Comparison of Gastric Inflammation in Balb/c Inoculated by Three Unique EPIYT Motif CagA Gene Helicobacter pylori Bali Isolates Based on Update Sidney System Criteria

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ABSTRACT

Helicobacter pylori infection (H. pylori) is associated with gastric carcinoma and various of gastrointestinal disease. The prevalence of H. pylori infection in Indonesia is still controversial. There is no data about H. pylori Bali isolates and the Cag A virulence factor from patients with dyspepsia in Bali. H. pylori Bali isolates provided from Laboratorium of Microbiology, Biomedical Research Unit, West Nusatenggara General Hospital, which have been isolated from gastric antral biopsies collected from 3 gastric ulcer Balinese patients. This research revealed that all of three H. pylori Bali isolates examined positive for CagA (Cytotoxin associated antigen A) gene. Based on the result of the gel electrophoresis, a 640 bp single band observed similar in each sample. All H. pylori Bali isolates have EPIYA-ABD motif with unique motifs EPIYT on second EPIYA sequence. Three of H. pylori Bali isolates named as BLI 01, BLI 02 and BLI 03 that have a CagA genes. All the H. pylori Bali isolates have an unique EPIYT motifs (substitution A to T) in CagA genes. The degree of gastric inflammation is more severe in the 4th week of the study. There was a significant difference of gastric inflammation degree in all of three groups of mice. H. pylori BLI 03 isolate with positive CagA gene, have a greatest degree of inflammation.

Keyword: H. pylori, Cag A, EPIYA.

INTRODUCTION

Helicobacter pylori infection (H. pylori) is associated with gastrointestinal diseases including gastritis, peptic ulcer, gastric carcinoma and gastric mucosa-associated lymphoid-tissue lymphoma (MALT Lymphoma). H. pylori bacteria have been classified into class 1 carcinogen in humans by the International Agency for Research on Cancer Consensus Group since 1994.¹ Based on the involvement of inflammatory cells in the gastric mucosa, histologically, and onset of occurrence, the gastritis can be divided into acute gastritis and chronic gastritis. The main cause of chronic gastritis and peptic ulcer is infection of H. pylori.² H. pylori infects half of the world’s population.³,⁴ The prevalence of H. pylori infection in Indonesia still controversial. Studies by Syam AF et al in dyspepsia patients in Java, Papua, Sulawesi, Borneo and Sumatera found that the prevalence of H. pylori infection was 22.1%. People from Papua, Batak and Bugis ethnics have a higher risk H. pylori infection than Dayak, Chinese and Javanese.⁵ There is no data about H. pylori Bali isolates and the Cag A
virulence factor from patients with dyspepsia in Bali. The research purposes were to obtain H. pylori Bali isolates, and to study the development of gastric inflammation caused by H. pylori Bali isolates in Balb/c.

MATERIAL AND METHODS

Preparation of H. pylori Bali Isolates

We used Fujinon 2500 series to do esophagogastroduodenoscopy. Biopsies performed in 7 patients with gastric ulcer diagnoses, biopsies were performed 2 in anthrums and 2 in gastric corpus. We collected 3 Bali isolates of H. pylori of that patients. Biopsy results are inserted into CarryBlaire transport media, then sent to Biomedical Research Unit of West Nusa Tenggara Provincial Hospital for cultures. H. pylori cultures were performed using a blood agar plate with 7% of sheep blood, adding Skirrow supplements and Dent supplements buried using an microaerophilic anaerobic jar. H. pylori Bali isolates provided from Laboratorium of Microbiology, Biomedical Research Unit, West Nusatenggara General Hospital, which have been isolated from gastric antral biopsies collected from 3 Balinese patients (Gastric ulcer) stored at -80°C Celsius. Medical records of patients provided from Endoscopy Unit, Wangaya Hospital, Denpasar, Bali.

