Phylogenetic Analysis of *Helicobacter pylori* 16S rRNA Gene in Gastric Biopsy from Patients with Dyspepsia

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**Objective**: To investigate the relationships between *H. pylori* strains from samples with other countries. **Methods**: Biopsy was taken from antrum and corpus in 42 patients with dyspepsia. Of 42, only 8 samples that could be used for DNA sequencing analysis. The sequencing results are analyzed by BLAST nucleotides to show similarities with *H. pylori* strains in GenBank. Phylogenetic analysis was performed to determine the relationships among *H. pylori* strains from the sample with reference sequences from several other countries. Phylogenetic tree construction was done by paired sequence alignment using MEGA software v.6. **Result**: Although phylogenetic analysis showed different patterns to 18 other countries, but the homology of all strains had a high similarity among strains or other strains. **Conclusion**: Sequencing and phylogenetic analysis results showed that our strains were closely related to Taiwan, India and Australia strains.

**Keywords**: *H. pylori*, Gastric Biopsy, Phylogenetic Analysis.

1. INTRODUCTION

*Helicobacter pylori* is gram negative bacteria that colonizes the human gastric mucosa of approximately 50% of the human population.¹ The prevalence of *H. pylori* infection ranges from 25% in developed countries to more than 80% in the developing regions.² Indonesia as developing country, the prevalence is known around 22.1%.³ Chronic infection of *H. pylori* may develop into pathologies such as peptic ulcer diseases, precancerous lesions and ultimately induces gastric cancer.⁴

*Helicobacter pylori* infection is thought to be the result of direct human-to-human transmission via oral, fecal or both.⁵ Infection increases gradually with age.⁶ *Helicobacter pylori* is commonly transmitted from mother to child.⁶ Genetic studies have revealed that the diversity is geographically and ethnically structured.⁷ Genetic diversity of *H. pylori* appears to be a reflection of evolution through thousands of years even before migration out of Africa that lead to geographic spread throughout the world.¹ Ethnic differences and the spread between communities are an important mechanism for risk factors of *H. pylori* infection. In addition, the spread of *H. pylori* infection is also influenced by the migration between countries. *Helicobacter pylori* exhibits a high diversity of alleles and genetic diversity that results in infected individuals carrying different strains.⁸ Therefore, the most probable place for genetic recombination is human gastric mucosa and it is possible that during the long-term colonization the *H. pylori* strains may undergo adaptive changes.⁹ One of the genes that can be used as specific targets to confirm *H. pylori* infection is 16S rRNA, which is known to be specific and also used to analyze closely related strain. This study aimed to investigate the relationships between *H. pylori* strains from samples with other countries.

2. MATERIALS AND METHODS

2.1. Patients and Clinical Specimen

Forty-two patients with dyspepsia were recruited from among patients scheduled for Esophagogastroduodenoscopy (EGD) at Cipto Mangunkusumo General Hospital Jakarta, Indonesia, from February to May 2017. Informed consent was obtained from all participants, and the protocol was approved by the Ethics
Committee of Cipto Mangunkusumo General Hospital (Jakarta, Indonesia). Four gastric biopsy specimens were collected during each endoscopy session: two samples from the lesser curvature of the antrum approximately 3 cm from the pyloric ring and two samples from the greater curvature of the corpus. Two antral and corporal specimens were used for real-time PCR and histological examination. Biopsy specimens for real-time PCR were immediately placed in 0.9% NaCl.

2.2. DNA Extraction
DNA extraction was done on another study (not yet published). Briefly, two hundred milligrams of biopsy sample were extracted by using QIAamp DNA Mini Kit in accordance with manufacturer’s instructions (Qiagen, Germany). DNA was eluted in 40 μl elution buffer. The pure DNA was stored at −35 °C not more than 1 week until used for real-time PCR testing.

2.3. Real-Time PCR
Real-time PCR was performed on 42 subjects from another study (not yet published). Eleven positive samples from 42 samples based on real-time PCR were used in this study.

2.4. DNA Sequencing
Conventional PCR was performed by using primers (forward: 5’-GCTAAAGATACGCTATGTCC-3’ and reverse: 5’-GTCGAATCAGCCTATGTCCAA-3’), specific for 16S rRNA of H. pylori, as reported by Santhosh et al.10 PCR was performed in a 40 μl reaction volume containing 10 μl of DNA, 1 x PCR buffer, 1.6 mM MgCl2, 0.8 mM each dNTP, 5x Q solution, 1.2 μM each specific primer, 2.5 U of Taq polymerase (Hotstar Taq DNA Polymerase Qiagen) and 10 μl of distilled water. PCR products were conducted with the following thermal cycler conditions: for 95 °C for 15 min (activation); 40 cycles (amplification) at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min; and 72 °C for 10 min (extension). The DNA of bacterial control was obtained from a specimen that was positive of H. pylori, as reported by Santhosh et al.10 PCR was performed in a 40 μl reaction volume containing 10 μl of DNA, 1 x PCR buffer, 1.6 mM MgCl2, 0.8 mM each dNTP, 5x Q solution, 1.2 μM each specific primer, 2.5 U of Taq polymerase (Hotstar Taq DNA Polymerase Qiagen) and 10 μl of distilled water. PCR products were conducted with the following thermal cycler conditions: for 95 °C for 15 min (activation); 40 cycles (amplification) at 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min; and 72 °C for 10 min (extension). The DNA of bacterial control was obtained from a specimen that was positive of H. pylori, as reported by Santhosh et al.10

2.5. Phylogenetic Tree
To analyze the phylogeny, we downloaded 18 H. pylori 16S rRNA gene sequences from other countries, available in the NCBI database. The sequences were subjected to multiple sequence alignment using the MEGA software v.6. The MEGA software program v.6 was used in subsequent phylogenetic tree constructions and analysis. Phylogenetic trees were constructed by using the Maximum Likelihood method based on the Jukes-Cantor model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. All positions containing gaps and missing data were eliminated.

