

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 gaster 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 antrums and 2 in gastric corpus. We collected 3 Bali isolates of *H. pylori* of that patients. Biopsy results are inserted into CarryBlair 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 -80o Celsius.⁶ 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 TAACAGGCAAGCTTTTGGAGG-32) and P2 (52 CTGCAAAGATTGTTTGGCAG-32) used to amplification of the *cagA* 32 variable region. The amplification steps were done under the conditions: 94°C for 1min; 34 cycles of 94°C for 1min and 55°C for 1min, 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. Substitution A àT in EPYA 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* Bali 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.

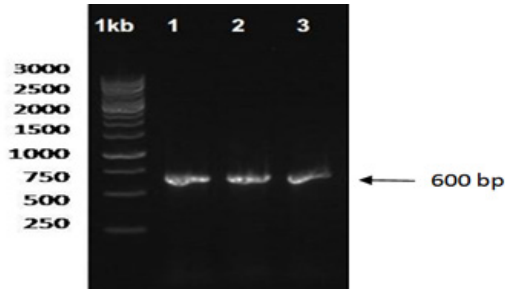


Fig. 1: 2% Agarose gel electrophoresis of 640 bp *cagA* PCR products amplified from *H. pylori* genomic DNA Bali isolates. 1kb DNA ladder; lane 1, BLI01; lane 2, BLI02 and lane 3, BLI03

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

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

Species/Abbrv	*****	*****	*****	**	*	*****	*****	**	**
1. <i>H. pylori</i> _BLI-01	•NGLKNS	EPIYA	QVNKKK	AGQAAS	PEEPI	Y	QVAKK	VNARID	RLNK
2. <i>H. pylori</i> _BLI_02	•NGLKNS	EPIYA	QVNKKK	AGQAAS	PEEPI	Y	QVAKK	VNARID	RLNK
3. <i>H. pylori</i> _BLI_03	•NGLKNS	EPIYA	QVNKKK	AGQAAS	PEEPI	Y	QVAKK	VNARID	RLNK
4. GU173854_(Philippines)	•NGLKNN	EPIYA	QVNKKK	AGQAAS	PEEPI	Y	QVAKK	VSAKID	QLNE
5. FJ458163_(Korea)	•NGLKNNT	EPIYA	KVNKKK	AGQAAS	PEEPI	Y	QVAKK	VSAKID	QLNE
6. EU369652_(Indian-Malay)	•NGLKN--	EPIYA	QVNKKK	AGQAAS	HEEPI	Y	QVAKK	VNAKID	RLNQ
7. giAB017923_(Jepang)	•NGLKNNT	EPIYA	QVNKKK	AGQATS	PEEPI	Y	QVAKK	VSSKID	QLNE
8. KF028589_(China)	•NGLKNNT	EPIYA	QVNKKK	AGQATS	PEEPI	Y	QVAKK	VSAKID	QLNE
9. DQ306710_(Central_China)	•NGLKN--	EPIYA	QVNKKK	AGQATS	PEEPI	Y	QVAKK	VSAKID	QLNE
10. LC007101(Indonesia)	•NGLKNNT	EPIYA	QVNKKK	IGQVAS	PEEPI	Y	QVAKK	VSAKID	QLNE
11. GU173879_(Thailand)	•NGLKN--	EPIYA	KVNKKK	TGQAAS	LEEPI	Y	QVAKK	VNAKID	RLNQ



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.⁷ To predict structure of the C-terminal CagA protein we use SWISS-MODEL program.⁸

Histopathological assessment with update Sidney system

Each gaster biopsi assessed for histopathology. The measurement focus on: *H. pylori*,

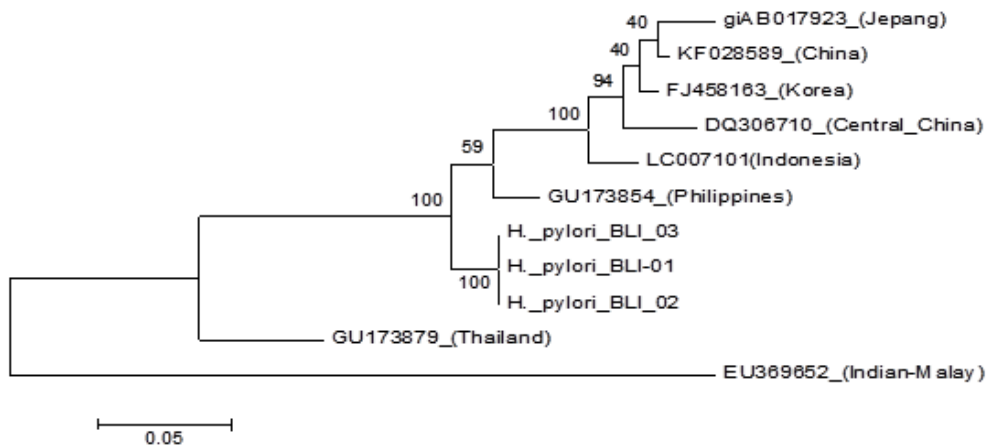


Fig. 3: Phylogenetic tree based on the *cagA*3' variable regions of *H. pylori* Bali isolates compared with 8 reference strains. Maximum Likelihood trees of 5% partial conservation sequence of *H. pylori* isolate Bali BLI_01, BLI_02 and BLI_03 compared with isolates from several countries, namely strains with access numbers GU173854 (Philippine), LC097101 (Indonesia / North Sulawesi), DQ306710 (Central China), FJ458163 (Korea), AB017923 (Japan), KF028589 (China), GU173879 (Thailand) and EU369652 (India-Malaysia)