Amplification of the 3' Region of the cagA Gene

DNAZol Kit (Invitrogen) used to extraction DNA Genomic according to the manufacturer's instructions. Primers P1 (52-GA TAAACGGCAAGCCTTTTGAAG-32) and P2 (52 CTGC ACTAAGTTGGCAG-32) used to amplification of the cagA 32 variable region. The amplification steps were done under the conditions: 94°C for 1 min; 34 cycles of 94°C for 1 min and 55°C for 1 min, and 72°C for 1 min. Final extension was done at 72°C for 5 min. The mixture was stored at 4°C. PCR products were separated by 2% agarose gel electrophoresis and examined under UV illumination. The PCR products were then sequenced.

Prevalence of CagA gene

All of the three H. pylori Bali isolates examined were positive for CagA gene. Based on the result of the gel electrophoresis, a 640 bp single band were similar in each sample. Once confirmed by PCR that the growing isolate was H. pylori, it was then sequenced in Singapore to determine the sequence of the DNA (Deoxyribo Nucleic Acid) and the EPIYA motifs of CagA of the three H. pylori Bali isolates. Three of H. pylori Bali isolates named as BLI01, BLI 02 and BLI 03.

Alignments of the deduced amino acid sequence of the C-terminus of CagA between the BLI01, BLI02 and BLI03 strain and the representative East Asian CagA genotype shown at table 1. Subtitution A àT in EPIYA motif indicated in red eclipse. Alignments of the deduced amino acid sequence of the C-terminus of CagA between the BLI01, BLI02 and BLI03 and the representative East Asian CagA genotype shown at Figure 1. It was found that low variation of the amino acid sequence C-terminus contained EPIYA motif of CagA H. pylori Bali isolates. All H. pylori Bali isolates have EPIYA-ABD motif with unique motifs EPIYT on second EPIYA sequence.

Inoculation H. pylori Bali isolates in Balb/c

Three of H. pylori Bali isolates were used to study the gastric inflammation in Balb/c mice. H. pylori Bali isolates were inoculated into three groups of Balb/c to determine the degree of gastric inflammation. We used twelve males Balb/c aged 2-4 months. Mice were fasted for 24 hours before inoculation. Inoculation was done after the mice were given orally 0.25 ml of 0.2 M NaHCO3 solution to neutralized gastric acid. Each group of mice were inoculated with different H. pylori isolates. The first group was inoculated with H. pylori BLI 01, the second group was inoculated with H. pylori BLI 02 and the third group was inoculated with H. pylori BLI 03. Mice were previously given H. pylori 1x1010 CFU/ml given as much as 3 x intermittent one day. Each group consisting of 4 mice. All mices feces cultured were positive infected by H. pylori after 2 weeks. Three mice were killed, one in each a group at first week, 2nd week, 4th week to see the development of gastric inflammation.

RESULTS

Patient characteristics

The H. Pylori Bali isolates cultured from gastric biopsies of patients undergoing endoscopic examination at Wangaya Hospital, Denpasar, Bali Province. All patients were Balinese people.
One was male and two were females. The upper gastrointestinal diagnosis was gastric ulcer in all patients. The average age was 45 years.

**H. pylori Bali isolates comparison with neighbouring countries**

Phylogenetic analysis of 3’ variable regions of cagA gene of H. pylori Bali isolates were compared with isolates from Japan, China, Korea, Philippines, Malaysia and Thailand. It was showed that there was a different clustered between Bali isolates than other countries. (Figure 3)

The sequenced amino acid from DNA sequences show EPIYA motifs of cagA H. pylori Bali isolates have an unique EPIYT motifs (substitution A 'T), compare with the others countries. Three of H. pylori Bali isolates have a cagA virulence factor.

