (96%), 16/25 (64%) and 14/25 (56%), respectively. A study by Ciesielska et al[15] has also shown that PCR assay with the target gene of 16S rRNA would have a 38% more positive result compared with the ureC genes. The 16S rRNA gene has been known to be selective for the *H. pylori* species alone; therefore, it can be used as PCR target for *H. pylori* detection.[16]

The sensitivity of real-time PCR assay based on DNA concentration in our study was 46 bacteria. Our result was similar to those of Saez et al[17] who used a real-time PCR test with a sensitivity of 40 bacteria per sample. Another study conducted by Ottiwet et al[18] using conventional PCR and Nested PCR showed that the detection limit were 70 and 15 bacteria, respectively. Their study utilized TaqMan probe for real-time PCR. The advantage of TaqMan probe includes its high sensitivity and specificity,

which has been demonstrated by the lower detection limit found in the study. These results bring us to a conclusion that real-time PCR using TaqMan Probes assay has fewer minimum detection limit, which indicates that the assay is more sensitive than histopathology.[19]

The specificity of the PCR real-time test in our study was evaluated against other microorganisms that could potentially lead to false-positive results. These microorganisms include *Enterococcus faecalis, Proteus vulgaris, Klebsiella pneumoniae, Escherichia coli, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Pseudomonas aeruginosa, Candida albicans, Haemophilus influenzae, Campylobacter jejuni, Herpes simpex virus, Varicella Zooster virus, Cytomegalovirus* and *Epstein virus Bar*. The specificity of the real-time PCR demonstrated that there was no cross-reaction with other microorganisms; therefore, the real-time PCR test can be considered specific.

Based on real-time PCR test results on antral biopsy specimens we found 20% positive results; while on the corpus specimens, it was at 16%. Our result is similar to that of Rune et al,[10] Saez et al[17] and Lee et al,[20] which suggest that PCR is more sensitive to gastric biopsy specimens in antrum than corpus.

A study conducted by Rune et al using a real-time PCR test showed that *H. pylori* DNA concentrations in the antrum was 1.83 times higher than the concentration in the corpus.[10] It might be explained by the pathogenesis of *H. pylori* infection as the colonization of the bacteria is present in the antrum. The bacteria colonize the gastric antrum to avoid parietal cells secreting acid in the corpus; however, in patients receiving PPI treatment, the pattern of *H. pylori* colonization is different.[10] To improve the positive results, sampling in our study was conducted in two sites, i.e. the antrum and corpus. When collecting sample, the same biopsy forceps were used to obtain sample specimens from gastric antrum and corpus. Contamination from both antrum and corpus samples may occur. Therefore, comparisons of real-time PCR results between specimens obtained from the gastric antrums and corpuses were presented

as additional results of the study, which were not included in the study objectives. In seven subjects, who had positive results of their specimens obtained from both gastric antrum and corpus, the *H. pylori* is probably present in both sites due to the large number of bacteria.

Out of 64 subjects, we found that 16 (25%) subjects showed positive results of *H. pylori* based on the real-time PCR test. The results of our study are similar to those of Kobayashi et al[22] that utilized a real-time PCR test with a 16S rRNA gene. Their study detected 11/43 (25%) positive samples. There were 8/64 (13%) positive subjects based on the real-time PCR and negative test results based on histopathological results. It may occur since *H. pylori* has an invisible coccoid morphology during histopathologic microscopic examination, which can be detected using PCR assay.[22] The change in bacterial morphology to coccoid form may be due to nutritional deficiencies, antibiotic treatment or PPI medication.[23] Furthermore, improper biopsy procedure in the *H. pylori* colony area, small biopsy size, lower number of *H. pylori* and patchy distribution of *H. pylori* in the gastric mucosa may have caused negative results found on histopathological examination.[11]

Histopathology examination in our study revealed that there were 14% subjects with positive results. We found different positive results between histopathological examination and real-time PCR test in 13% subjects. Such difference is similar to that of Tankovic et al[19], which showed different results in 12% subjects. It indicates that the real-time PCR test may improve the diagnosis by 11%. To avoid false-positive results, the risk of contamination during the real-time PCR testing stage had been minimized. Positive and negative controls were always included on each step of real-time PCR test procedure. DNA templates for positive controls were added after adding all sample templates and the procedure was performed in a separate room to avoid contamination.

In this study, we found that real-time PCR possesses a sensitivity and specificity of 88.9% and 85.5% respectively, which can be compared to results from other studies. The PPV and NPV were also 50% and 97.9% respectively. Macin et

al. reported sensitivity and specificity of 97.4% and 100% respectively.[24] Tankovic et al [19] found 98.2% and 87.8% for specificity and sensitivity, respectively.

However, there was 1 (2%) subject who had positive results on histopathology, but negative result on PCR assay. Such result is similar to that of Sukla et al[12] study who used real-time PCR that also found 1/57 (0.02%) negative result on their PCR assay, but positive result on histopathology. Moreover, a study by Triana et al[11] also showed positive result on histopathology, but negative result on conventional PCR in 1/30 (3.3%) subject.

Such results may occur due to inaccurate biopsy site, low concentration of DNA *H. pylori* which might be lost during the extraction procedure and the presence of Taq polymerase inhibitors.[10] In addition, false-positive results may be found due to the presence of other bacteria that have similar morphology to *H. pylori* on histopathologic examination. Uneven distribution of bacteria resulting in poor biopsy specimen collection for either histopathology or PCR assay that may lead to different outcomes. However, in our study, the prevalence of *H. pylori* at Cipto Mangunkusumo hospital was quite low. Further studies involving more subjects in a multicenter setting are necessary.

CONCLUSION

A real-time PCR assay can improve diagnosis of *H. pylori* infection by 11% compared to histopathology. Such information should be conveyed to clinicians to increase their awareness that real-time PCR can be used as an alternative tool to detect *H. pylori* infection. Furthermore, our study supports continuing development of diagnostic method for *Helicobacter pylori* infection.

CONFLICT OF INTEREST

The authors state that they have no conflict of interest, and no affiliation or connection to or with any entity or organization, which may raise a question of bias in discussion and conclusion of the manuscript.

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