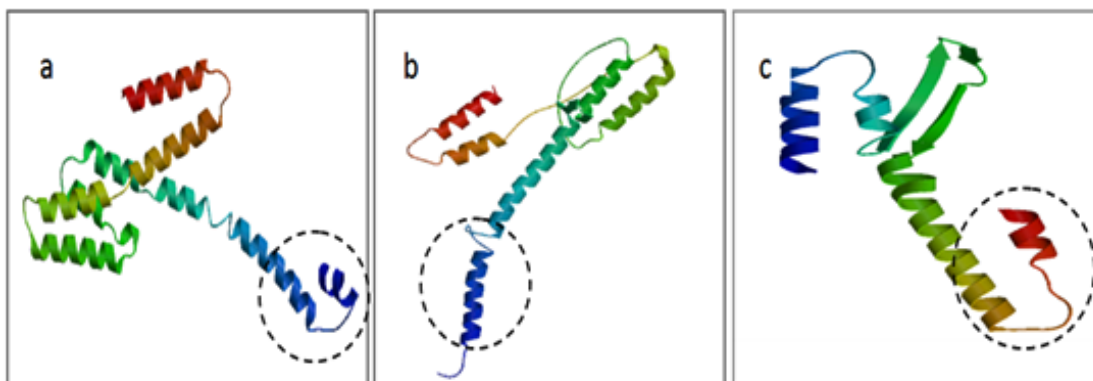


Fig. 4: Prediction of protein structure of the C-terminal part CagA *H. pylori* (a) Bali isolate BLI-01 strain has the motive EPIYA-ABD (East Asian) of patients with gastric ulcer. (b) Western strain EU369652 have EPIYA-ABC type of gastritis patient and (c) Chinese isolate KF028589. Part of C-terminal tail (marked by a circle broke up) is thought to be the site for interaction with SHP-2

Table 2: Inflammatory score base on update Sidney system

Variable	First week			Second week			Fourth week				
	BLI 01 Cor	BLI 02 Ant	BLI 02 Cor	BLI 03 Cor	BLI 01 Ant	BLI 01 Cor	BLI 02 Ant	BLI 02 Cor	BLI 03 Ant	BLI 03 Cor	BLI 03 Ant
H. Pylori	0	0	0	0	0	0	0	0	0	1	1
Neutrofil Activity	0	0	0	0	1	1	1	1	1	2	3
Chronic inflammation	1	1	1	1	1	>1	>1	2	2	3	3
Atrofi	0	0	0	0	0	0	0	0	0	0	0
Intestinal metaplasia	0	0	0	0	0	0	0	0	0	0	0

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 analogue scale.⁹The 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.

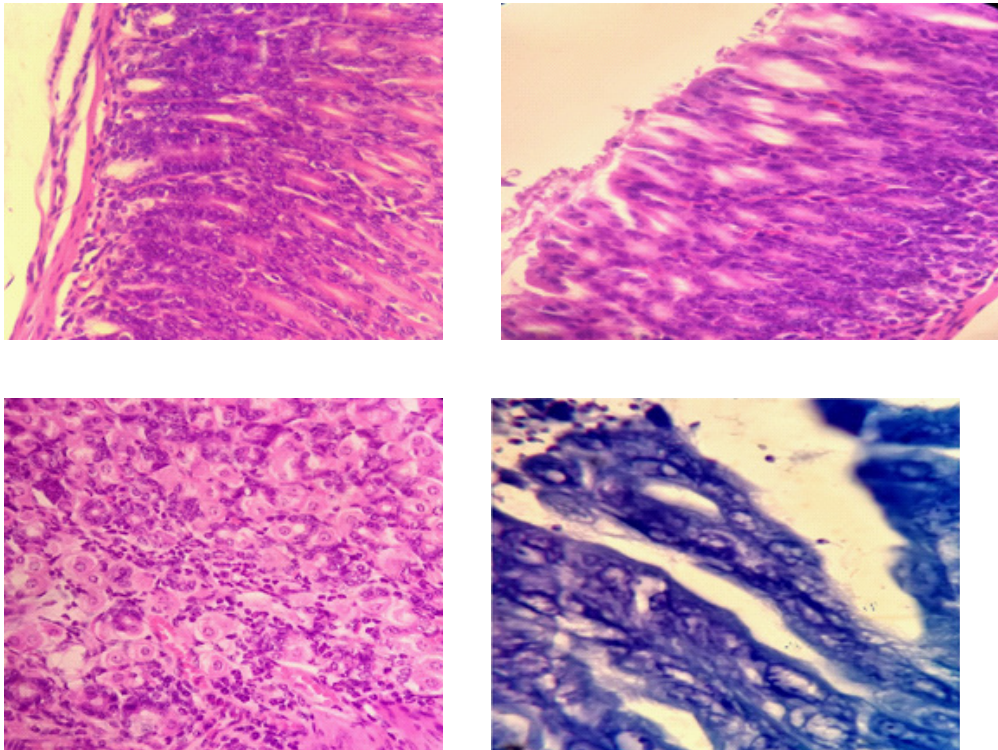
DISCUSSION

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.^{3,10,11} 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

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.¹² 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.¹³

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.³ 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, 50pP-glutamyltranspeptidase (gamma GT), the duodenal ulcer-promoting gene (dupA), or



- A. Lymphocyte cell and plasma cell, no activity yet, at the first week (HE, 400x)
- B. Small activity of neutrophil, at the second week (HE, 400x)
- C. Robust activity of neutrophil extra and intraepithelial, at the fourth week (HE, 400x)
- D. *H. pylori* colony in antrum 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.^{15,16,17} 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 a 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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