**Phylogenetic Analysis and Determination of the EPIYA Pattern**

The analysis included cagA 32 region nucleotide sequences of 3 isolates of which 3 Bali isolates and 8 from other countries retrieved

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Table 1: EPIYA motifs of CagA virulence factor from H. pylori Bali isolates

<table>
<thead>
<tr>
<th>Species/Abbrv</th>
<th>1. H. pylori_BLI-01</th>
<th>2. H. pylori_BLI_02</th>
<th>3. H. pylori_BLI_03</th>
<th>4. GU173854 (Philippines)</th>
<th>5. FJ458163 (Korea)</th>
<th>6. EU369652 (Indian-Malay)</th>
<th>7. giAB017923 (Jepang)</th>
<th>8. KF028589 (China)</th>
<th>9. DQ306710 (Central_China)</th>
<th>10. LC007010 (Indonesia)</th>
<th>11. GU173879 (Thailand)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
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<td>NGLKNSEPIYTQVKNKKQGAASPEPIYTQVAKKNARIDRINK</td>
</tr>
</tbody>
</table>

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**Fig. 2: Bali Island map**
from GeneBank. To determine the phylogenetic relationship between Lombok isolates and those from other countries the nucleotide sequences of cagA 32 region was multiple aligned and Maximum Likelihood phylogenetic tree constructed using the CLUSTALW program. The translated 32 region sequences of our Balinese isolates were then aligned using multiple alignments with MUSCLE program. Unrooted phylogenetic trees depicting relationships among CagA amino acid sequences were built using ML algorithm in MEGA v6 software. To predict structure of the C-terminal CagA protein we use SWISS-MODEL program.

**Histopathological assessment with update Sidney system**

Each gaster biopsy assessed for histopathology. The measurement focus on: *H. pylori*,...
Table 2: Inflammatory score base on update Sidney system

<table>
<thead>
<tr>
<th>Variable</th>
<th>First week</th>
<th>Second week</th>
<th>Fourth week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BLI 01</td>
<td>BLI 02</td>
<td>BLI 03</td>
</tr>
<tr>
<td>Neutrophil Activity</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td>1</td>
<td>1</td>
<td>&gt;1</td>
</tr>
<tr>
<td>Atrophy</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intestinal metaplasia</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
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the number of polymorphonuclear inflammatory cells (activity) and lymphocyte inflammatory cells (chronicity), present of gland atrophy and intestinal metaplasia as the update Sidney system classification. Scores of all variables are summed to obtain gastritis degrees base on visual analoque scale. The assessment is more detailed in Table 2.

**Macroscopically**

In the first week, 3 gaster size in all groups still in normal size. The size of the gaster becomes maximal at the second week. At fourth week, the size of the gaster back shrinks even smaller than the first week. In the first week, the gaster rugae infected by BLI 01 and BLI 02 isolates still normal appeared, while the gastric mice rugae inoculated by BLI 03 appeared thinning since the first week. All stomach rugae thinned as well as by the fourth week all the gaster rugae thins out at fourth week.

**Microscopically**

At the first week, chronic cells inflammation appeared in all of 3 gastric biopsies infected by *H. pylori* Bali BLI01, BLI 02 and BLI 03 isolates. At the second week, Acuta and chronic inflammatory cells appeared in all gaster mice inoculated by *H. pylori* BLI01, BLI 02 and BLI 03 isolates. The most inflammatory cells were found in gastric mice inoculated by *H. pylori* BLI 03 isolate. At the fourth week, one mice in group 2 dead, so the analysis only done in the group 1 and group 3. We found colony of *H. pylori* by Giemsa staining with equal density in the group 1 and group 3. Robust inflammatory cells found at the fourth week, acuta and chronic cell inflammations. Neither metaplasia intestinal nor atrophy in gaster mice until fourth week of the study.