### 3. RESULTS

Of 42 samples, eleven was positive for H. pylori. Of 11 positive samples, only 8 samples (taken from 6 subjects) could be amplified by PCR for DNA sequencing of 16S rRNA gene (Figs. 1 and 2). In this study, we show the results of each antrum and corpus. Eight samples used for sequencing showed different positive results between antrum, corpus or both (Table I).

Therefore, we analyzed 8 Jakarta strains and 18 other strains available in Genbank database. Based on phylogenetic analysis, 26 H. pylori strains involved in this study were clustered into 4 clades (Figs. 1 and 2). Eight Jakarta strains (strains detected in this study) were clustered into 4 clades (I, II, III, and VI). Three Jakarta strains (41A, 41K, 42A) were closely related to strains from Taiwan and India. Two Jakarta strains (9K, 35K)

### Table I. Positive results based on the site of the biopsy

<table>
<thead>
<tr>
<th>No. of subjects</th>
<th>Site of biopsy</th>
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<tbody>
<tr>
<td>9</td>
<td>Corpus</td>
</tr>
<tr>
<td>13</td>
<td>Antrum</td>
</tr>
<tr>
<td>23</td>
<td>Antrum</td>
</tr>
<tr>
<td>35</td>
<td>Antrum and corpus</td>
</tr>
<tr>
<td>41</td>
<td>Antrum and corpus</td>
</tr>
<tr>
<td>42</td>
<td>Antrum</td>
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</tbody>
</table>
were closely related to a strain from India. Other Jakarta strains (13A, 23A) were closely related to a strain from Australia. Interestingly, two strains (35A and 35K) detected in antrum and corpus collected from one patient were clustered into different clades. It indicates that one patient could be infected by different bacterial strains.

Samples were collected from Jakarta, Indonesia. Of the six subjects, consisting of 2 tribes, Batak and Java. From six subjects, four subjects included in Batak tribe. Batak tribe originally lives in North Sumatra, while Java tribe lives in Java Island.

4. DISCUSSION

The 16S rRNA gene is one of the well-characterized genes that is used in the identification of microorganisms. This gene also has a hypervariable region that is characteristic of each microorganism. Primers used for detection of *H. pylori* on clinical samples have a shorter size (75 bp), so the results are less informative for sequencing. A longer primer for sequencing (522 bp) is considered to be sufficient for species identification. Therefore, for sequencing we used a different primer for detection of *H. pylori*.

Positive samples that had been detected with real-time PCR were then analyzed conventional PCR with the same target genes, but using longer amplicon size. Samples with Ct value of 20–25 can be successfully amplified by conventional PCR. This indicates that the amount of DNA in the sample is quite high and can be detected directly with a conventional PCR. While, samples with Ct value close to 40 must be re-runned using conventional PCR until showed visible target DNA bands. This can be the amount of DNA in the sample is too low. As we know, conventional PCR is 10 times less sensitive than real-time PCR. Therefore, not all samples can be confirmed by sequencing.11

In this study, phylogenetic analysis was based on the characteristics of the nucleotide of 16S rRNA gene. The 41A and 41K strains are detected from the same patient. This indicates that on the same individual, the same bacterial strain could infect different sites (antrum and corpus). The phenomenon is different for 35A and 35K strains, in which the strains were known to be originated from one patient, but they do not clustered into one clade. This may be due to a mix infection of different strains in one patient. The finding agreed with study conducted by Raymond et al.,12 who found that in one individual can be infected by several different strains.

For 41 and 42 strains, the strains were detected from sibling patients and clustered into one clade. This indicates that there is a potential bacterial transmission and/or lateral gene transfer occurring in one family. The transmission was more frequent among close relatives or individuals living in the same house.13

Strains 41 and 42 were closely related to strain from Taiwan and India. We also observed relatedness of strain 9 and 35 to strain from India. Strains 13 and 23 were closely related to strain from India and Australia, respectively. Indonesia is geographically adjacent to Australia, allowing the spread of *H. pylori* infection through cross-country visits. In addition, the countries of Taiwan and India are included in the Asian region which allows the spread of strains to Indonesia. Migrations of *H. pylori* were accompanied by two distinct populations, called hpSahul and hspMaori. According to archaeological history, hpSahul split from Asian populations of *H. pylori* 31,000 to 37,000 years ago. The hpSahul populations in New Guinea and Australia have diverged sufficiently to indicate that they have remained isolated for the past 23,000 to 32,000 years. The second human expansion from Taiwan occurred in 5000 years ago with one of several hspMaori clades into Melanesia and Polynesia. The sequence
of branching events within the Pacific clade is consistent with sequential migrations from Taiwan via the Philippines and island Melanesia to Polynesia. However, Indonesia as a wide country with spans almost 2 million square kilometers between Asia and Australia and consists of 300 distinct native ethnic groups. Other studies showed that Batak tribe has a higher risk greater than Javanese. In this study, we found that the ethnic majority is Batak. This can be caused by their diet habit, such as consumption of spicy and salty foods. A higher prevalence of the infection was reported among patients eating spicy and salty foods. A high salt concentration in the stomach destroys the mucosal barrier, favors colonization by H. pylori, and leads to inflammation and damage-causing gastritis.

5. CONCLUSION
The phylogenetic analysis showed that Jakarta strains were closely related to Taiwan, India and Australia strains. Additional studies are required on larger sample size to screen prevalent strains in Indonesia, determine their characteristics and relatedness, and make data available for clinical application.

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

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References and Notes

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