**DISCUSSION**

*H. pylori* infection manifestation are associated with a complex combination of host susceptibility, environmental factors and bacterial virulence. Infections involving *H. pylori* strains that possess the virulence factor CagA have a worse clinical outcome than CagA negative strains. CagA-positive *H. pylori* increase the risk for gastric cancer than CagA negative. CagA behaves as a bacterial oncoprotein playing a key role in *H. pylori*-induced gastric cancer. There are 2 types of *H. pylori* isolates: CagA gene-positive strains and CagA-negative strains.
gene-negative strains. Almost all *H. pylori* isolates from East Asia are CagA positive, and approximately 20% to 40% of isolates from Europe and Africa are CagA negative. CagA is an oncoprotein that play a key role in *H. pylori*-induced gastric cancer. Activation of oncogenic signaling pathways and inactivation of tumor suppressor pathways are two crucial events in the development of gastric cancer. CagA shows the ability to affect the expression or function of vital protein in oncogenic or tumor suppressor signaling pathways via several molecular mechanisms.

All the *H. pylori* Bali isolates in this study have a CagA gene positive, and this results need to reevaluate in the further study to know the development to gastric cancer. CagA gene could mainly classified into 2 types (East Asian type and Western type) according to the sequence located in the of CagA and classified the repeat regions into 2 types, the first repeat and the second repeat, and found that the sequence of the second-repeat region was considerably different between East Asian strains and Western strains. Each region contains the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, which includes a tyrosine phosphorylation site. Recently, it has been more common to name the first-repeat regions as EPIYA-A and EPIYA-B segments and the second-repeat region in Western and East Asian strains as EPIYA-C and EPIYA-D segments, respectively. We found different level of gastric inflammation due to 3 Bali isolates *H. pylori* in this study. It may due to another virulence factor or bacterial determinants between three of Bali isolates *H. pylori* that we do not explore yet, such as Vac A, 5Øþþ-glutamyltranspeptidase (gamma GT), the duodenal ulcer-promoting gene (dupA), or

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A. Lymphocyte cell and plasma cell, no activity yet, at the first week (HE, 400x)
B. Small activity of neutrofil, at the second week (HE, 400x)
C. Robust activity of neutrofil extra and intraepithelial, at the fourth week (HE, 400x)
D. H pylori colony in anthrum at fourth week.

**Fig. 5: Histopathology examination**
peptidoglycan have been also shown to be important inducers of gastric inflammation.

Zhang et al. reveal a strong non-random distribution of the EPIYA B motif polymorphisms (including EPIYT and EPIYA) in Western H. pylori isolates, and provide evidence that the EPIYT are significantly less associated with gastric cancer than the EPIYA. By constructing a series of H. pylori cagA isogenic mutants and isogenic complementation plasmids, generating specific antibodies, co-culturing with human AGS cells, performing biochemical and modeling analysis, they demonstrate that CagA B-motif phosphorylation status is essential for its interaction with host PI3-kinase during colonization and that CagA with an EPIYT B-motif had significantly attenuated induction of interleukin-8 and the hummingbird phenotype, had higher affinity with PI3-kinase, and enhanced induction of AKT compared to the EPIYA.

Gastric biopsy after a few days of infection show signs of inflammation, appearance of acute inflammatory cells such as neutrophils. Two weeks after infection occurs infiltration of lymphocyte and monocyte cells. Within 4 weeks there was an increase in the number of CD4⁺ and CD8⁺ lymphocytes that indicated the onset of an adaptive immune response. In our research, the lymphocyte cells appearance in the first week, although still lack of the inflammation activity due to polymorphonuclear. The adaptive immune response due to H. pylori infection in mice is similar to that occurring in human. The hallmark of the immune response to H. pylori infection is the presence of gastric mucosal infiltration by T cells, plasma cells, and neutrophil cells in the gastric mucosa simultaneously.17, 18. Our study found the same appearance of inflammatory cells, that the acute and the chronic inflammatory cells found simultaneously in the gastric mucosa.

CONCLUSIONS

We obtained three H. pylori Bali isolated named as BLI 01, BLI 02 and BLI 03 that have a CagA genes. All the H. pylori Bali isolates have an unique EPIYT motifs (substitution A to T) in CagA genes. The degree of gastric inflammation is more severe in the 4th week of the study. There was a significant difference of gastric inflammation degree in all three groups of mice. H. pylori BLI 03 isolate with positive CagA gene, have a greatest degree of inflammation.

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